University of Modena and Reggio Emilia

PhD COURSE OF CLINICAL AND EXPERIMENTAL MEDICINE

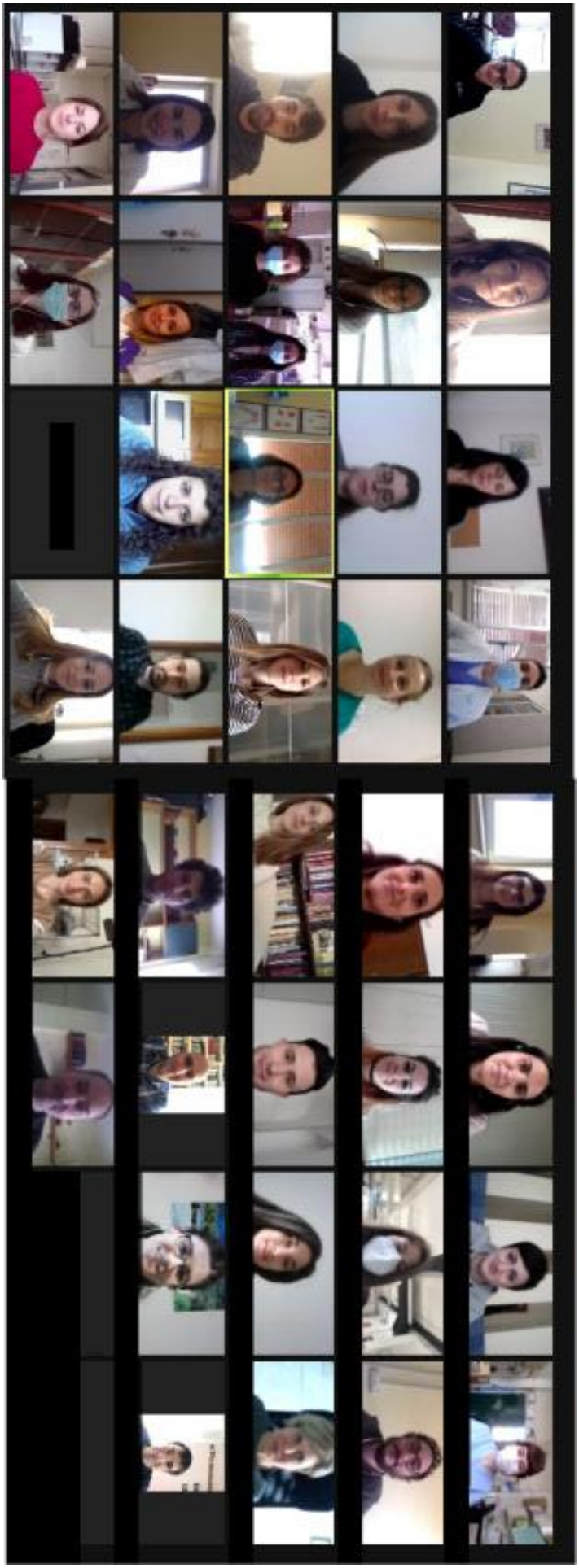

# PhD DAY 2021

# Abstracts

**June 16-23-30 (9:00 a.m.)**
**Virtual meeting – Dept. Biomedical, Metabolic and Neural Sciences**
**(287 Campi street, Sect. Physiology and Neural Sciences - Modena)**

# Covid-19 pandemic crisis

The PhD programme in Clinical and Experimental Medicine (CEM) had to cope with the emergency generated by Covid-19, then renamed as SARS-CoV-2. Thanks to professors managing our courses, and to students attending the PhD programme, we were able to regularly perform all the programs with a little delay. Just aside, a screenshot of students attending a virtual lecture of Dr. Chiara Gabbi on communicate science.



The International Doctoral Programme 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) 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*

# XXXIV cycle

***Dr. Tommaso Filippini***

*CEM Curriculum: Public Health*
*Tutor: Prof. Marco Vinceti*

## LIGHT-AT-NIGHT EXPOSURE AND RISK OF EARLY AND LATE ONSET DEMENTIA: A COMPARATIVE CASE-CONTROL STUDY IN MODENA, NORTHERN ITALY

**Background**

Dementia is an emerging leading cause of death and a chronic disease causing severe impairment of social and working activities, as well as an extremely high family burden. Genetic susceptibility might play an etiologic role in some cases, nevertheless known dementia mutations causing dementia onset are few. Therefore, environmental and occupational risk factors, as well as lifestyle and dietary habits might be involved in disease etiology. In particular, in modern society over 80% of the population worldwide and close to 100% in Western countries live under light-polluted skies and outdoor environments. Thus, individuals are exposed to an increased amount of artificial light at night (LAN), and emerging evidence suggest that LAN exposure may have a detrimental effect on humans. Biological plausibility for a LAN-dementia link have been provided by laboratory studies that showed that LAN exposure may induce DNA damage and oxidative stress, and alter hormone synthesis and metabolism as well other metabolic functions. Another possible mechanism involved in dementia risk is disruption of circadian rhythm. In particular, it has been shown in a tauopathy/Alzheimer's disease model of Drosophila flies that nighttime exposure affects sleep-wake cycles due to increased pTau proteins and neurodegeneration.

**Objectives**

In the present study, we aimed at investigating the role of an emerging environmental factor, exposure to light-at-night, in the etiology of both early-onset (EOD) and late-onset (LOD) dementia in an Italian population.

**Methods**

We carried out a case-control study in Modena province, Northern Italy. We recruited dementia cases referred to the Cognitive Neurology Centers at the Modena Policlinico-University Hospital and Carpi Hospital in the period October 2016-October 2019. Inclusion criteria were diagnosis of dementia, either EOD or LOD, respectively before and after 65 years, and residence in the province of Modena. Exclusion criteria were coexisting diagnoses of pervasive developmental disorders, major psychiatric disorders, or cognitive impairment in the context of other neurological disorders (e.g. cerebrovascular disease with severe motor disability or multiple sclerosis). As referent population, we recruited the caregivers of these dementia patients. Each subject received a questionnaire tailored to record anamnestic and lifestyle factors potentially related to dementia onset, as well as residential history. We geocoded residential address preceding the time

of diagnosis provided that the participant had been living there for at least five years. If subjects changed their residence within five years from the recruitment, the previous address was considered for exposure evaluation. Using within a geographical information system the QGIS software (version 3.16.6-Hannover), we estimated LAN exposure through 2015 average annual data available from the NASA-Joint Polar-orbiting Satellite System, having a pixel resolution of 500mx500m.

We used a multivariate unconditional logistic regression model adjusted by sex, age and educational attainment to calculate odds ratio (OR) and 95% confidence intervals (CI) of EOD and LOD associated with increasing tertile of LAN exposure using, according to distribution in controls. We also implemented a restricted cubic spline model with three knots (10th, 50th and 90th percentiles) to assess the shape of the association between LAN exposure and dementia risk, accounting for non-linearity. Median value was used as reference point. We used 'logit', 'mkspline' and 'xblc' routines of the Stata-16.1 statistical package (Stata Corp., College Station, TX, USA, 2021) for data analysis.

**Results**

We recruited 148 participants, including 59 (male/female: 26/33) EOD cases, 34 (male/female: 15/19) LOD cases, and 55 (male/female: 24/31) controls. Mean age at diagnosis was 59 and 74 years for EOD and LOD, respectively. High school educational attainment level or higher was achieved in 60% of controls, while the corresponding figures for EOD and LOD were 37% and 26%, respectively. Median annual LAN exposure was 28.0 nW/cm2/sr (interquartile range-IQR: 11.1-39.1), ranging from 0.8 to 57.8 nW/cm2/sr. Median exposure was considerably higher in LOD cases (35.6 nW/cm2/sr, IQR: 26.8-41.7) compared to both EOD cases (25.8 nW/cm2/sr, IQR: 9.0-38.1) and controls (25.8 nW/cm2/sr, IQR: 6.4-38.1). EOD risk according to increasing tertile of LAN exposure (based on distribution in control population) showed an unclear relation, with ORs of 1.26 (95%CI 0.49-3.20) and 1.17 (95% CI 0.45-3.04) in the second and third tertile, respectively. Conversely, for LOD there was a dose-response association between LAN exposure and risk, with ORs of 1.37 (95% CI 0.15-12.35) and 3.48 (95% CI 0.47-25.80) in the middle and top tertile, respectively. In spline analysis according increasing LAN exposure, we do not find any indication of increased EOD risk, while a nearly linear increased in risk can be noted for LOD, although estimates were statistically imprecise.

**Conclusions**

In this study, we found scant evidence for an association between lightening exposure during night and risk of early onset dementia, while an indication of increased risk for late-onset dementia in association with LAN emerged, although the limited sample size and the high imprecision of the estimates suggest caution in the interpretation of results.

***Dr. Lucia Marchetti***

*CEM Curriculum: Nanomedicine, Medicinal and Pharmaceutical Sciences*
*Tutor: Prof. Davide Bertelli*
*CoTutor: Prof. Federica Pellati*

## DEVELOPMENT OF PARTICLES AS MULBERRY DNJ DELIVERY SYSTEM FOR A PROLONGED HYPOGLYCAEMIC EFFECT

**Background**

Mulberry 1-deoxynojirimicin (DNJ) has shown important anti-hyperglycaemic and anti-obesity effects, by inhibiting α-glucosidase and regulating glucose metabolism pathways. Hence, it is a promising candidate for the prevention and management of diabetes mellitus (T2DM). Unfortunately, DNJ systemic effects are impaired by its short half-life due to the high hydrophilicity and fast renal clearance.

**Objectives**

The study aimed at the optimization of mulberry leaf extraction with a view to obtain a standardised product, whose bioactivity was evaluated in-vitro as enzymes inhibitory potential. As a continuation of the project, different strategies for the encapsulation and controlled release of the active constituent DNJ were attempted to achieve optimal therapeutic results.

**Methods**

DNJ was determined in 12 Italian mulberry varieties by HILIC–ESI/MS analysis and the comprehensive characterization of extracts was carried out by reverse phase HPLC-MS2. The freeze-dried extracts (FDEs) were tested on porcine α-amylase and yeast α-glucosidase, IC50 were compared to acarbose as positive control. Particles at different concentrations of polymer (P1 and P2) loaded with FDE were obtained and DNJ release was evaluated (sink conditions) in simulated gastric and intestinal fluids. NMR measurements of proton relaxation rates in presence and absence of polymer were used to calculate interaction constants between DNJ and the polymer.

**Results**

DNJ mean content ranged from 0.47±0.01 to 1.79±0.2 □g/mg FDE. A significant inhibition was observed on α-glucosidase, even stronger than pure DNJ, conversely, only a limited activity was observed against α-amylase. Encapsulation efficiency equal to 54.0±0.4 and 55.2±0.3% was calculated for P1 and P2, respectively. DNJ was released in gastric fluid with a burst effect, then the concentration remained constant up to 2 h. In intestinal fluid, for both P1 and P2 the cumulative DNJ release did not exceed 40% in 3h.
The NMR relaxometry study revealed a significant decrease in relaxation times for DNJ protons in presence of the polymer. The encapsulation of FDE into particles could provide new insights into a rational use of

mulberry extract as a new therapeutic approach for T2DM and its complications, with limited side effects compared to conventional hypoglycaemic oral drugs.

***Dr. Ivana Castaniere***

*CEM Curriculum: Translational Medicine*
*Tutor: Prof. Enrico Clini*

## INSPIRATORY EFFORT AND LUNG MECHANICS IN SPONTANEOUSLY BREATHING PATIENTS WITH COVID-19 ACUTE RESPIRATORY FAILURE: A MATCHED CONTROL STUDY

**Background**

The respiratory mechanics of patients with COVID-19 pneumonia developing acute respiratory distress syndrome (CARDS) are still subject to debate, and probably represent a model of lung injury different from typical acute respiratory distress syndrome (ARDS). In particular, data with regard to the lung mechanical behavior of CARDS in the early phase of its onset are lacking.

**Objectives**

To investigate the pathophysiological characteristics in the early phase, we compared the respiratory mechanics and physiological characteristics of spontaneously breathing patients with CARDS with a historically matched cohort of ARDS individuals who were candidates for a non-invasive mechanical ventilation (NIV) trial.

**Methods**

Thirty-five consecutive COVID-19-pneumonia patients developing CARDS, spontaneously breathing and undergoing a NIV trial, were compared with a historic cohort of ARDS patients 1:1 matched by PaO2/FiO2 level. In the two groups, respiratory mechanics and respiratory drive were recorded at baseline and 2 hours after the start of NIV. Correlations between positive end-expiratory pressure (PEEP) levels and changes in lung mechanics and PaO2/FiO2 ratios were also assessed. To build the ARDS group, a one-to-one matching procedure was performed with the nearest-neighbor method without replacement. The Student's t-test assessed the difference between group means when data were distributed normally; otherwise, the Wilcoxon test was used. Comparison between dichotomous variables was performed with the $\chi 2$ test or Fisher's exact test, where appropriate. The relationship between PEEP and relative change in dynamic compliance and PaO2/FiO2 ratio 2 hours after starting NIV was tested with the Pearson correlation coefficient and assessed through linear regression. Statistical significance was set at $p=0.05$.

**Results**

At baseline, CARDS patients presented significantly lower respiratory drive activation (esophageal pressure swing [ΔPes] 12 vs 34 cmH2O $p<0.0001$, respiratory rate [RR] 23 vs 28 breaths per minute [bpm] $p<0.0001$, Vte 9.1 vs 11 mL/kg of predicted body weight [PBW] $p=0.003$) than ARDS patients. Moreover, dynamic compliance (57 vs 24 mL/cmH2O $p<0.0001$) was more preserved while dynamic mechanical power (27 vs 99

J/min p<0.0001) was lower in CARDS compared with ARDS. Two hours after starting NIV, percent variation in transpulmonary pressure (ΔPL 47% vs 3% p=0.0003) and in dynamic mechanical power (76% vs 16% p=0.01) was higher in CARDS than in ARDS. PEEP levels were inversely correlated with both dynamic compliance (r=–0.41, p=0.01) and PaO2/FiO2 ratio (r=–0.08, p=0.7) in CARDS, while a direct positive association with the same variables (r=0.45, p=0.01 and r=0.3, p=0.06, respectively) was found in ARDS.

**Conclusions**

In the early phase of its onset, patients with CARDS present atypical physiopathological characteristics, with a resultant mechanical behavior that is different from comparable forms of typical ARDS.

***Dr. Annalisa Tameni***

*CEM Curriculum: Translational Medicine*
*Tutor: Dr. Alessia Ciarrocchi*

## LNCRNA BLACKMAMBA-DNA HELICASE HELLS AXIS DRIVES ALK-ALCL PROLIFERATION BY ORCHESTRATING A COMPLEX TRANSCRIPTIONAL PROGRAM

**Background**

Anaplastic Large Cell Lymphomas (ALCLs) are a group of neoplasms arising from the transformation of mature T-cell. The presence of chromosomal rearrangements involving the ALK gene stratifies ALCLs in ALK+ and ALK- identifying two distinct diseases with different clinical behavior and prognosis. Clinically, ALK-ALCLs display a poorer outcome as compared to ALK+ ALCL patients. The ALK- subtype is extremely heterogeneous and displays many genetic alterations among which STAT3 hyperactivation has been recently demonstrated as key player in cancer development and progression by regulating long noncoding RNAs (lncRNAs). Still, the molecular mechanisms leading to ALK-ALCL transformation remain quite elusive. LncRNAs are transcripts longer than 200 nucleotides that have key pleiotropic functions such as controlling gene expression and cell identity. More than 8000 lncRNAs are aberrantly expressed in cancer, making them ideal tumor-specific biomarkers and putative targets for therapeutic interventions. We recently discovered and characterized the chromatin-associated lncRNA BlackMamba, specifically overexpressed in ALK-ALCL patients. Mechanistically, BlackMamba is regulated via STAT3 and its expression is required to sustain ALK- ALCL cell proliferation, clonogenicity and morphology. Indeed, short-hairpin mediated BlackMamba knockdown (KD) led to delay of cell proliferation, increased polynucleation and actin cytoskeleton rearrangements. We demonstrated its key role in promoting ALK-ALCL neoplastic features by regulating a set of genes involved in migration, integrin-mediated cell adhesion and regulation of actin cytoskeleton. Among the BlackMamba targets genes we found the lymphoid helicase HELLS which has been implicated in cancer progression both for its helicases and transcriptional activities. In ALK-ALCL samples, the expression of BlackMamba and HELLS are significantly correlated in ALK-ALCL patients. Besides, we showed that BlackMamba and HELLS interacts suggesting a high functional relationship between these two molecules. Collectively, our preliminary data indicated a previously unknown tumorigenic role of STAT3 via an lncRNA-DNA helicase HELLS axis and reveal an undiscovered role for lncRNA in the maintenance of the neoplastic phenotype of ALK–ALCL.

**Objectives**

The aim of this project was to characterize the molecular mechanisms through which the axis BlackMamba-HELLS regulates ALK–ALCL neoplastic phenotype. We pursued this aim through: i) the characterization of BlackMamba-HELLS-dependent gene expression program in ALK-ALCLs, posing attention to the target genes involved in cytokinesis and cytoskeleton-organization because of the phenotype observed in Black Mamba-

HELLS KD cells. ii)The identification of BlackMamba-HELLS transcriptional partners that cooperate in the regulation of ALK-ALCL transcriptional program.

**Methods**

SiRNA and stable downregulation shRNAs techniques were used to characterized HELLS molecular functions and to assess cytoskeleton and cytokinesis-related target genes biological properties. To identify HELLS target genes, we performed RNA-Sequencing on HELLS KD ALK-ALCL. To analyze the effect of HELLS and each of the tested downstream target (RHOA,ANLN,PAK2,RHOU) we performed cell proliferation assay, evaluated polynucleated cells and F-actin rearrangements by using May-Grunwald Giemsa and Immunofluorescence (Phalloidin, DAPI) staining in KD cells. To identify BlackMamba-HELLS transcriptional partners, we performed bioinformatic analysis using JASPAR and PROMO 3.0 tools. qRT-PCR was used to quantify transcriptional partner expression in ALK–ALCL cell lines. Co-Immunoprecipitation (CO-IP) experiments were employed to investigate physical interaction between BlackMamba-HELLS and predicted transcription factors (TFs). Chromatin Immunoprecipitation experiments were performed to verify BlackMamba-HELLS partners binding on common target genes promoters. Transient downregulation experiments with siRNA were used to characterize YY1 molecular functions and to assess its overlapping HELLS-related gene signature.

**Results**

**BlackMamba-HELLS transcriptionally coordinate a panel of cytoskeleton and cytokinesis-related genes.**To characterize HELLS molecular and biological functions in ALK–ALCL, we silenced its expression by doxycycline inducible shRNA in two ALK-ALCL cell lines (MAC2A and TLBR-2). Loss of HELLS led to reduction of cell proliferation and clonogenicity, and increased the number of polynucleated cells and F-actin cytoskeleton rearrangements. Next, we performed RNA-sequencing experiments in TLBR-2 cells in which the expression of HELLS has been downregulated by shRNA. We observed that HELLS heavily conditions the transcription program of ALK-ALCL affecting the expression of 1334 genes (554 genes upregulated and 780 genes downregulated upon HELLS KD). Among the downregulated genes, we found the significant enrichment of cytoskeleton-remodeling and cytokinesis pathways (6) indicating that the increased polynucleation observed upon HELLS KD could be linked to defect in cytokinesis. We further validated this set of genes confirming that RHOA, RHOU, PAK2, ANLN, PITPNM1, KLHL21, CDC42SE2, PLK1, AURKB and KIF20A are also BlackMamba targets. Next, we focus on the functional study of PAK2 RHOA, RHOU and ANLN as functional downstream mediators of BlackMamba/HELLS signaling. Each of these genes, was silenced by doxycycline inducible shRNA and siRNA in two ALK-ALCL cell lines (MAC2A and TLBR-2) obtaining at least a 60% knockdown (KD) for 72h. Phenotypic analysis showed that Pak2KD did not affect significantly cell growth or the formation of polynucleated cells. Morphologically, loss of Pak2 caused the reorganization of actin filaments increasing the production of lamellipodia. RhoUKD resulted in a slight reduction of cell growth and an increase of percentage of polynucleated cells… TRUNCATED ABSTRACT

***Dr. Sara Castellano***

*CEM Curriculum: Translational Medicine*
*Tutor: Prof. Enrico Tagliafico*

## GENOMIC CHARACTERIZATION OF CHRONIC MYELOPROLIFERATIVE NEOPLASMS

**Background**

Myeloproliferative neoplasms (MPNs) are a group of related hematologic cancers that include polycythemia vera (PV), essential thrombocythemia (ET), and myelofibrosis (MF). PV and ET can progress to myelofibrosis giving rise to post-PV (PPV) and post-ET (PET) myelofibrosis. However, MF can also occur without pre-existing conditions (primary MF). Currently, risk stratification of MF patients is based mainly on clinical features and the presence of driver mutations. None of the existing models for MF integrates transcriptomic data. Therefore, there is a need to better characterize the transcriptomic profile of these disorders in order to add more robustness to current scoring systems.

**Objectives**

The main scope of this project is to identify a molecular signature and to build a robust classification model able to distinguish "high risk" MF patients with inferior overall survival from "low risk" ones.

**Methods**

We analyzed the gene expression profile of granulocytes isolated from 114 MF patients to identify survival-related transcripts, by performing a Cox regression analysis. Nearest shrunken centroids, subsequent iterations, and k-fold cross-validation were used to build, optimize and validate a robust classification model starting from survival-related transcripts. To assess if our model was able to improve the prognostic power of current scoring systems, we designed two new combined models by integrating information from our gene expression-based classification within two existing scores (DIPSS and MIPSS70). The Akaike information criterion (AIC) score was used to compare models for prediction of survival.

**Results**

Cox regression analysis led to the identification of a list of 832 probesets, which was then optimized, obtaining a classification model based on 273 probesets. Classification of the 114 samples of our dataset with this model resulted in 54 high-risk and 60 low-risk samples. We observed significant enrichment of patients' clinical features associated with worse prognosis within the high-risk group (e.g., levels of hemoglobin, leukocytes, circulating blasts, driver and high molecular risk mutations). Strikingly, several patients belonging to the low and intermediate-1 categories of existing prognostic scores were classified as high-risk with our model. These patients were deceased or leukemia transformed earlier than the prognostic class reference

median survival. Furthermore, our model showed good performance particularly in distinguishing high-risk and low-risk patients within DIPSS and MIPSS70 intermediate categories. It is noteworthy that intermediate-risk classes represent the most challenging patients' categories, for whom determining the optimal therapeutic strategy is more difficult. Additionally, both our new combined models showed better AIC values than DIPSS and MIPSS70 alone, thus suggesting that prognostication in MF patients might be ameliorated through the evaluation of granulocytes' gene expression profiles.

**Conclusions**

These results suggest that our gene expression-based model can add prognostic information to the existing scores for MF risk stratification that are based on clinical features and driver mutations. It can improve the identification of MF patients' subgroups characterized by poor prognosis, allowing these patients to be directed towards the most appropriate therapeutic option. These results should be validated in an independent dataset to confirm their predictive power.

***Dr. Federica Violi***

*CEM Curriculum: Public Health*
*Tutor: Dr. Roberto Grilli*
*CoTutor: Prof. Marco Vinceti*

## 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 reorganisation of primary care, largely based upon the principles of the Chronic Care Model and the adoption of formally structured clinical pathways. This reorganisation departs from the traditional hospital-centred models of care towards new innovative clinical and organisational 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 organisation 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*).
- To compare different approaches towards the implementation of A&F interventions.

**Methods**

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 has been 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. Process and outcome indicators measurable on administrative data available has been selected, also relying on a review of the literature in the field. Moreover, a qualitative analysis, through the construction of focus groups and interviews, has been conducted among health professionals and patients, to identify the determinants and the dynamics that regulate the hospital-territory continuity of care and to allow the identification of additional information and indicators required in an optimal A&F intervention for the management of effective clinical pathways. Furthermore, a structured questionnaire was administered to different Italian regions implementing A&F interventions to improve clinical practice with the aim of analyzing and comparing different regional initiatives in terms of type of competences and responsibilities involved, characteristics of target clinical behaviors of the interventions and type of indicators and data sources used.

**Results**

When asked which indicators should be included in A&F, the most quoted indicators from professionals were process indicators (i.e. n. of patients enrolled in the clinical pathways and variation over time, n. of visits from specialists or GPs and exams provided to enrolled patients, adherence to the scheduled time for visits and exams), outcomes indicators (i.e. n. of access to emergency and hospitalizations for CHF, glycemic control, glycated hemoglobin and n. of diabetes complications for DM), indicators on quality of life and on patients' perceived pathway quality. The analysis allowed the identification of additional information and indicators required in an optimal intervention for the management of effective clinical pathways, as well as optimal feedback options. Regarding the questionnaires submitted to different A&F regional interventions, preliminary findings showed that, when implementing an A&F intervention, the clinical behavior identified as target should be clearly defined and not to be used as a "generic reminder" to pay attention to the quality of the services provided.

**Conclusions**

Within that context, we had the opportunity, relying on a qualitative research approach, to investigate what health professionals dealing with those diseases think it is important to them to know about the process and outcomes of the care provided.

***Dr. Giulia Cassone***

*CEM Curriculum: Translational Medicine*
*Tutor: Prof. Carlo Salvarani*
*CoTutor: Dr. Marco Sebastiani*

## TREATMENT OF GIANT CELL ARTERITIS PATIENTS WITH ULTRA-SHORT GLUCOCORTICOSTEROIDS AND TOCILIZUMAB: THE TOPAZIO STUDY

**Background**

Giant-cell arteritis (GCA) is the most common form of vasculitis in patients over 50 years old. Extracranic large vessel involvement (LVI) has emerged in recent decades, especially with the development of new imaging tools such as PET-TC, MR-Angiography (MRA) and CT-Angiography (CTA). It is unknown, however, how effective these methods are for assessing disease activity while patients are under treatment.

Actually, GCA treatment is mainly based on long term use of glucocorticosteroids (GCs), with subsequent high risk of side effects. Tocilizumab has recently been approved for the treatment of GCA. However, it is often use in combination with GCs.

**Objectives**

The aim of our study is to evaluate clinical and functional/morphological imaging variations in a series of patients with GCA treated with ultra-short glucocorticosteroids (GCs) and tocilizumab (TCZ) s.c. We will also evaluate effectiveness and safety of TCZ mono-therapy as a maintenance treatment in GCA.

**Methods**

Tin this monocentric observational prospective study, we enrolled patients with GCA satisfying ACR criteria, or patients presenting with only systemic manifestations and a C-reactive protein (CRP) > 25 mg/L and a large vessel vasculitis assessed by MRA, CTA or PET/CT.

All patients received high-dose pulse intravenous methylprednisolone (500 mg) for 3 consecutive days (Day 0-1-2) and subsequently they were treated weekly with tocilizumab 162 mg s.c..

Each patient underwent functional and morphological imaging (PET and MRA or CTA) at baseline and at 24 and 52 weeks, and then observed for a total follow-up of 76 weeks per subject.

Statistical analyses were performed using the SPSS statistical software, version 17.0 (SPSS Inc., Chicago, IL, USA). Results were expressed as median and interquartile range (IQR) for continuous numeric variables or as percentage for dichotomous variables. Categorical variables were analysed by means of chi square test or Fischer exact test. Continuous variables were compared using unpaired or paired nonparametric tests (Mann Whitney or Wilcoxon test, respectively).

**Results**

The study was approved by the local ethical committee in April 2019.

To date, 20 GCA patients were enrolled (F/M 15/5, mean age 70,7 years). 9 patients had a new diagnosis of GCA, while 11 patients had a relapse.

Until May 20, 9/20 patients completed W52-follow-up and 6/20 patients completed W76-follow-up. Between 9 patients who complete W52-follow-up, all reported clinical improvement, namely improvement of symptoms and normalization of acute phase reactants. In 8 patients an improvement or stabilisation of PET and MRA or CTA findings were detected, while one patient had vasculitis relapse with 18FDG uptake grade 2 on Meller's visual *grading scale* in the right common carotid artery.

8/20 patients withdrew the study before W52: 6 patients had a disease relapse, while in 2 patients tocilizumab was discontinued because of adverse effects (severe neutropenia in one case, allergic skin reaction in the other one). Even if 8/20 patients had a past clinical history of diverticulosis, no other side effects were detected.

Of note, one patient experienced a severe disease relapse on W24: PET findings were suggestive for active aortic vasculitis (grade 3 on Meller's visual *grading scale*) and MRA revealed the presence of thoracic and abdominal aortic aneurysms, deserving surgical treatment.

**Conclusions**

In these preliminary results, MRA, CTA and PET/CT seems to be useful methods for assessing disease activity in GCA patients during treatment.

TCZ demonstrated a good safety profile in patients with GCA, however its potential effect in stabilize or resolve large vessels inflammation without the concomitant use of GCs has yet to be demonstrated in large randomized clinical trials.

Further multicentric prospective studies are needed to clarify the role of functional and morphological imaging for assessing disease activity in GCA and to evaluate the role of TCZ monotherapy in such patients.

***Dr. Maria Luisa Introviane***

*CEM Curriculum: Nanomedicine, Medicinal and Pharmaceutical Sciences*
*Tutor: Prof. Fabio Prati*
*CoTutor: Prof. Emilia Caselli*

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

**Background**

In the field of antimicrobial resistance (AMR), one of the most important mechanisms of resistance is the production of β-lactamases, bacterial enzymes that open the β-lactam ring of antibiotics and deactivate the drug. Classes A, C, and D β-lactamases utilize for such deactivation a serine residue, while class B metal-enzymes require one or two zinc ions. Boronic Acid Transition State Inhibitors (BATSIs) are inhibitors of β-lactamases, that inhibits the enzymes by forming a tetrahedral adduct with them.[1]

**Objectives**

Selectivity and high potency of specific BATSIs towards β-lactamases have been proved in several studies, by means of changing the substituents on the carbon atom attached to the boron. In particular, α-acylamidoboronic acids are characterized by the presence of an amide side chain bearing substituents typical of commercially available β-lactams.[1] Starting from α-acylamidoboronic acids we replaced the amide group with different bioisosters to gain activity against all classes of β-lactamases.

**Methods**

To improve the activity against class A and C we synthesised and tested on ADC-7 (class C) and on KPC-2 (class A) α-acylamido-β-triazolylboronic acids and 1,2,3-triazolylmethaneboronic acids. Against class D enzymes we synthesized β-triazolylboronic acids and α-sulfonamideboronic acids and we tested them on OXA-23 and OXA 24/40 enzymes. To inhibit class B metal enzymes, the synthesis of α-triazolylboronic acids substituted with zinc binding groups was performed and the activity on VIM-2 and IMP-1 was measured. In addition, we coupled a molecule active against class A and class C with a drug able to inhibit metal β-lactamases, synthesizing a co-drug.

**Results**

Among the α-acylamido-β-triazolylboronic acids (Figure 1, A) compounds S02030 and MB076 demonstrated the best activity against class A (KPC-2, IC50 0.084 μM and 0.132 μM) and class C β-lactamases (ADC-7, Ki 0.044 μM and 0.020 μM). They overcame the in vitro tests and in vivo tests on animal models are currently performed. To improve the activity and pharmacokinetic profile we synthesised several analogues of these compounds. A library of 26 1,2,3-triazoles 1,4-disubstituted (B) was synthesized and characterized via kinetic

and microbiological assays. They demonstrated an extraordinary inhibitory activity against ADC-7 ($K_i$ values spanning from 0.090 μM to 33 μM). Additionally, the X-ray crystal structures of ADC-7 in complex with 5 of these compounds confirmed the expected interactions of the inhibitor in the active site demonstrating that the triazole is an effective amide bioisoster.[2] Fourteen of these boronic acids were tested against resistant strains of *K. pneumoniae* producing KPC carbapenemases. All compounds show very good inhibition of KPC-2 ($K_i$s ranging from 1 nM to 1 μM) and most of them were able to restore cefepime (FEP) activity. Docking studies of the inhibitor-enzyme complexes were also performed: α-triazolylboronic acids are accommodated very well into the active site of the KPC-2 enzyme, even if these studies didn't allow a clear interpretation of the observed affinities and crystal structures are pursued.[3] Class D β-lactamases are different from other enzymes because they possess a hydrophobic bridge (formed by a Tyr or Phe on one side, and a Met or Trp on the other) across the top of the active site and inactivate last-resort drugs such as doripenem. Compound CR167 is a α-sulfonamidoboronic acid, good inhibitor for class A (Amp-C, $K_i$= 0,0013 μM) and class C (ADC-7, $K_i$= 0.160 μM) enzymes, that demonstrated to inhibit class D β-lactamases with nanomolar affinity (OXA 24/40, $K_i$= 0.300 μM).[4] Starting from the analysis of its binding mode, we synthesised a series of chiral sulfonamidoboronic acids (C) where a little R2 hydrophobic chain was inserted to improve the binding affinity. Compound PCF022 demonstrated to interact efficiently with OXA enzymes (OXA23, IC50= 6.7 μM; OXA 24/40, IC50= 0.8 μM), while the others were not so active in in vitro analysis. Also β-triazolylboronic acids (D) were synthesised to overcome the hydrophobic bridge of class D enzymes by the insertion of a carbon atom between the triazole and the boronic acid. A series of 10 molecules was tested on OXA 23 and OXA 24/40. Class B metal β-lactamases possess one or two zinc ions in the catalytic site. α-Triazolylboronic acids substituted with zinc binding groups (E), such as phthalic acid, glutamic and aspartic acid and 3-mercaptopropanoic acid, were synthesised and tested for their ability to chelate metals. Preliminary *in vitro* tests on purified enzymes were performed and the best compound of the series, PCF036, demonstrated a very good activity on VIM-2 (IC50= 7.7 μM). Finally, we tried to couple the activity against class A and C of an α-acylamido-β-triazolylboronic acid with L-Captopril (F), an antihypertensive drug which demonstrate inhibitory activity against class B enzymes. We succeed in the coupling of these molecules using two different linkers and we are ready for *in vitro* analysis… TRUNCATED ABSTRACT

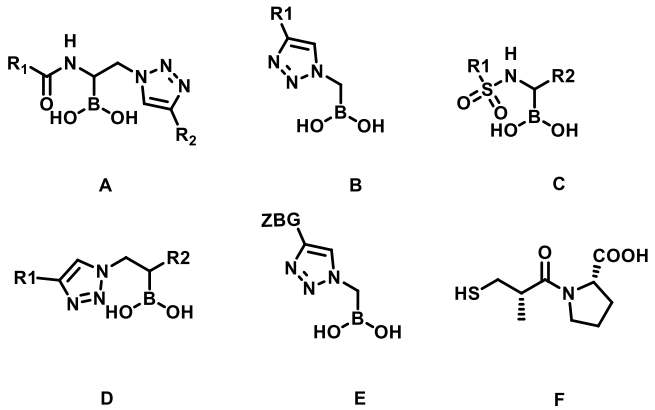



**Figure 1**. Schematic representation of compounds.

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## DATA-DRIVEN TECHNOLOGIES FOR DRUG REPURPOSING: USE OF MACHINE-LEARNING MODELS TO PREDICT THE SELECTIVITY PROFILE OF KNOWN MOLECULES

**Background and Objectives**

Drug repurposing (the identification of novel therapeutic indications for known drugs) provides several advantages with respect to de novo drug discovery, such as reducing times, risks and costs (1). Moreover, in recent times the increase in biological, clinical and chemical data paved the way to the large-scale use of efficient and cost-effective data-driven in silico strategies (2).

Therefore, the main focus of the PhD project covers two main areas of in silico drug repurposing:

- the application of tailored protocols for specific repositioning campaigns;
- the development of novel methods and general approaches.

Here, a machine learning (ML)-based study will be presented, aimed at efficiently predicting the selectivity profile of potential drug repurposing candidates able to inhibit human Carbonic Anhydrases (hCAs)(3).

**Methods**

The training datasets were built using hCAs bioactivity data from ChEMBL (release 26), with the use of flexible bioactivity thresholds resulting in well-balanced inactive and active classes. ML models were built to assess hCA activity profiles at given activity thresholds using 118 molecular descriptors. Python Scikit-learn modules were used to build, fine-tune and validate all ML models. The Accuracy and Matthew Correlation Coefficient (MCC) metrics were used to evaluate performances.

**Results**

A total of 360 models were built for three hCA isoforms, employing 10 different classification algorithms, 12 sampling sizes, and 3 feature selection methods. The best performing models were able to classify molecules activity profile with excellent performances (Accuracy > 90% and MCC > 67%). Remarkably, results proved to be better than those obtained by other traditional approaches. Furthermore, this approach can be applied in ultra-fast screenings of commercial databases for experimental testing.

**Conclusions**

In conclusion, results show data-driven in silico approaches can be efficiently used for drug repurposing (3).

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## PI3K/AKT/MTOR INHIBITION TO SENSITISE FLT3-ITD ACUTE MYELOID LEUKAEMIA (AML) CELLS TO TARGETED THERAPY USING RECEPTOR TYROSINE KINASE (RTK) INHIBITORS

**Background**

Acute myeloid leukaemia (AML) has a very poor 5-year survival of ~20% in Europe. The internal tandem duplication (ITD) mutation of the Fms-like receptor tyrosine kinase 3 (FLT3) (FLT3-ITD) is the most frequent mutation (~25%) in normal karyotype AML. In recent clinical studies, few patients display prolonged remissions with receptor tyrosine kinase (RTK) inhibitors, such as FLT3 inhibitors (FLT3i) therapy, highlighting a substantial unmet need for novel effective treatment. Persistence of leukaemia stem cells (LSC) drive AML leukemogenesis, responsible for drug resistance and disease relapse following conventional chemotherapy. Growing evidence recognizes that FLT3-ITD mutation leads to the constitutive activation of FLT3 kinase and its downstream pathways, including PI3K/AKT/mTOR signalling, strongly associated with LSC survival and crosstalk between LSC and stromal cells associated bone marrow (BM) tumour environment (TME). The TME provides protection of FLT3-ITD AML cells against FLT3 inhibitors. Thus, the PI3K/AKT/mTOR pathway may represent as a putative target for FLT3-ITD AML. In this study, we wish to test our hypothesis that PI3K/AKT/mTOR inhibition could sensitise FLT3-ITD AML cells to RTKi-lead targeted therapy using human AML cell lines and primary patient blasts.

**Objectives**

- Confirm the phenotype of FLT3-ITD versus FLT3 wildtype (wt) AML cell lines and primary patient blasts following treatment with PI3K/AKT/mTOR inhibitors and FLT3 inhibitor.
- Evaluate the efficacy of combination therapy of FLT3 inhibitor with PI3K/AKT/mTOR inhibitors in FLT3-ITD AML cell lines and primary patient blasts.
- Assess the contribution of bone marrow stromal cells (to mimic TME conditions) on protection of FLT3-ITD AML primary patient blasts against FLT3 inhibition and evaluate whether PI3K/AKT/mTOR inhibition potentiates the efficacy of FLT3 inhibitors.

**Methods**

Human AML cell lines MOLM-13 cells (FLT3-ITD) and THP1 cells (FLT3 wt) are cultured in RPMI 1640 medium supplemented with 10% FBS and 1% L-glutamine. Cells were treated with a range of selected concentrations of drugs (pan-PI3Ki BAY-806946 and FLT3i quizartinib) around their respective IC50 for 48h to assess cell growth, cell cycle state and apoptosis. Cell growth was measured by resazurin-based assays. Briefly, cells

were incubated with resazurin for 4 hr before fluorescence signal was measured at 590nm using a plate-reader (MDC SpectraMax Gemini XS). Cell cycle state and apoptosis was determined using flow cytometry PI/RNase staining and Annexin V/DAPI staining. Combination indices (CI) were calculated using CompuSyn. FLT3-ITD AML patient-derived xenografts (PDX) and primary patient samples were co-cultured with/without MS-5 stromal cell line in StemSpan medium supplemented with recombinant human cytokines G-CSF, IL-3 and TPO (1ng/mL). Following overnight incubation to allow niche interactions, cells were treated with BAY-806946 and quizartinib alone or in combination for 48h. Cell viability of the bulk AML blasts, LSC-like (CD34+CD38-) population was assessed by flow cytometric analysis using cell surface markers and Annexin V/DAPI staining. Finally, cell growth was assessed using counting beads for flow cytometry.

**Results**

Combination of quizartinib (0.72nM) with BAY-806946 (110nM) at respective IC50 concentration displayed synergistic inhibition of cell growth (78.5%) in MOLM-13 cells compared to quizartinib alone (45.0%) (n=4, p=0.0005, unpaired t-test). Assessment of cell cycle state indicated enhanced G1 cell cycle arrest, where G1/G2SM ratio was 1.9 for quizartinib, 1.7 for BAY-806946 and 9.0 for combination (n=4, p=0.0274, unpaired t-test). Combination treatment also showed enhanced induction of apoptosis compared to quizartinib alone (14.0% for quizartinib, 13.8% for BAY-806946 and 47.9% for combination) (n=4, p=0.0033, unpaired t-test). At the protein expression level, following 2h drug treatment, combination treatment did not further enhance inhibition of p-AKT, p-ERK, and p-rpS6 levels, indicative of PI3K/AKT/mTOR and FLT3-ITD AML signalling. Furthermore, we observed following 24h drug treatment, reactivation of AKT and ERK/MAPK but sustained inhibition of mTOR signalling (n=4). Further evaluation of our drug combination in PDX FLT3-ITD AML samples co-cultured with MS-5 stromal cells, revealed that quizartinib (5nM-5µM range) inhibits cell growth in a dose-dependent manner (35% inhibition with 5µM quizartinib respective to vehicle control; n=2, p=0.65, unpaired t-test) and is not apoptotic.

**Conclusions**

In summary, I have shown in MOLM-13 cells that combination treatment of quizartinib and BAY-806946 exerts enhanced inhibition of cell growth. This effect was mainly caused by G1 cell cycle arrest and induction of apoptosis. At the protein expression level, combination treatment did not further improve pathway inhibition, but displayed sustained inhibition of mTOR signalling. Evaluation of quizartinib in FLT3-ITD AML PDX samples has shown inhibition of cell growth but quizartinib was not apoptotic. We are currently evaluating the effect of combination treatment in our co-culture system.

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## THE ASSOCIATION BETWEEN COLORECTAL ADENOMAS, METABOLIC SYNDROME, ANXIETY AND DEPRESSION: PRELIMINARY RESULTS OF A CROSS-SECTIONAL STUDY AMONG OUTPATIENS ACCORDING TO A PSYCHO-NEURO-IMMUNO-ENDOCRINOLOGICAL (PNEI) PERSPECTIVE

**Background**

Many studies have suggested the association between Colorectal Cancer (CRC), Metabolic Syndrome (MetS) and psychiatric disorders, especially anxiety and depression. All these conditions could be related to an impaired quality of life, through still unclear mechanism. A chronic low-grade inflammation state could mediate these associations, according to the psycho-neuro-endocrine-immune (PNEI) perspective.

**Objectives**

Primary aim was to measure prevalence and association between colorectal adenomas, MetS and anxious-depressive symptoms in outpatients. Secondary aim was to investigate the possible role of unhealthy lifestyle in these conditions. Third aim was to assess the possible role of chronic and subclinical inflammation as a mediator of these conditions.

**Methods**

In this cross-sectional study, prevalence and associations between colorectal adenomas, MetS, anxious-depressive symptoms, unhealthy lifestyle and inflammatory markers were measured among outpatients of both gender, aged between 18 and 80 years, referred to Modena University Hospital for colonoscopy because of either a history or new evidence suspicious for colorectal neoplastic disease (e.g. positive fecal occult blood), or non-specific abdominal symptoms. For each enrolled patient, demographic (age, gender) and anthropometric characteristic (height and waist circumference in centimetres, body weight in kilograms) were collected. BMI (body weight in kilograms/height in metres2) was then calculated manually. Systolic and diastolic arterial blood pressure (mmHg) were measured by means of a manual sphygmomanometer at the right arm. Dichotomous data (yes/no) on daily alcohol consumption, smoking habit and sedentary lifestyle were also collected. Blood samples were drawn before performing colonoscopy, immediately processed for serum extraction by centrifugation and stored frozen at -80°C until test execution. The following biochemical analyses were performed: high sensitive (hs) CRP (mg/L); glycaemia (mg/dL), total cholesterol(mg/dL), low-density lipoprotein (LDL) cholesterol (mg/dL), high-density lipoprotein (HDL) cholesterol(mg/dL), very low-density lipoprotein (VLDL) cholesterol (mg/dL), triglycerides (mg/dl), serum cytokines (IL-1, IL-6, TNF-a, IL-10, Kynurenine). MetS was diagnosed according to the International Diabetes Federation criteria (2005). Symptoms of anxiety and depression were measured by means of the Hospital Anxiety and Depression Scale

(HADS). Descriptive statistics were performed as means, frequencies, standard deviations and ranges. Correlations analysis between variables were measured via the Pearson' coefficient. Statistical significance was set at $p<0.05$. Statistical analysis was performed with the software STATA 14.2 (College Station, TX, USA).

**Results**

Eighty-eight patients were recruited (M/F 44/44), with a mean age of 61.9 ± 9.8 years. The mean BMI of the sample was 27.1 ± 4.8 kg/m2; mean Systolic Blood Pressure was 144.9 ± 18.2 mmHg; mean Diastolic Blood Pressure was 83.5 ± 10.2 mmHg. The mean level of blood glycaemia was 94.9 ± 18.9 mg/dl; mean total cholesterol was 200.2 ± 30.8 mg/dl. Thirty-seven participants were affected by at least one colorectal adenoma (42.1% of the total sample). MetS was diagnosed in 40 participants (45.5% of the sample). Clinically significant anxiety and depressive symptoms were detected in 20 (22.7%) and 18 (20.4%) subjects; 9 (10.2%) patients suffered from combined anxiety-depressive symptoms. Adenoma correlated to male sex ($r=0.25$; $p=0.01$), BMI ($r=0.21$; $p=0.04$), CRP ($r=0.41$; $p<0.01$) and alcohol use ($r=0.21$; $p=0.04$). MetS correlated with age ($r=0.30$; $p<0.01$), CRP ($r=0.45$; $p<0.01$), symptoms of depression ($r=0.24$; $p<0.01$) and sedentary lifestyle ($r=0.33$; $p<0.01$).

**Conclusions**

Hints of evidence were collected about the hypothesis of a link between a proinflammatory status, psychological traits, unhealthy lifestyle, increased mucosal inflammation and metabolic parameters in a sample of outpatients screened for adenomas. Our preliminary results suggest that the integrated PNEI approach could help clinicians in the management of these multimorbid patients. Such a comprehensive assessment could be further developed in future projects with larger samples.

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**EFFECTS OF DYSMETABOLISM ON CARDIOVASCULAR SYSTEM: 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.

Moreover, MS can also lead to organ fibrosis, mainly in cardiac and renal parenchyma, being associated with adverse long-term outcomes. However, data relating to this process in pregnancy are limited.

In our clinical project we previously demonstrated that a lifestyle intervention constituted of a hypocaloric, low- glycemic, low-saturated fat caloric restriction associated to a constant PA, started early in pregnancy, could be a strategy to limit the detrimental role of dysmetabolism related to obesity and MS in the delicate "fetal programming" process by improving body composition in MS women at term of pregnancy and reducing the rate of large for gestational age infants. Moreover, we hypothesize that the supplementation with natural insulin sensitizing compounds, specifically inositols (INOs) will improve metabolic and cardiovascular profile during pregnancy and reduce maternal organ fibrosis.

**Objectives**

1. The primary objective is to evaluate whether a combination of insulin sensitizer compounds myoinositol and D-chiro-inositol (INOs) will synergistically improve maternal cardiovascular and metabolic profile in pregnant mouse models of MS and obesity.
2. The secondary objective is to evaluate the effect of MS on maternal markers of fibrosis, and to assess INO supplementation effects.

**Methods**

Female heterozygous endothelial nitric oxide synthase–/+ mice with moderate hypertension were placed on a high-fat diet for 4 weeks to induce a MS phenotype. Similarly, wild-type C57BL/6 mice were placed on a high-fat diet for 4 weeks to induce a murine obesity model. Mice were then bred with wild-type males. On gestational day 1, dams were randomly allocated to receive either a mixture of myoinositol/D-chiroinositol

in water (7.2/0.18 mg/mL, respectively) or water as control. At term (gestational day 18), maternal weights, systolic blood pressure, and a glucose tolerance test were obtained. Dams were then sacrificed, pups and placentas were weighed, maternal blood and tissues collected. Serum levels of metabolic biomarkers relevant to diabetes and obesity (ghrelin, gastric inhibitory peptide, glucagon-like peptide 1, glucagon, insulin, leptin, resistin) were measured by a multiplex enzyme-linked immunosorbent assay. Analysis was done comparing MS INOs–treated vs MS control mice and obese-INOs–treated vs obese control mice.

Maternal cardiac, renal and liver tissues were stained with Masson's trichrome to assess connective tissue deposition. ELISA was used to measure serum level of TGF- b, a fibrogenic biomarker; PICP and PIIICP, biomarkers of collagen types 1 and 3 synthesis, respectively. For the maternal fibrosis experiment, WT and eNOS-/+ dams on regular control diet were used as control. One-way or two-way ANOVA used for statistical analysis.

**Results**

Mean systolic blood pressure was lower in MS pregnant mice treated with INOs compared with control (CTR). MS mice treated with INOs showed lower glucose values during the glucose tolerance test and in the area under the curve (INOs: 17512.5 ± 3984.4 vs CTR: 29687.14 ± 8258.7; P = .003), but no differences were seen in the obese pregnant mice. Leptin serum levels were lower in the MS INOs–treated mice compared with the CTR group (INOs: 16985 ± 976.4 pg/dL vs CTR: 24181.9 ± 3128.2 pg/dL, P = .045). No other differences were seen in any of the remaining serum metabolic biomarkers. Maternal weight gain was not different in the pregnant MS dams, whereas it was lower in the obese INOs–treated dams compared with the CTR group (INOs: 10.9 ± 0.5 g vs 12.6 ± 0.6 g, P = .04).

Percentage of cardiac and renal fibrosis was higher in obese and MS dams vs CTR dams and reduced by INOs treatment in MS dams. Liver fibrosis was higher in MS dams vs obese dams and reduced by INOs. Level of TGF-b was higher in MS and obese dams vs CTR and not affected by INOs. PICP was higher in CTR vs MS and obese dams, and not affected by INOs. PIIICP was higher in MS and lowered by INO.

**Conclusions**

INOs treatment during pregnancy improves blood pressure, glucose levels at the glucose tolerance test, and leptin levels in pregnant dams with MS but not in obese pregnant dams. In addition, INOs treatment was associated with lower gestational weight gain in the obese but not in the MS dams. MS established before pregnancy induces maternal fibrosis in cardiac and renal parenchyma during pregnancy. These tissues findings correlate with higher serum levels of TGF-b and collagen-type 3 in MS dams. In the setting of MS, maternal cardiac and renal organ fibrosis was decreased by INOs treatment.

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## THE ROLE OF EARLY METABOLIC RESPONSE IN FOLLICULAR LYMPHOMA PATIENTS: A SUBSET ANALYSIS FROM FOLL12 FIL TRIAL

**Background**

Follicular lymphoma mostly displays the characteristics of indolent lymphoma. However, in a small but significant portion of patients (about 20%), the clinical behavior reflects the features of aggressive lymphomas. This suggests the presence of a high-risk group of patients, with unfavourable outcome despite the standard chemoimmunotherapy approaches and irrespective of initial standard prognostic stratification. Among newer available prognostic tools, the length of response and metabolic response (MR) after completion of immunochemotherapy (ICT) (fPET) play a relevant role and have been confirmed with a strong correlation with both Progression Free survival (PFS) and overall survival (OS)

So far, only few data are emerging to define the role of an earlier assessment of MR during the initial ICT in FL. The use of FDG PET for the intermediate evaluation during the course of induction therapy (Interim PET) can assess chemo-sensitivity of the disease but currently it does not predict treatment success sufficiently well to enable treatment modification.

An accurate and early definition of high risk disease should be considered as a key research priority in the indolent lymphoma field to introduce the use of risk-adapted strategies aimed to increase treatment efficacy in high risk patients, and to minimize the toxicity of therapy in the low risk population.

**Objectives**

In the FOLL12 randomized trial we evaluated the efficacy of a response adapted post induction management of patients with FL responding to initial ICT. The platform included both molecular and metabolic tolls (MRD and fFDG). In a significant proportion of patients MR was also assessed as intermediate evaluation during the administration of ICT for a significant proportion of patients.

This project describes the details of early assessment of MR and its correlation with patient outcomes.

**Methods**

The FOLL12 trial enrolled 807 treatment naïve adult patients with grade 1-3a, stage II-IV, and a high tumor burden FL. Complete metabolic response (CMR) was centrally assessed at End of Induction (EOI; fPET) using the 5-point Deauville scale (DS). In this study we included only the patients for whom MR was also assessed during ICT between cycle 4 and 5 (iPET). iPET results were defined on the basis of the local report and centrally reviewed applying standard DS. The primary endpoint was 3-year Progression Free Survival (PFS).

**Results**

iPET was performed in 211/807 patients enrolled in the trial and local report was available in 186 cases.
48% percent of patients were older than 60 years and 37% had a high-risk disease according the clinical FLIPI2 score. Based on local report, iPET was considered positive in 38/186 patients (20%). iPET and fPET were both available for comparison in 174 cases. Regarding the 38 iPET+, a fPET- was achieved in 23 cases (68%). In univariate able analysis the 3-year PFS was lower for the iPET+ patients compared to the iPET- (52% vs 87%: HR of 2.73 95%CI 1.51 – 4.95). Considering both iPET and fPET, a positive iPET was associated with an increased risk of progression also if a negative PET at the end of induction was achieved (HR 2.09: 95% CI3.22 – 19.5). iPET was also associated with a different 3year OS rate (99% vs 89% for iPET – vs +; p=0.035). In multivariate analysis the prognostic role of iPET for PFS was confirmed (HR 2.60 (1.41 – 4.79) and was independent from FLIPI2 (0-2 vs 3-5 HR 1.88 (1.05 – 3.35)), and for chemotherapy regimen administered (RB vs R-CHOP HR 1.39).

**Conclusions**

Interim metabolic response is confirmed with a strong prognostic role for PFS in patients with advanced stage FL treated with standard ICT. Considering the higher rates of iPET+ cases compared to fPET, iPET may better contribute to anticipate the identification of FL patients at different risk of progression and might be used to define a novel generation of response adapted trials in FL in a context of precision-medicine.
This abstract was also selected for e- poster presentation at 16 ICLM, International Conference on Malignant Lymphoma that will take next June.

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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 II is an X-linked recessive disorder caused by lysosomal enzyme iduronate-2-sulfatase (I2S) deficiency. The lack of I2S causes glycosaminoglycans (GAGs) such as chondroitin sulfate (CS), dermatan sulfate (DS) and heparan sulfate (HS) to accumulate in all body tissues, causing organs abnormalities. In severe cases, this leads to death during the teenage years. In MPS II, enzyme replacement therapy (ERT) appears to be the most effective treatment. Patients treated with ERT show clinical improvement. However, ERT is unable to cross the blood-brain barrier and shows several limitations. 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.

**Objectives**

This project is focused on GAGs qualitative and quantitative characterization in a murine MPS II model (I2S 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.

**Methods**

GAGs were extracted from plasma, urine and liver samples of 225 mice, according to a standardized protocol. The crude GAGs fraction was digested with specific enzyme to isolate HA, CS, DS and HS disaccharide units. Disaccharides were lyophilized and tagged with fluorophore. Finally, derivatized disaccharides were separated and quantified by capillary electrophoresis interfaced to laser induced fluorescence.

**Results**

Compared to wild type (WT), the I2S KO samples of plasma, urine and liver, showed from the first weeks significant differences for all the analyzed parameters. In these subjects, CS-DS, HS and HA concentration, as well as total GAGs concentration, increased significantly compared to WT subjects and remains high during all the analyzed weeks. We observe, higher GAGs concentration, CS / HS imbalance towards HS, increase in charge density, and alteration in the sulfation profile. In particular, CS-DS was mainly sulfated in 2,4s position, compared to 6s position which was prevalent in WT subjects. These differences are particularly pronounced

in liver samples, suggesting a higher GAGs accumulation in solid organs. As for the therapies, we can see how ERT is able to reduce many of the altered parameters, but it is unable to restore the WT condition completely. Genistein treatment, instead, showed no significant alterations, either quantitatively or qualitatively, compared to non-treated I2S KO subjects.

**Conclusions**

Comparing the results obtained from WT mice and I2S KO mice we observe higher values of each index considered, typical manifestations of the pathological condition under study. We can also evidence how the increase in disaccharides sulfated in position 2,4s indicates an accumulation of DS in plasma, urine and liver, specific of MPSII. From these results we can propose the analysis of 2,4s sulfation index in plasma, as a useful tool in the diagnosis of this pathology. Moreover, as in MPSII patients, ERT therapy is able to reduce significantly GAGs accumulation. These findings further strengthen the coherence of this animal model to MPSII pathology. However, ERT is not able to induce total normalization of the parameters. On the contrary, genistein therapy did not show significant differences in I2S KO subject, in any of the parameters considered. Therefore, this therapy would not seem to benefit the patient. These results support the I2S KO mouse model as a good experimental model for MPSII study. In the next stage we will also analyze different organs. Furthermore, we will have more indications on the effectiveness of different therapies in reducing GAGs accumulation, providing the basis for future clinical trials on humans.

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## CIRCULATING MUCOSAL-ASSOCIATED INVARIANT T CELLS LEVEL IS ASSOCIATED WITH RESPONSE TO IMMUNE INHIBITORS (ICI) IN MELANOMA

**Background**

Treatment of metastatic melanoma has been revolutionized by monoclonal antibodies that block the activity of molecules present on the surface of activated T cells, defined immune checkpoint inhibitors (ICI). Despite observations of durable responses to ICI therapy, not all patients respond to the treatment, and attention should be paid to identifying the mechanisms at the basis of this phenomenon.

**Objectives**

T lymphocytes patrol and kill cancer cells, and this activity is exerted in particular by CD8 T cells. Our project is focused on identifying alterations that occur in CD8 T lymphocytes of metastatic melanoma patients during treatment with anti-PD1, in order to understand how and why some patients respond or not to ICI therapy.

**Methods**

The study cohort comprised 28 patients with metastatic melanoma, 17 of which were defined responders (R) whereas 11 non-responders (NR), according to the RECIST classification. Cryopreserved PBMC, obtained prior to initiating therapy (T0) after the first (T1) and second therapy cycles (T2), was studied by high-dimensional flow cytometry and single-cell RNA sequencing (scRNA-seq). A 30-parameter/28-color flow cytometry panel was optimized to broadly characterize CD8 T cell using following markers CD3, CD4, CD8, CD45RA, CCR7, CD28, CD27, CD127, CD95, CD98, CD71, CD25, HLA-DR, CD38, CD39, CXCR6, CCR4, Ki67, T-bet, granulysin, PD-1, BTLA, CD244, ICOS and viability marker live-dead. ScRNA-seq was performed on living CD8+ T cell, following by cell-encapsulation and library preparation. A 18-parameter/16-color second flow cytometry panel was used to fine characterize the phenotype of mucosal associated invariant T cell (MAIT) using CD3, CD8, CCR7, CD45RO, CD25, CD127, CD95, CXCR4, CD69, CD38, HLA-DR, CD161, TCR7.2 and promokine viability marker. MAIT cells were stimulated to detect intracellular production of granzyme (GRZM)-A, GRZM-B, TNF and IFN-γ production. *In silico* analysis was performed to confirm the presence of MAIT cells in the metastasis and primary tumor site. High dimensional data analysis and scRNA-seq analysis was performed on Rstudio v3.6.3 using custom script or using FlowJo software version 10.0.

**Results**

We found 28 clusters spanned among naïve, T stem cell memory ($T_{SCM}$), central memory (CM), effector memory (EM) and effector memory re-expressing CD45RA (EMRA). The longitudinal comparison highlights, in responder patients, an increase of proliferating EM expressing high level of Ki67, ICOS, CD95, HLA-DR, CD71, CD98, CXCR6, granulysin and CD38, both before and after first and second cycle of ICI therapy. scRNA-

seq revealed the presence of 8 clusters characterized by different transcriptome assets. Three clusters were identified as naïve, two were memory, one identified as transitional memory and the other one as mature memory. We also identified two clusters of cytotoxic cells, the first one more differentiated cells, i.e., cytotoxic EMRA, while the other one formed by activated EM. Finally, MAIT cells with homing properties were identified. At percentage level we found increasing level of activated EM cells only after two ICI cycles in responder patients. Instead, MAIT cells were higher in responders compared to non-responders, before and after ICI therapy. No other differences were reported in term of percentages across the rest of clusters. Besides, to deepen previous results, we re-analyzed the MAIT cluster. We identified two different types of MAIT cells with differential expression of genes related to T-cell activation or effector functions, suggesting their probable different role and function. Responder patients showed increasing level of MAIT cells specifically within the activated cluster, both before and after the anti-PD1 therapy. We found an increased percentage of peripheral MAIT cells and higher proportion of cells producing IFN-y and GRZM-B in responder patients, before starting therapy but not after. *In silico* analysis of public dataset revealed the presence of MAIT cells within primary and metastatic lesion and their increased level within lesions regressing after ICI. Finally, to associate our finding with clinical outcomes, we correlated the median level of circulating MAIT cells obtained using flow cytometry with the therapy response. The overall median value was 1.7%. Using this value as a threshold, we reported that patients who exhibited MAIT frequency above the threshold showed a better response to therapy ($p= 0.0363$, Log-rank Mantel-Cox test; $N<1.7\% = 4$; $N > 1.7\% = 8$).

**Conclusions**

During the past decade, many studies have reported that CD8+ T cells of melanoma patients are characterized by an exhaustion phenotype, and that ICI therapy reactivates the immune system. Despite that, many patients do not respond to this treatment. We observed that ICI therapy induces the priming of CD8+ T cells, increasing cytotoxicity in responder patients, both at the gene and protein level, as demonstrated by scRNA-seq and flow cytometry analysis. The main finding of our study identifies MAIT cells as positive predictive marker of clinical responses to ICI in metastatic melanoma patients. While some aspects of these results diverge from the current literature in other cancers, the strikingly improved ICI response of patients with more than 1.7% of circulating CD8+ MAIT cells provides a strong rationale for further investigation regarding the potential role of MAIT cells in antitumor immunity. These cells were also found at relatively high levels within primary and metastatic tumor sites of responder patients, suggesting their recruitment through chemokine receptor like CXCR4 within cancer microenvironment. Overall, these data highlight that MAIT cells not only are present in high percentage in the periphery and in the tumor site of responder patients, but also show higher cytotoxic capability, producing more GRZM-B and IFN-y. Despite the small single-center cohort and the inherent bias, our results remark the potential role of MAIT cells in antitumor immunity in metastatic melanoma patients.

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## FEASIBILITY AND SAFETY OF PHYSICAL EXERCISE IN PROSTATE CANCER PATIENTS RECEIVING ANDROGEN DEPRIVATION THERAPY AND RADIOTHERAPY

**Background**

Androgen deprivation therapy (ADT) and radiotherapy (RT) increase survival in selected patients with prostate cancer (PCa). Nevertheless, ADT causes loss of bone mineral density (BMD) and skeletal muscle mass as well as alterations in body composition and cognitive function that together lead to an increased risk of accidental falls and fractures. In addition, half of the patients receiving RT suffer from chronic fatigue. Preliminary evidence suggests that physical exercise (PE) can be proposed as a valid strategy to lessen the adverse effects of ADT and RT in men with PCa.

**Objectives**

Aim 1. To conduct a systematic literature review on the effectiveness, feasibility, and safety of PE on bone health in patients with PCa receiving ADT.

Aim 2. To describe the lifestyle of Italian men with recently diagnosed PCa, with focus on their PE levels, habits and preferences. Furthermore, we also investigate the barriers and motivation to change towards healthier habits in this population.

Aim. 3. To evaluate the feasibility and safety of PE in patients with PCa receiving ADT and RT.

**Methods**

Aim 1. A systematic literature review was conducted. We searched MEDLINE, EMBASE, CINAHL, and the Cochrane Library. Eligible studies included randomized controlled trials (RCTs) investigating the effectiveness of PE programs in preventing accidental falls and fractures and/or preventing BMD loss in patients with PCa receiving ADT. The systematic review also investigated the feasibility of PE, which was measured through recruitment, retention, dropout, and adherence rates, and the safety of PE which was measured through the number, type, and severity of adverse events. The components, setting, intensity, frequency, and duration of PE programs were also extracted.

Aim 2. A cross-sectional study is currently ongoing in an Italian Hospital setting. Men newly diagnosed with PCa were consecutively invited to participate in an interview conducted either in person or by telephone and transcribed verbatim.

Aim 3. A feasibility, pilot study is currently ongoing. A single group of patients with PCa receiving ADT and RT are invited to participate in a multicomponent supervised and unsupervised PE program. Approximately 25 patients are expected to be enrolled in 24 months. The primary outcome is the feasibility and safety of the

PE program. We are also recording the number of accidental falls and fractures occurring during the intervention and at follow up as well as muscle strength, balance, cognitive function, quality of life, and symptoms of fatigue, mood disturbances, and patients' satisfaction.

**Results**

Aim 1. Nine RCTs were included, none of them focused on the risk of accidental falls and fractures. Only two trials reported beneficial effects of PE for lumbar spine, hip or femoral shafts BMD. Eight RCTs were included in the PE feasibility and safety analyses. The recruitment rate ranged from 10.9% to 73.1%, and the retention rate ranged from 71.9% to 96.1%, with more dropouts in the control group than in the intervention one (24.5% vs 16.1%). Patient adherence to the prescribed structured exercise regimen varied from 43.0% to 93.3%. Adverse events occurred in men performing football training (n.5) and resistance exercise (n.3). PE consisted of a combination of aerobic, resistance, and impact-loading exercise or in football training.

Aim 2. Patient recruitment started in September 2019. The mean age of the first thirty-four patients included was 71.6 ± 7.1 (range 50-84). Most participants (68%) reported to be physically active but 35% of them did not reach the recommended PE level. Despite that, 38% of participants would be interested in participating into exercise. Regarding eating, smoking, and drinking habits, almost all participants were not willing to change their habits, despite 62% were overweight/obese, and 50% declared themselves aware of being overweight.

Aim 3. Do to Covid-19 restriction the study has just initiated the recruiting. To date two patients were included, had completed the baseline assessments and are attending the program.

**Conclusions**

To date, the available evidence is too weak to recommend PE to preserve bone health in patients with PCa receiving ADT. Multicomponent PE or football training seem to be promising exercise modalities for bone health. However, adverse events should be systematically recorded. Future research is required to confirm these results on clinically relevant outcome measures by testing PE programs targeted to coordination and balance as well as to muscle strength. The preliminary results of the observational study suggest that half of patients newly diagnosed with PCa do not meet the recommended level of PE for cancer survivors. Nevertheless, half of the patients interviewed would be interested receiving exercise support. Helping patients to overcome barriers to PE and identify individual facilitators may allow to implement feasible and acceptable PE programs, encouraging long-term adherence to them. It may also be appropriate to develop effective strategies to promote healthy eating, drinking and smooking behaviors. The results of this study (Aim 3) will add proof of evidence to the feasibility and acceptability of PE integrated into the daily routine of patients with PCa.

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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. 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 is associated with a clinical "cholinergic" phenotype dominated by axial symptoms, early cognitive deterioration and cerebral Amyloid-β (Aβ) deposition. However, there are no studies that have analysed the correlation between axial symptoms, Aβ deposition and cognitive alterations in STN-DBS patients.

**Objectives**

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

**Methods**

At first, we are evaluating retrospectively data from 30 PD patients operated on with bilateral STN-DBS from January 2012 to December 2018. Demographic variables, PD characteristics, cognitive and clinical-instrumental data have been collected by reviewing medical records. Each patient has been reevaluated two

to seven years after surgery: axial symptoms has been studied applying a standardized clinical-instrumental approach with the contemporary analysis of speech, gait and postural parameters. Disease severity was assessed using the four parts of the Unified Parkinson's Disease Rating Scale (UPDRS) score and subscores. Each patient has been studied in different stimulation and drug conditions in order to evaluate the selective influence of the two treatments on axial symptoms: preoperative off-medication and on-medication conditions; postoperative on-stimulation/off-medication, off-stimulation/off-medication and on-stimulation/on-medication conditions (both single and dual task). 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.

**Results**

21 patients were recruited from September 2019 to March 2021 (70% males; mean age: 63 years, sd ± 5.69) with a mean follow-up after surgery of 5.1 years (sd ± 1.55 years). Comparing the three postoperative conditions, both stimulation alone and the combination of stimulation and medications led to a statistically significant improvement of global motor score and subscores. Concerning the gait analysis, both the 10-meter walk test (IWalk) and the Timed Up and Go Test (ITug) showed a similar trend with the longer duration in the off-stimulation/off-medication condition and a significant reduction in the on-stimulation/off-medication and on-stimulation/on-medication conditions meaning that both stimulation and stimulation plus levodopa statistically improved gait. We did not find significant differences between the on-stimulation/off-medication condition compared to the on-stimulation/on-medication condition meaning that the effects of levodopa on gait are limited. The dual task condition performed in the on-stimulation/on-medication condition severely affected the duration of the tests that tend to be similar to the off-stimulation/off-medication condition. On the contrary, both stimulation and levodopa had an heterogenous effect on speech parameters. We found a significant improvement of the following parameters: mean intensity of sustained phonation and spontaneous speech, maximum phonation time of sustained phonation, shimmer of sustained phonation, jitter of sustained phonation and syllable repetition. Five patients undergone [18F] flutemetamol PET and in none of them brain Aβ deposition was detected. The complete neuropsychological assessment was performed in 13 patients: in 8 of them a clear worsening of cognitive function was found compared to the preoperative values while in the remaining five patients the assessment was comparable to the preoperative evaluation.

**Conclusions**

Even if in a preliminary analysis, our data highlights that STN-DBS could improve both motor scores and gait parameters in the long-term after surgery, with mixed effect on speech parameters.

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## IN VITRO MODULATING EFFECT OF TAUROLIDINE AND EDTA AS PERITONEAL CATHETER-LOCKING SOLUTIONS ON BIOFILM FORMATION AND SECRETORY PROFILE OF PSEUDOMONAS AERUGINOSA

**Background**

Pseudomonas (P.) aeruginosa peritonitis is the most concerning complication in peritoneal dyalisis because it is associated with relapsing infections resulting with catheter removal and temporary hemodialysis. The pathogenicity of P. aeruginosa is related to biofilm formation and secretion of virulence factors.

**Objectives**

The objectives of this study were to (i) measure in vitro antimicrobial activity of taurolidine and EDTA against planktonic cells of P. aeruginosa, (ii) evaluate in vitro antimicrobial activity of taurolidine and EDTA against planktonic and sessile cells of P. aeruginosa forming a 24- and 72-hour-old biofilm onto contaminated peritoneal dialysis catheter and lastly, (iii) verify the impact of taurolidine and EDTA on the secretory profile of P. aeruginosa.

**Methods**

Sodium calcium edetate (EDTA) (0.25%,0.75% and 2.5%) and taurolidine (0.125, 0.25% and 2%) are two non-antibiotic solutions with antimicrobial activity. These agents were tested alone or in combination against BLI-P. aeruginosa grown on segments of a sterile peritoneal dialysis catheter. BLI-P. aeruginosa is an engineered strain of P. aeruginosa. Bacterial concentration was measured in CFU/ml afterward the conversion of the bioluminescence signal measured at 595 nm using the spectrophotometer

The effects of EDTA and/or taurolidine were investigated on P. aeruginosa growth on segments of peritoneal dialysis catheter after the incubation with a lock solution lasting 24 hours.

**Results**

Taurolidine showed its potent effect in significantly reducing the viability of P. aeruginosa planktonic cells after one hour of treatment with all the tested concentrations of solutions. Conversely, EDTA had a delayed inhibitory effect on P. aeruginosa since a significant reduction of the microbial load occurred after 6 and 24 hours of treatment.

Taurolidine resulted more effectively than EDTA in reducing planktonic and sessile forms of P. aeruginosa living within 24 and 72-hour-old biofilm. This inhibitory phenomenon was transient since lock solution

removal allowed full recovery of microbial growth. Interestingly, the biofilm reorganization appeared delayed during the first phase of bacterial growth.

The secretory profile of P. aeruginosa biofilm was affected by the exposure to the two lock solutions. In particular, as established by comparing the peak areas of treated and untreated samples, the levels of four molecules, namely 3-oxo-C12-HSL, C4-HSL, PQS and IQS, decreased transiently with the use of EDTA and taurolidine, either alone or in combination

**Conclusions**

Taurolidine alone or in combination with EDTA reduced significantly the microbial load of P. aeruginosa on 24- and 72- hour-old biofilms cultured on peritoneal catheter and yielded a destructuration of the biofilm structure. This effect was accompanied by a transient change in the level of virulence factors secreted by the bacteria.

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## THE PROGRESSION OF ACTINIC KERATOSIS TO SQUAMOUS CELL CARCINOMA FOLLOWING A "DIFFERENTIATED PATHWAY": A METABOLOMIC AND HISTOPATHOLOGICAL STUDY

**Background**

Actinic Keratosis (AK) are sun-induced precancerous lesions that can progress to squamous cell carcinoma (SCC). Historically, it was proposed a step progression from AK to SCC through the "classic pathway" from AK grade I (AKI) (characterized by atypical cells localized in the basal cell layer of epidermis) to AK grade III (AKIII) (identified by atypia that involves all the layers of epidermis up to the corneum), with an intermediate passage through AK grade II (AKII) (in which cells atypia involves basal, granular, and eventually spinous layer). Recently it has been demonstrated that AK with atypical cells in the basal layer (AKI) is the most common precursor of invasive SCC, arising from a "differentiated" pathway.

Metabolomics is the global and systematic assessment of metabolites existing in a biological system, aimed at selecting potential predictive disease biomarkers that are able to provide insights into the underlying pathophysiology. Advancement of analytical technique, such as nuclear magnetic resonance (NMR), has enabled the quantitative identification of a wide range of metabolites using a small volume of sample.

**Objectives**

The main aim of this study is to evaluate the progression from AK to SCC from the histopathological and metabolomic point of view, evaluating and comparing both metabolomic profiles and histopathological features of SCCs and AKs (grade I, II and III).

**Methods**

The study was an experimental, prospective, monocentric, non-randomized, case-control trial.

Patients affected by AK and SCC and healthy controls volunteers were enrolled in the study to collect specimens for metabolomic analysis and histopathological examination. The inclusion criteria for the enrollment were: majority age and clinical diagnosis of AK or SCC confirmed by the histopathological examination. The histopathological study of AK and SCC was extended to other cases randomly and retrospectively selected.

Metabolomic analysis was made through High Resolution Magic Angle Spinning (HR-MAS) Nuclear Magnetic Resonance (NMR) spectroscopy. The whole spectral profiles were analysed through multivariate statistical analysis using MetaboAnalyst 5.0.

Histologic examination was performed on sections stained with hematoxylin and eosin and statistical analysis was performed using STATA software version 14.

**Results**

A group of 36 patients affected by AK and SCC and 10 healthy controls were enrolled for metabolomics analysis. Histopathological analysis was conducted on 170 specimens of SCC and AK (including the ones that underwent metabolomic analysis). SCCs and all AKs of different grade (I, II, III) showed a general increase in metabolite content compared to healthy controls, and moreover it turned out that the same metabolites play a crucial role for all diseases categories (SCC and all grade of AKs).

From the histopathological point of view, we found a statistically significant association between AKI and SCC. Moreover, in the logistic regression model, the presence of parakeratosis in AK appeared to be a protective factor, while AKs with hypertrophy had two-time higher risk to develop SCC.

**Conclusions**

From the results of the metabolomic and histopathological analysis of our study, emerged that all grade of AKs can have a potential of malignancy evolving into SCC. Interestingly AK I seem to be more frequently associated to SCC, therefore supporting the hypothesis of a "differentiated pathway".

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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 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 membrane receptor expression in 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, and increase of luteinizing hormone (LH) receptor (LHCGR) 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). These data suggest the capability of estrogens to modulate FSH/FSHR-dependent apoptotic signals and LH/LHCGR-dependent signalling cascades, suggesting new mechanisms regulating the selection and rescue of dominant follicles in an individual-specific manner. The interaction of those receptors may be relevant to understand pathological conditions linked to gonadotropin action such as the polycystic ovary syndrome (PCOS), characterized by altered LH signaling.

**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. Also, I will study the involvement of LHCGR/GPER expression ratio in PCOS.

**Methods**

LHCGR and GPER dimerization were analyzed in HEK293 by bioluminescence resonance energy transfer (BRET) and by proximity ligation assay (PLA). The presence of LHCGR-GPER heterocomplexes was identified by PLA on the surface of human granulosa cells (hGLC) as well. Downstream effects of LHCGR-GPER heteromers were studied in LHCGR or LHCGR-GPER transiently transfected HEK293 cells, analyzing the intracellular $Ca^{2+}$ release and the cAMP accumulation by BRET, and IP1 accumulation by homogeneous time-resolved fluorescence (HTRF). Several endpoints were evaluated in hGLCs, which were depleted of GPER expression by siRNA, such as cAMP and IP1 accumulation by HTRF, pERK1/2, pCREB and p38 map kinase activation by Western blotting and progesterone production by immunoassay. Cell viability has been

evaluated in HEK293 and in hGLC by MTT assay. All the endpoints analyzed will be studied in hGLC extracted from PCOS patients as well. In FSHR-GPER co-expressing HEK293 cells heteromers internalization and cAMP activated by endosomal compartment were evaluated by BRET and PLA in presence and absence of a dynamin-dependent internalization inhibitor.

**Results**

In previous experiments we demonstrated that FSHR forms heteromers with GPER at the cell surface of HEK293 cells overexpressing the two receptors. The role of FSHR/GPER heteromers may be relevant to inhibit FSH-induced death signals, since increasing GPER expression levels in HEK293 cells co-expressing FSHR results in displacement of the Gαs-protein to FSHR, blockade of FSH-induced cAMP production and inhibition of apoptosis, through activation of the anti-apoptotic AKT-pathway via a Gβγ-dependent mechanism. I found by photoactivated localization microscopy that GPER is capable to heterodimerize with LHCGR on the surface of HEK293 as well (cell=3) and then I confirmed these data in HEK293 by BRET ($r^2$=0.91; nonlinear regression; n=4) and PLA (cell=3). While the GPER/LHCGR complex does not affect the LH and hCG-induced cAMP production, it impedes the intracellular $Ca^{2+}$ release and IP1 accumulation in LHR-GPER co-expressing HEK293 cells upon LH and hCG compared to LHCGR-expressing cells. By PLA, I demonstrated that these heterocomplexes occur on hGLC surface as well (cell=3). Nevertheless, the treatment of hGLC with LH and hCG with or without estradiol showed no difference in terms of cAMP and IP1 accumulation and progesterone production in cells that naturally co-express LHCGR and GPER and in cells depleted of GPER expression by siRNA, but more replicates are needed to confirm the data. The western blot analysis showed an increased pERK1/2 activation and a lower CREB phosphorylation in hGLCs depleted of GPER, while no differences are observed in terms of Mapk p38 phosphorylation upon LH and hCG treatment between hGLC treated with siRNA against GPER and the one treated with siRNA MOCK. Interestingly, hGLC extracted from PCOS patients showed a higher LH and hCG-induced pCREB and Mapk p38 activation compared to normal hGLC, suggesting a stronger activation of steroidogenic and pro-apoptotic pathways. My results show that GPER is capable to impact receptor internalization as well. FSHR-GPER co-expressing HEK293 cells showed a reduced kinetic of FSHR internalization through early and late endosomes both at basal level and upon FSH treatment. Moreover, FSHR expressing HEK293 cells, pre-treated with a dynamin-dependent internalization inhibitor, showed higher and more potent FSH-induced cAMP response compared to the untreated cells. Instead, in FSHR-GPER co-expressing cells, FSH-induced cAMP response is highly inhibited both in cells pre-treated or not with the internalization inhibitor.

**Conclusions**

According to these findings, estrogens are selectively involved in the regulation of pro- and anti-apoptotic signals and receptor internalization through FSHR/GPER complexes and in modulation of LHCGR-mediated signaling cascade.

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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 phenotypes, morphological patterns, and clinical presentation. The exceeding rarity (5–10% of all lymphoproliferative disorders) and the heterogeneity of PTCLs has made extremely difficult to investigate on them, and a satisfactory understanding of their clinical pictures, response to treatment and prognosis are still awaited. 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, where the most common subtypes are PTCL not otherwise specified (NOS; 25.9%), angioimmunoblastic (AITL; 18.5%), NKTCL (10.4%), adult T-cell leukemia/lymphoma (ATLL) 9.6%, anaplastic large-cell lymphoma (ALCL) ALK positive (6.6%) and ALCL, ALK negative (5.5%). PTCLs are 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**

In 2018, the International T-cell non-Hodgkin's Lymphoma Study Group decided to launch the T-cell Project 2.0, which adapts to changes made in diagnosis, classification, staging and response evaluation, in order 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 to have a contemporary, real-time understanding of the evolving landscape of T-cell lymphoma biology and treatment, together with the application of modern technologies to further identify a new therapeutic target.

**Methods**

Consecutive patients with newly diagnosed PTCLs according to the WHO2016 classification and satisfying inclusion criteria are prospectively registered at a dedicated website via secure HTTP protocols, and followed for up to 5 years. Two-year Progression free survival has been chosen as primary endpoint.

**Results**

Since the beginning of the study on May 2018, 738 patients with newly diagnosed PTCLs were registered by 93 active centers across 14 countries. Of these data on, 694 cases have been validated by the centralized trial

office. Overall, PTCL-NOS, ALCL ALK-negative, and AITL, represent the most frequent subtypes, accounting on 31%, 19% and 13% of cases, respectively. As reported in Table 1, PTCL-NOS represents the most frequent subtype worldwide, whereas Adult T-cell leukemia/lymphoma was more frequent in Brazil, AITL and ALCL ALK-negative in Australia/India, and ALCL ALK-positive in North America and Europe. Of note, extranodal NK/T-cell lymphoma, nasal type, was relatively frequent in Brazil and quite rare in the other Latin America Countries. Finally, many sub-types represent less than 5% of cases in all geographic areas.

**Conclusions**

The TCP2.0 continues to recruit very well, despite the difficulties linked to the COVD-19 pandemic. It may represent a powerful source of data for better assessing the clinical relevance of the 2016 WHO Classification, the role of FDG-PET in staging and response assessment, the prognosis of different entities, the genomic landscape of different subtypes. Moreover, it will help to investigate on the most adequate treatment strategies for these neoplasms in the real-world setting.

**Table 1.** The most frequent histological T-cell lymphoma subtypes registered in the TCP 2.0 according to 2016 WHO Classification

| Subtypes (ICD-O code) | Total N (%) | Australia/India N (%) | Brazil N (%) | Hispanic America N (%) | North America/Europe N (%) |
|---|---|---|---|---|---|
| **PTCL, NOS (97023)** | **186 (31.3)** | **54 (32.3)** | **93 (31.4)** | **23 (33.8)** | **16 (25.4)** |
| Lymphoepitelioid lymphoma | 3 (0.5) | 0 (0.0) | 1 (0.3) | 2 (2.9) | 0 (0.0) |
| **ALCL, ALK – (97153)** | **112 (18.9)** | **40 (24)** | **53 (17.9)** | **12 (17.6)** | **7 (11.1)** |
| **AITL (97053)** | **80 (13.5)** | **27 (16.2)** | **32 (10.8)** | **8 (11.8)** | **13 (20.6)** |
| *PTCL-TFH (97023)* | 1 (0.2) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (1.6) |
| ENKTCL (97193) | 69 (11.6) | 19 (11.4) | 39 (13.2) | 3 (4.4) | 8 (12.7) |
| ATLL (98273) | 59 (9.9) | 5 (3.0) | 46 (15.5) | 6 (8.8) | 2 (3.2) |
| ALCL, ALK + (97143) | 52 (8.8) | 11 (6.6) | 24 (8.1) | 7 (10.3) | 10 (15.9) |
| HSTCL (97163) | 10 (1.7) | 5 (3.0) | 4 (1.4) | 1 (1.5) | 0 (0.0) |
| SPTCL (97083) | 10 (1.7) | 6 (3.6) | 2 (0.7) | 0 (0.0) | 2 (3.2) |
| EATL (97173) | 7 (1.2) | 0 (0.0) | 2 (0.7) | 4 (5.9) | 1 (1.6) |
| *MEITL (97173)* | 2 (0.3) | 0 (0.0) | 0 (0.0) | 1 (1.5) | 1 (1.6) |
| LGL leukaemia (98313) | 2 (0.3) | 0 (0.0) | 0 (0.0) | 1 (1.5) | 1 (1.6) |
| CLPD-NK (98313) | 1 (0.2) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (1.6) |
| **Total** | **594** | **167** | **296** | **68** | **63** |

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

**Background**

Gaucher disease (GD) is an inherited lysosomal storage disorder, characterized by deficiency of the lysosomal enzyme glucocerebrosidase (GBA) with consequent glycosphingolipids accumulation in macrophages of the reticulo-endothelial system. Hepato-splenomegaly, thrombocytopenia and bone disease represent the main features of type 1 GD. Moreover, GD patients present a peculiar metabolic profile characterized by an increased energy expenditure, peripheral insulin resistance, lipid metabolism disorders. Liver involvement is frequent, ranging from a benign condition to serious complications, such as liver fibrosis or cirrhosis. Enzyme replacement therapy (ERT) and substrate reduction therapy (SRT) have proven to be very effective on visceral and bone disease alterations and on improvement of expectancy and quality of life. Moreover, a significant weight gain in GD type 1 patients on stable ERT has been reported. So, aging GD patients, as for the general population, may be exposed to unhealthy lifestyle and highly prevalent metabolic risk factors with a potential impact on morbidity and mortality, especially on cardiovascular and liver-related complications.

**Objectives**

A few data are available about lifestyle, body composition, cardiovascular risk and liver disease burden in GD patients, thus the aims of this project are:

1. To characterize the metabolic profile and the liver disease burden in a large cohort of adult type 1 GD patients mostly on stable ERT/SRT;
2. To evaluate the role of GD severity, ERT/SRT and/or lifestyle on the metabolic profile and to identify the parameters associated with cardiovascular risk and liver disease;
3. To evaluate 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.

**Methods**

In this observational study we enrolled a cohort of adult patients with confirmed type 1 GD monitored at the Regional Referral Centre for Lysosomal Storage Diseases in Modena. The first part of this project presents a cross-sectional design (Aims 1-2). We carefully evaluated GD patients at baseline, collecting data about:

- GD severity with scores (GD1-DS3, SSI) and biomarkers (ACE, Lyso-GB1); history of splenectomy; ERT/SRT status and duration

- Anthropometric and metabolic parameters;
- Body composition assessed with DEXA (model Hologic Discovery);
- Lifestyle habits evaluated with standardized questionnaire ('three-day estimated food record' for dietary assessment, 'IPAQ' for physical activity, 'SF-36' for quality of life);
- Liver disease: abdominal ultrasound; liver transient elastometry (Fibroscan ®) for non-invasive liver fibrosis and steatosis assessment;
- Cardiovascular risk: cardiovascular risk score; carotid and cardiac doppler ultrasound

The second part of the project is a prospective longitudinal study (Aim 3). GD patients enrolled at baseline are evaluated every 6-12 months with reassessment of clinical, anthropometric, biochemical, metabolic, nutritional and lifestyle parameters and imaging data listed above, with a follow-up period of at least 2 years.

**Results**

We enrolled 22 adult type 1 GD patients. Each patient underwent a complete baseline assessment with GD severity, anthropometric and metabolic features and liver involvement. Twelve patients were males; median age was 53 [21-84] years. The majority of patients had mild-moderate disease and were on stable therapy with a median ERT duration of 109 [0-275] months. Metabolic comorbidities were widely represented: 40% was overweight/obese, 50% had arterial hypertension, 15% presented insulin-resistance and 20% fulfilled criteria for the diagnosis of metabolic syndrome (MetS). Analysis of the dietary questionnaires showed a prevalence of unbalanced and pro-inflammatory diet compared to TLC diet. In particular, most patients had an excessive intake of saturated fat, cholesterol and oligosaccharides. Still considering lifestyle habits, only 3 patients presented a high level of physical activity and 31% of patients was considered "inactive" according to IPAQ questionnaire. Interestingly, GD patients presented an increased fat mass and decreased bone and lean mass. Regarding liver involvement, 40% of patients had significant steatosis and 20% significant fibrosis. GD variables (disease severity, genotype and therapy) were not significantly associated with metabolic parameters, dietary habits and liver steatosis. Conversely, significant liver fibrosis was associated with GD severity and a short time of ERT; but, considering only GD patients on stable ERT, liver fibrosis was also significantly associated with the MetS components. Interestingly, we observed an association between unhealthy lifestyle and cardio-metabolic variables. Specifically, the level of physical activity was inversely related with BMI, waist circumference, insulin-resistance, liver steatosis and MetS. Moreover, patients with significant steatosis showed a higher prevalence of overweight/obesity, insulin resistance and MetS than the counterpart. The collection of follow-up data for the prospective part of the project is still ongoing.

**Conclusions**

Metabolic comorbidities and liver steatosis are prevalent in our cohort. These alterations seem to be mainly related to unhealthy lifestyle.

***Elia Paradiso***

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## 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. Both gonadotropins LH and FSH are necessary to ensure steroidogenesis, gametogenesis and reproduction. hCG acts during pregnancy via the same receptor for LH, the LHCGR, to stimulate progesterone production by the corpus luteum and maintaining pregnancy. In addition, gonadotropins are growth and differentiation factors, modulating cell proliferation, survival and apoptosis. The interplay between the two gonadotropins FSH and LH is complex. Follicular growth is an example of the strict cooperation between LH and FSH, indeed, the receptors for the two hormones are even co-expressed on the same granulosa cells at late maturation stages. Both hormones stimulate similar signal transduction pathways. The role of S1P 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 GPCRs, known as S1PR1-5. S1PR1 and S1PR3 are expressed in human primary granulosa lutein cells (hGLC). 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. S1PR1 heterodimerization to LHCGR/FSHR and GPER and the kinetics of LH- and hCG-mediated G proteins and β-arrestin 2 coupling to LHCGR were evaluated, as well as, the activation of related second messengers and kinases in vitro. Moreover, the role of gonadotropins-induced LHCGR internalization will be also evaluated.

**Methods**

HEK293 cell line was transiently transfected with the LHCGR-encoding plasmid, together with BRET biosensors for monitoring G proteins, β-arrestin 2 or pERK1/2 activation. Cells were treated with LH and hCG and the coupling to Gαs, Gαq, Gαi proteins and β-arrestins, as well as the cAMP, pERK1/2 and Ca2+ activation were evaluated, under native conditions or under LHCGR internalization blockade by "Dynasore". LHCGR internalization induced by gonadotropins was evaluated by BRET and Proximity Ligation assay (PLA). S1PR1 heterodimerization to LHCGR/FSHR and GPER, and intracellular signaling pathways activation were assessed

by BRET. The role of S1P/gonadotropins-dependent steroid hormones synthesis and gene expression were analyzed by immunoassay and real time PCR.

**Results**

S1P induces a potent activation of pCREB occurring even in the presence of the PKA inhibitor H-89, in hGLC, although no cAMP production was detected. Complete inhibition of pCREB occurred by blocking either S1P2 or S1P3 with the specific receptor antagonists JTE-013 and TY52156, or under PLC/PI3K depletion. S1P-dependent CREB phosphorylation did not induce steroidogenesis, is not linked to expression of genes encoding steroidogenic enzymes and pro/anti-apoptotic molecules while induced FOXO1 and the EREG expression in granulosa cells. In another experimental set, the kinetics of LH and hCG-mediated G proteins and β-arrestin 2 coupling to their receptor, and the activation of related second messengers and kinases were evaluated by BRET. hCG induces Gαs-, Gq and β-arrestin 2 coupling to LHCGR more effectively than LH, while the latter is more potent than hCG in promoting Gi coupling. Under receptor internalization blockade by Dynasore, hCG maintains similar kinetics, but not LH, which needs LHCGR endocytosis for inducing receptor coupling. These data reflect hormone-specific kinetics of downstream effector activation related to G proteins and β-arrestin 2. Indeed, LH induced a rapid (within 5-10 min) cAMP increase up to the plateau, which is achieved upon hCG treatment only after 10-20 min. Moreover, LH is more potent than hCG in activating ERK1/2 phosphorylation over 20 min (Kruskal Wallis test; $p<0.05$; N=4; LOWESS data fitting). Interestingly, the kinetic of hCG-induced intracellular Ca2+ increase depends on LHCGR internalization; the ion concentration slowly increases achieving the plateau in 150 s before fading over further 150 s, while it rapidly increases and is maintained constant in the presence of Dynasore. LH failed in inducing intracellular Ca2+ increase, consistently with weak Gq recruitment. Also, it was evaluated the interaction between LHCGR and the small protein Rab5, a marker of early endosomes used to estimate LHCGR internalization mediated by gonadotropins. hCG is more potent than LH in promoting LHCGR internalization.

**Conclusions**

I demonstrated that S1P may induce a cAMP-independent activation of pCREB in granulosa cells, although this is not sufficient to induce intracellular steroidogenic signals and progesterone synthesis. S1P-induced FOXO1 and EREG gene expression suggests that the activation of S1P-S1PR axis may cooperate with gonadotropins in modulating follicle development. It has been created a human granulosa cell model to control the expression of the receptors for LH and FSH. LHCGR internalization is fundamental for modulating LH- and hCG-specific signals, impacting G proteins and β-arrestin 2 coupling, and the downstream signaling cascades.

***Dr. Laura Turco***

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## THE ROLE OF HVPG IN THE MODERN ERA OF CIRRHOSIS: NEW ETIOLOGIES AND A TARGET PATIENT POPULATION TO EXPLORE

**Background**

Nonselective b-blockers (NSBB), by reducing portal pressure gradient (measured by HVPG), may prevent disease complications. However, all studies carried out so far combined patients with compensated and decompensated cirrhosis and were conducted in an era where the majority of patients had virus related cirrhosis. Nowadays the majority of patients have a NASH-related cirrhosis, but their portal hypertension seems not to be fully captured by HVPG, therefore HVPG may be not able to predict their decompensation.

**Objectives**

First study. To assess the benefits of NSBBs-HVPG response in patients with compensated or decompensated cirrhosis separately. Second study. To evaluate the agreement between HVPG and direct measure of portal pressure gradient (PP) in patients with NASH cirrhosis compared to those with alcohol or HCV-related cirrhosis. Third study. To examine the relationship between HVPG and hepatic decompensation in patients with NASH-cirrhosis compared to those with HCV-related cirrhosis.

**Methods**

First study. A meta-analysis with data from patients with cirrhosis included in studies that assessed outcomes in HVPG responders and non-responders divided in patients with compensated or decompensated cirrhosis. Second study. Multicenter cross-sectional study including all patients with NASH cirrhosis treated with TIPS from 2010 to 2019, matched with two controls with alcohol or HCV-related cirrhosis. Third study. Multicenter study including all patients with NASH-related cirrhosis with an HVPG measured from 2014 to 2018 compared to those with HCV related-cirrhosis in terms of etiology, HVPG and decompensation.

**Results**

First study. Data from 15 studies (1113 patients) of prophylaxis of variceal bleeding that had reported on outcomes in HVPG responders vs. non-responders to NSBB. In patients without ascites, responders had lower odds of events than nonresponders (OR, 0.35; 95% CI, 0.22–0.56) and death/liver transplant (OR, 0.50, 95% CI, 0.32–0.78). In patients with ascites, responders had lower odds of events than nonresponders (OR, 0.27; 95% CI, 0.16–0.43) and death/liver transplant (OR, 0.47; 95% CI, 0.29–0.75). Second study. Patients with decompensated NASH cirrhosis (n=40) compared with matched patients with decompensated cirrhosis due to alcohol (n=40) or HCV (n=40). Correlation between wedged hepatic venous pressure (WHVP) and PP was

excellent in the control group (R: 0.92; ICC: 0.96; p<0.001). Agreement was only moderate in the NASH group (R: 0.61; ICC: 0.74; p<0.001). At multivariate analysis, NASH etiology was independently associated with this disagreement [OR: 4.03 (95% CI 1.60–10.15); p=0.003]. Third study. Patients with NASH-related and HCV-related cirrhosis had similar age, gender, CPT and MELD. Median HVPG was lower in NASH group (13 vs 15 mmHg) beside rates of clinical decompensation and high-risk varices were higher in NASH group (32% vs 25% p=0.019 and 32% vs 27%, p=0.103). NASH group had lower HVPG (17 vs 19mmHg p=0,001) and higher prevalence of decompensation for any HVPG threshold.

**Conclusion**

Firββst study**.** Patients with cirrhosis, with or without ascites, who have NSBB induced- HVPG reduction are at lower risk for adverse events or death. Second study. In patients with decompensated NASH cirrhosis, WHVP underestimates PP. Third study. Patients with NASH cirrhosis have higher prevalence of decompensation at any value of HVPG compared to patients with HCV-cirrhosis.

# XXXV cycle

***Dr. Carolina Castro Ruiz***

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## COMPARISON BETWEEN THE TNM-AJCC 8th EDITION AND THE JAPANESE (JSCCR) GRADING SYSTEMS IN COLON CANCER

**Background**

Surgery remains the most efficient therapeutic approach in colon cancer. Its main targets are the treatment of the primary tumor, determine the lymph node status, and the treatment of metastatic disease if present. Lymph nodes (LNs) are a significant prognostic factor in predicting disease-free survival (DFS) and overall survival (OS) in patients without metastatic disease.

LN metastases are a risk factor for disease recurrence and the development of metastatic disease. Furthermore, they determine whether or not the patient should undergo adjuvant therapy. A recent study has stated that the prognosis is not only determined by the number of positive LNs but that their topographic distribution may carry an important role.

Currently, we apply the AJCC-TNM classification, in which a correct nodal sampling is based on the retrieval of at least 12 LNs regardless of their location. On the other hand, the JSCCR (Japanese Society for Cancer of the Colon and Rectum) classification takes into consideration the topographic distribution of the positive LNs. At this moment there are no studies that determine the superiority of one system over the other in terms of predicting 3-year disease recurrence and OS. Due to the important prognostic value that the LN status has, its correct staging is a largely debated argument.

**Objectives**

Primary aims:

- Applicability of the JSCCR classification to our population. The outcome is described as the percentage of cases in which this classification probes to be completely applicable (Note: for the TNM system, is by definition 100%)
- Agreement between disease stages applying both staging systems

Secondary aims:

- Evaluate if the JSCCR system can highlight recurrence risk subcategories based on the topographic distribution of positive LN's with a 3-year follow-up.

- Assess if the JSCCR system can detect a different mortality rate in subcategories based on the topographic distribution of positive LN's.

**Methods**

This is a monocentric prospective study that aims to confront these two grading systems applying both of them to the same population.

We have determined the main differences and similarities between both staging systems. We developed collaboration with the pathology department and the oncology department of our institution.

We will enroll patients with diagnosis of colon cancer in a 12-month period. We excluded patients with rectal cancer due to its different metastatic pattern and because often undergo neoadjuvant therapies, which modify the LN status. We will include patients >18 years old with diagnosis of colon cancer and CT-scan negative for metastatic disease who guaranty to continue the follow-up period in our Institution. We will exclude patients with synchronous solid tumors or oncological hematologic diseases, patients who have undergone neoadjuvant therapies or have a diagnosis of recurrent/metastatic disease.

After the extraction of the specimen, a surgeon will dissect the lymph node stations according to the JSCCR classification that will be sent to the pathology department numbered according to such classification. The pathologist will analyze the specimen and deliver an accurate report of each lymph node station. Both staging systems will be applied for each patient.

Following surgery, the patients are referred to an Oncologist that will determine whether they are suitable for further therapies otherwise he/she will begin the follow-up period.

**Results**

We started the enrollment phase on January 11th. So far we have enrolled 24 patients, 17 males, and 7 females. Tumor topographic distribution so far is as follows: cecum (6 patients), ascending colon (9 patients), left colic flexure (1 patient), descending colon (1 patient), and sigma (6 patients).

We expect to enroll 100 patients, the enrollment phase will be completed on February 2022 and the 3-year follow-up phase will begin. At the end of the enrollment phase, we will be able to answer our primary aims.

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## ANTICONVULSANT EFFECT OF NON-PSYCHOACTIVE CANNABIS SATIVA L. OILS IN A MODEL OF 6-HZ-CORNEAL STIMULATION

**Background**

*Cannabis sativa L*. is an herbaceous plant in the Cannabis genus and belongs to the family of *Cannabaceae*. It contains a characteristic class of isoprenylated resorcinyl polyketides compounds called phytocannabinoids to distinguish them from synthetic and endogenous cannabinoids. Cannabis has been cultivated for over 6000 years to treat pain and insomnia and used since the 19th century to suppress epileptic seizures. Epilepsy contributes to approximately 1% of the global disease burden. By affecting especially young children as well as older persons of all social and racial variety, epilepsy is a present disorder worldwide. Currently, only 65% of epileptic patients can be successfully treated with antiepileptic drugs. For this reason, alternative medicine receives more attention. Cannabinoids have been shown to regulate the excitability of neuronal circuits involving the endocannabinoid system and associated ligands and receptors. Recently, non-psychoactive *Cannabis sativa L*. extracts are in the focus of the latest investigation in alternative medicine, indicating a higher potency and fewer side effects than single cannabis derivatives.

**Objectives**

The aim of this project is to investigate the anticonvulsant properties of newly composed *C. sativa* extracts using the 6-hz corneal stimulation mouse model. This model is used to screen novel anticonvulsant molecules to overcome the challenge of drug refractoriness. Until now, over 200 terpenes have been identified in cannabis so far, inducing anti-inflammatory, antioxidant, and anticonvulsant activities. For this reason, one decarboxylated *C. sativa* extract in combination with the naturally occurring terpenes (K2) and one extract without the terpenes (K1) were prepared to examine their pharmacological interaction regarding a possible potentiation of their anticonvulsant properties. These extracts plus pure CBD oil were screened using a mouse model of 6-Hz corneal stimulation.

**Methods**

Sixteen male CD1 mice (Charles River, Calco, Italy) of 15-20 g body weight were used in this study. In total, 12 mice received electrode implantation to perform electrocorticographic (ECoG) recording of the induced seizures. First, we evaluated if the Cannabis extracts and CBD oil could modify the duration and severity of induced seizures. Notably, the compounds (K1, K2, CBD 4,5mg/ml in olive oil) were administered 5 times every 24h by oral gavage 60-75 minutes before the corneal stimulation. The duration of the convulsive

seizures was determined by ECoG recordings, while the severity of convulsive seizures was detected by behavioural observing using a modified Racine scale. Second, the mice were perfused immediately after the last stimulation to perform immunohistochemical staining to finally characterize the immunoreactivity of FosB/ΔFosB and p-ERK1/2. As previously shown FosB/ΔFosB and p-ERK1/2 levels were remarkably increased in the lateral amygdala and hippocampus after induced seizures.

**Results**

We analyzed duration and severity of seizures induced by 6-Hz corneal stimulation. Overall seizure duration (non-convulsive and convulsive measured in sec) appeared to last longer after the 2nd stimulation in CBD-treated mice (n=3, 295%), but to decrease after the 5th stimulation (57%) compared to the control group (n=3). Interestingly, a positive progress was found in the mice treated with K1 compound after the 5th stimulation (n=3, 48%). The K2-treated mice showed a reduction in seizure duration after the 3rd, 4th, and 5th stimulation (n=3, 82.6%, 66.7%, 78%, respectively). However, the control and CBD groups present a similar progression in increased seizure severity. Contrary, mice treated with K1 counteracted the seizure development after the first 3 days of stimulation (50%, 62.5%, 54.5%). Moreover, K2-treated mice appear to reduce the seizure duration after the 3rd, 4th, and 5th day of stimulation (45.5%, 50%, 60%, respectively).
The findings of the change in immunoreactivity of FosB/ΔFosB and p-ERK1/2 are still in progress.

**Conclusions**

Our results support the significance of *C. sativa* extracts as an anticonvulsant therapy. Combining *C. sativa* extracts with their naturally occurring terpenes indicate a wide range of treatment possibilities, which need to be further explored. Future outcomes will help to understand the neurophysiological interaction between phytocannabinoids and terpenes with diverse excitatory and inhibitory receptors and changed levels of immunoreactive markers.

***Dr. Alessandra Odorici***

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## Antimicrobial effects of microRepair and probiotics over oral microorganisms

**Background**

The microRepair consists of laboratory-created carbonate-hydroxyapatite-zinc crystals. Thanks to the presence of free phosphate and calcium ions, the biomimetic hydroxyapatite of the microRepair interacts with tooth's hydroxyapatite, persistently binding to it, penetrating enamel's lesions, repairing and remineralizing the tooth. In addition, the microRepair releases zinc ions, which are known to exert antibacterial activity and to restore pH towards physiological levels. Furthermore, in the last decade, the use of probiotics to improve oral health has been increased considerably; in particular, Lactobacilli and Bifidobacteria have been found to counteract the growth of pathogens and to reduce the formation and persistence of oral biofilms as well as.

**Objectives**

The aim of this in vitro pilot study was to evaluate the effectiveness of microRepairs combined with selected probiotics in counteracting microbial growth and biofilm formation of oral microorganisms on abiotic surfaces such as orthodontic elastics (OE).

**Methods**

Six healthy volunteers were selected and asked to collect separately two sets of their saliva after chewing, (for 20 minutes/each), two different chewable gums, traditional gum A and probiotic-containing gum P. The donors repeated their saliva donation in 3 successive sessions every 2 weeks. At the end, the two series of collected salivary samples were pooled to obtain Saliva A and Saliva P, that were subsequently used for the in vitro studies on orthodontic elastics (OE). Accordingly, the OE were contaminated with Saliva A or Saliva P by 1 h incubation at 37 °C, washed and further incubated for 23 h at 37 °C; then, each OE set was divided into two subgroups, one exposed to treatment with a supernatant conditioned by microRepair toothpaste (Tp-SUP) and the other with saline solution. Furthermore, the OE contaminated and treated or not treated with the Tp-SUP were incubated for up to 48 h. At time 0 h, 24 h and 48 h, several parameters were tested, including microbial load, adhesion to OE, formation and persistence of biofilm.

**Results**

Initially, the microbial load was found to be qualitatively and quantitatively similar in both saliva pools, the most represented bacterial species being *Streptococcus mitis*/*oralis* (67% Saliva A and 81% Saliva P). The levels of biofilm were lower on the OE exposed to Saliva P compared to Saliva A. Tp-SUP treatment drastically reduced the biofilm persistence, regardless of the saliva used for OE contamination. Notably, *Streptococcus mitis*/*oralis* predominated before treatment with Tp-SUP, while Lactobacillus spp overgrew after treatment in both Saliva A and Saliva P-contaminated OE.

**Conclusions**

Through this in vitro study we established that the microRepair biomimetic hydroxyapatite and probiotics profoundly influence the behavior of oral microorganisms by reducing their adhesion to abiotic surfaces and compromising the formation/persistence of biofilms. They also seem to promote the replacement of potential oral pathogens with beneficial microorganisms for an oral health.

FUTURE PERSPECTIVE: Thanks to these *in vitro* data, our future goal will be to select oral disease predisposed patients (i.e., diabetic individuals) and evaluate by the time the effectiveness of hydroxyapatite and probiotics administration in rebalancing the oral microbiota and reducing the risk of tooth decay and periodontal disease.

***Dr. Silvia Faccioli***

*CEM Curriculum: Translational Medicine*
*Tutor: Dr. Francesco Lombardi*

## POSTURAL MANAGEMENT TO PREVENT HIP LUXATION IN QUADRIPLEGIC CEREBRAL PALSY CHILDREN: COMPARING TWO APPROACHES IN A RANDOMIZED CONTROLLED TRIAL

**Background**

Cerebral palsy (CP) is the most common motor disability in childhood (prevalence 2-2.5/1000). CP children have increased risk to incur in progressive hip luxation. More severe non ambulant quadriplegic patients, classified as Gross Motor Function Classification System (GMFCS) 4 and 5, appear to be the most affected. Surgery is suggested in case of advanced luxation, but considering connected risks and burden, and high rate of recurrence, it is crucial to identify an adjuvant approach in these patients. General postural management, is recommended to prevent secondary deformity, and applied as usual care, nonetheless there is a lack of evidence about its role on hip luxation. Encouraging results have been reported, keeping children in a sitting position with the hips abducted, in order to reach the femoral head centering in the acetabulum.

**Objectives**

The aim of our study is to verify if keeping a sitting position centering femoral heads is more effective than usual postural management (sitting with symmetric trunk and pelvis), in preventing hip luxation in quadriplegic non-ambulatory CP children.

**Methods**

The study has been approved by the Area Vasta Emilia Nord Ethics Board (July 2020) and the protocol has been registered on ClinicalTrials.gov Register (ClinicalTrials.gov Identifier: NCT04603625). It is a multicenter randomized trial, involving thirteen Italian sites. Inclusion criteria are: quadriplegic CP, age 1-6 years; GMFCS 4 or 5; Migration Percentage (MP) <41%. Exclusion criteria are: anterior hip luxation, hip muscles' contractures, soft tissue surgery in the previous 12 months, previous reconstructive surgery. After recruitment, patients are randomized to usual or experimental sitting, that is required to be used at least 5 hours a day, for 2 years. The primary outcome will be the degree of luxation, measured by means of the MP, on pelvic radiography, at 12 and 24 months. Secondary outcomes will include compliance and Health Related Quality of Life (HRQoL), using validated tools, hip pain, device cost, MRI lesions, concurrent spasticity treatments (botulinum toxin and baclofen) and physiotherapy.

**Results**

Covid-19 emergency induced a relevant delay. An amendment has been requested, to extend the recruitment phase to December 2021. At present 28 patients have been recruited.

Preliminary results at 12-months evaluation will be available next year.

***Dr. Cecilia Botti***

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## THE EXTENT OF VESTIBULAR INJURY IN SUDDEN SENSORINEURAL HEARING LOSS

**Background**

Sudden sensorineural hearing loss (SSHL) is the loss ≥30 dB in three consecutive frequencies. The most widely acknowledged theories for explaining the pathogenesis of SSHL implicate vascular ischemia and viral infections. However, the exact mechanism underlying SSHL remains elusive. Several prognostic indicators of favourable or unfavourable outcome in SSHL have been proposed: patient's age, the interval between the onset of symptoms and the beginning of treatment, the presence of vertigo or tinnitus, audiometric patterns, the severity of hearing loss, the hearing level in the opposite ear, the amplitude of distortion product otoacoustic emissions, findings at vestibular evoked myogenic potentials (VEMPs). Also, abnormal caloric result (lateral canal paresis) is a significant negative prognostic factor. The importance of the assessment of vestibular function was evidenced by previous studies. While cervical and ocular vestibular evoked myogenic potentials (cVEMPs and oVEMPs, respectively) test otolith organs and their afferents, semicircular canal function can be reliably evaluated by the video head impulse test (vHIT) in all canal planes. A recent study showed that vHIT results were different in vestibular neuritis and SSHL with vertigo, suggesting different causes of vestibular neuritis and SSHL. Lateral canal function in SSHL without vertigo has been retrospectively studied by caloric test. However, to our knowledge, the function of all three semicircular canals in SSHL without vertigo has never been studied before by the means of the vHIT.

**Objectives**

The primary aim of the study is to describe the extension of macular and canal injury in patients affected by SSHL with or without vertigo, reporting results at c-VEMPs, o-VEMPS and vHIT. Moreover, the correlation between canal/macular function and the outcome of SSHL will be analysed. In particular, secondary aims are described in detail:

- To evaluate the correlation between macular and canal injury and the prognosis of hearing function;
- To evaluate the association between the patterns of inner ear injuries and ischemic vascular injuries at head MRI.

**Methods**

All consecutive patients with SSHL with or without vertigo who referred to the Otolaryngology and Audiology Units of Azienda USL – IRCCS di Reggio Emilia were consecutively recruited for one year, starting from the

approval of the local Ethic Committee Area Vasta Emilia Nord in September 2020. All patients gave informed consent for inclusion in the study. Inclusion criteria were: age ≥18 years, every sex, new diagnosis of SSHL. Exclusion criteria were: incomplete follow-up.

Patients were treated by the usual therapy and follow-up protocols, which consist in complete audio-vestibular evaluation with audiometric test, impedance, vHIT, c-VEMPs, and o-VEMPs at admission, followed by head-MRI and follow-up with audiometric examination. Demographic and clinical data (initial and final audiogram, c-VEMPs, o-VEMPs, vHIT, age, gender, comorbidity, associated symptoms, delay of treatment, ischemic vascular alterations at head-MRI) were collected. o-VEMPs, cVEMPs and vHIT results will be compared with hearing recovery and the presence of ischemic alterations at head MRI.

**Results**

Up to now, 79 patients met the inclusion criteria and were included in the study. We will complete the enrolment in August 2021. Preliminary results are reported for 75 patients who completed the audiological follow up.

Median age was 55 years (22-84). The male to female ratio was 1.14. The number of cardiovascular risk factors were ≤1 in 32.0% (24/75), and ≥2 in 68% (51/75) of patients. Previous hearing loss occurred in 25.3% (19/75), previous vertigo occurred in 28.0% (21/75). SSHL presented with associated symptoms such as vertigo (26.7%), dizziness (37.3%), tinnitus (81.3%), nausea (24%), headache (12.0%), or facial nerve palsy (1.3%). The degree of hearing loss was: mild (4/75, 5.3%), moderate (33/75, 44%), severe (23/75, 30.7%), profound (13/75, 17.3%), or complete (2.6%, 2/75). Hearing recovery was classified according to the American Academy of Otolaryngology: complete (28.0%, 21/75), partial (32.0%, 24/75), or no recovery (36.0%, 27/75). cVEMPs or oVEMPs abnormalities were found in 34.7% (26/75) and 38.7% (29/75), respectively; Both c- and oVEMPs abnormalities were present in 20.0% (15/75). Canal function was clearly altered at the vHIT as follows: lateral 14.7% (11/75), posterior 21.3% (16/75), or anterior canal 16.0% (12/75). Two canals were involved in 6.7% (5/75) (lateral canal was always involved); all three canals were altered in 4.0% (3/75). Patients with normal canal and macular function had complete recovery in 45.8% of cases, while patients with two or more canal or macular defects had complete recovery in only 12.5%. The presence of canal or macular dysfunction was associated with poorer outcomes ($p<0.05$).

A complete statistical analysis with multivariate analysis will be performed in the next months after the complete enrolment and radiological/audiological follow up of the patients.

**Conclusions**

Canal and macular injuries seem related to poorer outcomes in patients affected by SSHL. A complete functional assessment of vestibular receptors and afferents in SSHL with or without vertigo could help to better define the extension of the injury and give information about the prognosis.

***Dr. Roberto Tonelli***

*CEM Curriculum: Translational Medicine*
*Tutor: Prof. Enrico Clini*

## HOXB7 PROTEIN EXPRESSION IN PATIENTS WITH IDIOPATHIC PULMONARY FIBROSIS

**Background**

Idiopathic Pulmonary Fibrosis (IPF) is a chronic, progressive, fibrosing interstitial lung disease (ILD) of unknown etiology, with a median survival of 3 years from the time of diagnosis and no available therapies of proven efficacy. The interplay between alveolar cell regenerative deficits, tissue senescence and imbalance between pro-fibrotic and anti-fibrotic mediators is supposed to play a key role among the mechanisms governing fibrosis onset and progression. In this scenario, the myofibroblasts resulted the key effector cells of IPF, being stimulated by bFGF or TGFbeta to produce excessive extracellular matrix that increases lung structural rigidity and reduces alveolar gas exchange. Resident lung mesenchymal stromal cells (MSCs) resulted the main source of myofibroblasts, whose differentiation from stem cell progenitors was associated with Homeobox protein B7 (HOXB7) gene –a gene encoding for a nuclear transcription factor involved in cell proliferation and development– expression in non-small cell lung cancer. In this line, a potential role of HOXB7 in promoting myofibroblast proliferation from lung resident MSCs, thus favoring the onset of pulmonary fibrosis, has been hypothesized.

**Objectives**

With this prospective, proof of concept study we aimed at comparing the expression level of HOXB7 protein in patients with IPF as compared to patients without lung fibrosis. Moreover, we analyzed the correlation between HOXB7 protein expression and the severity of IPF.

**Methods**

Ten patients with established diagnosis of IPF from surgical lung biopsies were retrospectively selected among the Modena IPF Database and the related lung histological formalin-fixed and paraffin-embedded (FFPE) specimens were analyzed. Three patients with established diagnosis of lung cancer and no evidence of lung fibrosis from surgical lung biopsies served as controls. IPF severity was assessed through the Gender Age Pulmonary Function (GAP) index calculated at the time of surgical lung biopsy. Immunohistochemistry (IHC) assay with HOXB7 specific antibody was performed for all specimens and the expression level of the HOXB7 protein was quantified through Deconvolution 2 plugin on 20 different fields for each IHC image. T student test and ANOVA analysis with post-hoc Dunn multiple tests were used for comparison. The

expression level of HOXB7 protein was correlated with GAP index by means of Pearson correlation coefficient. Statistical significance was set at 0.05.

**Results**

According to the IHC quantification, the overall expression of HOXB7 intensity signal value was higher in IPF patients (median = 23259 procedure defined units [p.d.u], interquartile range [IQR] = 17566-74703 p.d.u) than in controls (median = 9784 p.d.u, IQR = 8926- 30456 p.d.u, $p<0.0001$). Eight out 10 IPF patients over-expressed HOXB7 protein as compared to the average HOXB7 intensity signal value of controls. The expression of HOXB7 protein resulted significantly associated with the severity of IPF as expressed by GAP index ($r=0.8$, $p=0.01$).

**Conclusions**

HOXB7 seems overexpressed in the lung of patients with IPF, begin correlated with disease severity. Further investigations on HOXB7 expression at transcriptomic and proteomic level are needed to clarify its role in the onset and progression of IPF.

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## ARTHROSCOPIC BANKART REPAIR WITH SOLID AND ALL-SOFT SUTURE ANCHORS: A RETROSPECTIVE COMPARATIVE STUDY OF PATIENTS' OUTCOMES AND MAGNETIC RESONANCE IMAGING FINDINGS

**Background**

Arthroscopic Bankart repair is considered the first-line surgical option in subjects with anterior shoulder instability. The solid anchors made of poly-laevo-lactic-acid (PLLA), currently used for shoulder stabilization, showed long time to complete degradation and are potential source of chondral damage and local osseous reaction; moreover, literature is lacking of studies demonstrating these implants are replaced by bone. All-soft suture anchor represents a new generation of implants developed in 2010 for the fixation of soft tissue to the bone. This device does not contain rigid components, is a coreless sleeve and suture construct, and has originally been designed to minimize complications of solid anchors.

**Objectives**

The scope of the project was to assess clinical outcomes and Magnetic Resonance (MR) findings in patients underwent arthroscopic shoulder stabilization with solid and all-soft suture anchors.

**Methods**

This was a retrospective comparative study of 37 patients (37 shoulders) (M/F:30/7, mean age: 25.8 years ± 7.1) underwent arthroscopic labral repair with solid (PLLA) and all-soft suture anchors from January 2014 to April 2018. Inclusion criteria were a preoperative diagnosis of traumatic anterior shoulder instability and a minimum 24-months follow-up. We used solid anchors in 19 consecutive subjects ("solid anchor group") (51 %) and soft anchors in 18 ("soft anchor group") (49%). Clinical outcome measures included the Italian version of the Western Ontario Shoulder Instability (WOSI) index and the Rowe score. High field MR (2.5 Tesla) was used to assess labral healing (slope and height) and anchor bone ingrowth (osseous reaction [OR] in both groups and drill hole consolidation [DHC] in solid anchor patients). A total of 58 anchors were analyzed. Preoperative vs postoperative clinical scores and any association between clinical, demographic and radiographic variables were assessed. Significance was set at $p < .005$.

**Results**

The mean postoperative follow-up was 39 ± 15 months in the solid anchor group and 41 ± 12 months in the soft anchor group. The two groups were similar for age, gender, body mass index (BMI), dominant side and number of preoperative dislocations. The preoperative and postoperative delta scores of WOSI were significantly different in both groups ($p < .0001$). Rowe score was excellent in both groups (> 90 points). The two groups showed similar values of postoperative WOSI and Rowe scores. The median values of MR variables of labral healing and anchor bone ingrowth failed to show difference in the two groups. The high values of slope (26 ±1.4 and 25.8 ± 1.5, in the solid and soft anchor group, respectively) confirm the good rate of labral healing in both groups. Low grade of of OR (grade 0-1) was found around solid and soft anchors.

**Conclusions**

Solid anchor made of PLLA and all-soft suture anchors are reliable systems for arthroscopic labral fixation. The good rate of labral healing and bone ingrowth found with all-soft suture anchors, make these devices a viable option to standard solid PLLA anchors.

***Dr. Luca Bedetti***

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## NEURO-DEVELOPMENTAL OUTCOME OF VERY LOW BIRTH WEIGHT INFANTS: AN ITALIAN AREA-BASED STUDY

**Background**

Preterm birth is a major global health problem. Although survival improved, the incidence of long-term neuro morbidities in premature infants is still high. Among extremely preterm neonates, neuro-developmental impairment affects up to 80% of children. These rates are of particular concern to both the public and professionals, and the identification of specific risk factors may lead to adopting preventive intervention measures. Therefore, it is of paramount importance that representative and recent outcome data are available. Anyway, updated data on the neuro-development of very low birth weight (VLBW) infants are scant and national networks on preterm neurological outcomes are still lacking in Italy.

**Objectives**

This study aims to evaluate the neuro-developmental outcome and risk factors for severe functional disability at 2 years of corrected age in an Italian cohort of VLBW infants.

**Methods**

During this multicentric prospective study, 9 Italian Neonatal Intensive Care Units (NICUs) joined the Neuroprem Network (a network on neurodevelopmental outcome of Italian VLBW infants). We enrolled VLBW infants born from 1st January 2016 to 31st December 2017 who completed neurodevelopmental follow-up at two years of corrected gestational age. To assess neurodevelopment, the Griffiths Mental Developmental Scales (GMDS-R) or the Bayley Scales of Infant and Toddler Development (BSDI III), and the neuro-functional evaluation (according to the International Classification of Disability and Health) were administered. The primary outcome measure was severe functional disability, defined as the presence of at least one between cerebral palsy, a BSDI III cognitive composite score <2 SD, a GMDS-R global quotients score <2 SD, bilateral blindness or deafness. Risk factors for severe functional disability (including gestational age, male gender, sepsis and periventricular-intraventricular hemorrhage) were assessed through multivariate logistic regression analysis.

**Results**

We enrolled 502 VLBW infants; 48 (9.6%) children presented severe functional disability, of which 27 had cerebral palsy (5.4%). Rates of severe functional disability and cerebral palsy were higher in neonates with a lower gestational age ($p<0.001$). Overall, 147 (29.3%) infants were referred to neuro-motor intervention. At multivariate logistic regression analysis, gestational age at birth (OR 0.79; 95% CI 0.67-0.90; p= 0.001) and periventricular-intraventricular hemorrhage (OR 2.51; 95% CI 1.19-5.26; p=0.015) were significantly associated with severe functional disability.

**Conclusions**

This study provides updated information on neuro-developmental outcomes of VLBW infants from a large Italian cohort. It confirms the higher risk of neurodevelopmental impairment in infants born at a lower gestational age and it underlines how these infants deserve special consideration during the assistance in NICU. Furthermore, these data support the planning of a national structured neonatal follow-up program.

***Dr. Sara De Vincentis***

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## MALE OSTEOPOROSIS, A STILL OVERLOOKED AND UNDERMANAGED ISSUE: AN IDENTIKIT OF PATIENTS SEEKING BONE HEALTH EVALUATION AT A TERTIARY ACADEMIC MEDICAL CENTRE

**Background**

As in women, osteoporosis is a major health and social burden in men, with an estimated lifetime risk of fracture for males aged 50 years or older between 13% and 30%. Moreover, the consequences of osteoporotic fractures in men are more severe than in women, both in terms of morbidity and mortality. Although the increasingly significant problem of bone health in men has begun to receive much more attention than in the past, male osteoporosis remains largely underdiagnosed and undertreated, even after the first fracture has occurred. Furthermore, it has been suggested that secondary causes of osteoporosis are generally underestimated in both sexes, especially in men. Among secondary forms of osteoporosis, those related to endocrine diseases, particularly androgen and/or estrogen deficiency, are of special interest since the role of sex steroids on human male skeleton has been only partly disclosed. Overall, the characteristics of men referring to health care system for bone evaluation remains partially unknown.

**Objectives**

To characterize on the basis of real-life data male patients seeking the first bone health evaluation at a tertiary academic medical center, referral for both andrological and bone diseases, over a 13-year observation period.

**Methods**

A retrospective, cross-sectional study was carried out. This cohort study consisted in recording the real-life clinical approach of male outpatients referring for the first time to the Endocrinology Unit due to bone health evaluation from 2007 to 2020. The only inclusion criteria were age > 18 years and signed informed consent, whereas no exclusion criterion was provided. The following variables were collected: age, weight, height, medication use, prior history of any fracture, parental history of fragility fractures, lifestyle habits (e.g. smoke, alcohol, physical activity) and medical history in order to rule in/out secondary causes of osteoporosis.

Osteoporosis and osteopenia were defined considering Dual-energy X-ray Absorptiometry (DXA) outcomes, according to WHO criteria: osteoporosis for T-score <-2.5, osteopenia for -2.5< T-score <-1.0, or normality for T-score >-1.0. Statistical analysis: Continuous and categorical variables were compared between sub-groups using ANOVA univariate and Chi-Square test.

**Results**

A total of 455 men (mean age 62.5+15.1years) were included: 42 aged 18-40 years, 57 aged 40-50, 79 aged 50-60, 109 aged 60-70, 122 aged 70-80, and 46 aged >80. Overall, 125 patients (27.4%) were already followed at our Centre due to endocrinological/andrological diseases that are known to increase fracture risk (94 men) or not (31 men); general practitioners and other specialists asked for bone evaluation for 226 (49.6%) and 101 (22.1%) men. DXA has already been performed for 354 patients. Prevalence of osteoporosis, osteopenia, and low bone mineral density for age were 25.9%, 26.4% and 13.2%, respectively. At least one fragility fracture has already occurred in 213 patients (46.8%), with higher prevalence in non-endocrinological than endocrinological patients (56% vs 24%, $p<0.001$). The most frequent site of fracture was lumbar spine (128 patients, 60%) followed by femoral neck alone or in combination with other sites (50 patients, 23.4%). A total of 344 patients (76%) was reported to present at least one comorbidity associated to bone loss, with higher prevalence in fractured patients compared to non-fractured ($p=0.036$). Considering fractured patients, 49 of them (23%) have never been treated with any anti-osteoporotic therapy, including calcium and vitamin D supplementation.

**Conclusions**

Male osteoporosis presents with a high rate of fragility fractures at first visit (about 50%) among patients referring to a tertiary academic medical center. The high prevalence of comorbidities associated to bone loss suggests that secondary forms of osteoporosis prevails in men, and they should be carefully investigated through an accurate anamnesis to identify patients at high risk of fracture. Most of fractured patients have not previously been evaluated by a clinician with expertise in bone diseases or properly treated, suggesting that awareness for male osteoporosis needs to be raises and reinforced in primary healthcare setting in order to prevent fractures. This disease remains still overlooked and undermanaged.

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## NOVEL NON-INVASIVE DIAGNOSTIC TECHNIQUES FOR BEDSIDE AND REAL-TIME DIAGNOSIS OF SKIN DISEASES

**Background**

Non-invasive diagnostic techniques in dermatology gained increasing popularity in the last decade. They can be, in fact, successfully used to confirm a clinical diagnosis of skin cancer and to follow up after treatment, instead of performing an invasive, expensive and time-consuming surgical biopsy. Optical coherence tomography (OCT) and reflectance confocal microscopy (RCM) have been widely used in dermato-oncology, but they have limitations, and their further applications must be investigated. Additionally, the novel device line-field confocal optical coherence tomography LC-OCT has not been systematically investigated yet.

**Objectives**

The study investigates new applications of conventional OCT as well as of the novel device LC-OCT for healthy skin, skin tumours and inflammatory skin diseases. The aims were: to evaluate novel standardized descriptors for healthy skin; to analyze allergic and irritant contact dermatitis compared to healthy skin; to describe the LC-OCT features of healthy skin, non-melanoma skin cancer, melanocytic lesions, psoriasis, eczema and bullous diseases in comparison with the gold standard histology and to assess the diagnostic sensitivity and specificity of the new device compared to the standard methods and histology.

**Methods**

Four-hundred skin lesions including healthy skin, melanoma, non-melanoma skin cancer, benign melanocytic and non-melanocytic tumours, inflammatory and bullous diseases were included in the study so far. Clinical and dermoscopic pictures were collected using Fotofinder©, RCM with Vivascope©, OCT with Vivosight© and LC-OCT with the prototype device DAMAE Medical©. When excision was indicated, the histological examination was performed. Diagnostic criteria were assessed and compared with the gold standard histology.

**Preliminary Results**

We were able to in-vivo evaluate healthy skin in a standardized manner at different time points with the new OCT parameters blood flow (BF), epidermal thickness, attenuation coefficient (AC) and skin roughness. We also described most common OCT features of acute contact allergic reactions: microvesicles, macrovesicles, coalescing vesicles, the latter useful in differentiating acute allergic from irritant dermatitis ($p<.05$). Objective

quantitative parameters correlated well with the severity grade: AC ($p<.05$) and BF at 0.2 and 0.35 mm ($p<.01$). We defined main LC-OCT features of basal cell carcinoma (BCC) and its histological subtypes: atypical keratinocytes, altered DEJ, tumour nests in the dermis, dark clefting, prominent vascularisation and white hyperreflective stroma for nodular BCCs, string of pearls pattern for superficial BCCs, shoal of fish pattern for infiltrative BCCs. We reported an overall BCC subtype agreement between LC-OCT and histology of 90.4 % (95% CI: 79.0, 96.8). The sensitivity, specificity and Cohen´s kappa values of LC-OCT for determining BCC subtypes in comparison to histology were: 96%, 96%, 0.98 (nodular BCCs), 82%, 100%, 0.88 (superficial BCCs), 100%, 98%, 0.90 (infiltrative BCCs), 91%, 95%, 0.93 (mixed BCCs). The multinomial regression with stepwise selection of variables identified following features as most useful in distinguishing BCC subtypes: epidermal thinning, atypical honeycombed pattern, prominent vessels/neoangiogenesis, shoal of fish pattern, string of pearls pattern and white hyperreflective stroma ($p<.01$).

**Conclusions**

Novel OCT-based diagnostic techniques can enrich the actual knowledge on the in-vivo analysis of healthy skin and skin diseases. Further analyses including the melanoma and skin field cancerization are needed to gain more experience in this yet unexplored field of dermatology.

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## STENTING VERSUS BALLOON DILATATION IN PATIENTS WITH TRACHEAL BENIGN STENOSIS

**Background**

Benign tracheal stenosis represents a major therapeutic challenge in patients who cannot undergo open surgery. As alternatives, two endoscopic techniques can be used to restore the tracheal patency: balloon dilatation (BA) through laryngoscopy, and tracheal stenting (ST) by rigid bronchoscopy.

**Objectives**

Purpose of this study is to compare the efficacy of BA and ST to cure benign tracheal stenosis not subjected to surgery. Secondary aim is to compare the onset rate of adverse events in the two treatment groups.

**Methods**

A retrospective, observational cohort study was carried at the University Hospital of Modena (Italy) from November 2012 to November 2017 in two single units (Diagnostic and Interventional Bronchoscopy Unit and Otolaryngology Unit). Patients were cured (primary outcome) if they did not present significant respiratory symptoms or re-stenosis in the long-term (2 years) following the procedure.

**Results**

Sixty-six patients were included (33 in BA and 33 in ST group, respectively). Unadjusted Kaplan-Meier estimates showed greater therapeutic effect of ST compared with BA at 2 years. After adjusting for confounders, stratified analyses showed that this effect was significant in patients with complex stenosis, idiopathic etiology, and degree of stenosis >70%. Compared with BA, ST showed a higher rate of adverse events.

**Conclusions**

This study shows that ST placement and subsequent removal after one year has a significant positive impact on stabilization on tracheal patency in complex benign tracheal stenosis, compared to BA technique. However, ST is burdened with a significantly higher number of adverse effects, that limit widespread use of this technique; multidisciplinary evaluation is often required. These findings warrant future prospective studies for confirmation.

***Dr. Cecilia Catellani***

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## ROLE OF MIRNAs IN PREDICTING GROWTH HORMONE (GH) RESPONSE IN CHILDREN WITH GROWTH HORMONE DEFICIENCY (GHD) AND THEIR RELATIONSHIP WITH ONCOGENESIS: GLOBAL PROFILING, IN SILICO AND IN VITRO STUDIES

**Background**

Growth hormone (GH) play a fundamental role in growth processes and metabolism. Pediatric subjects diagnosed with GHD undergo long-term GH replacement therapy at dosages that are currently not individualized. Measured growth rate does not always coincide with the expected rate and patient's basal conditions and on personal innate sensitivity to therapy. The role of GH in the regulation of cell proliferation, differentiation and apoptosis leads to consider a possible oncogenic effect. The SAGhE consortium aimed at evaluating the possible oncological risk in GHD patients who underwent GH replacement therapy. An increased risk of bone tumors has been reported in patients treated with GH in childhood. The data collected to date evidence the need for continuous surveillance. MiRNAs are regulators of gene expression, and are recognized as important regulators of biological processes such as body growth and have been extensively studied in cancer.

**Objectives**

AIM 1: to identify all circulating miRNAs varying on GH treatment using a miRNA profiling approach, and to evaluate whether they could be useful to predict the clinical outcome in terms of growth. The association between miRNA level variations (before and after treatment) and all additional recorded clinical parameters will be studied.

AIM 2: to evaluate the impact of these identified miRNAs on pathways related with cancer by using an *in silico* approach and to study the role of specific miRNAs in oncogenic processes in *in vitro* cell models.

**Methods**

AIM 1: The enrollment of 10 normal-weight, prepubertal patients with idiopathic isolated GHD (5 Males, 5 Females; CA:8.79±0.82yr; G&P:7.12±0.92; height SDS:-2.43±0.13) was performed at the Pediatric Endocrine Clinics in Reggio Emilia and Modena and it includes the collection of serum samples at two time points before the beginning of GH treatment and at 3 months on treatment. The patients were treated with GH, according to the indications of the Italian Regulatory Drug Agency (AIFA Note 39). Total RNA was extracted from serum samples (miRVana™ PARIS miRNA isolation Kit) and reverse transcribed to cDNA (TaqMan™ Advanced miRNA cDNA Synthesis Kit). MiRNA expression profiling was performed by using the TaqMan Advanced miRNA

Human Card A, which measures expression levels of 377 different human miRNAs in order to identify those miRNAs changing in response to treatment and changing by either a fold change ≥+1.5 or ≤-1.5 factor (either up- or down-regulated, respectively). MiRNAs showing a p-value≤0.05 in the 2 time points before treatment, varying independently from treatment, were excluded. The sample after 3 months on treatment was used to determine miRNAs that changed precociously on GH treatment. Then a selection of miRNAs underwent a validation step which was performed by using Taqman MicroRNA assays from RNA samples purified from serum samples from 25 prepubertal patients having isolated idiopathic GHD. Statistical analysis was performed by using GraphPad software as appropriate.

AIM 2: The selected miRNAs from the above analysis were subsequently analysed using miRNetv.2.0 platform for gene target and pathway analyses. A further subgroup of miRNAs, targeting genes involved in growth regulation and cancer, was identified. Single miRNA predicted target genes were evaluated using TargetScan.

**Results**

The profiling analysis showed that 16 miRNAs were up-regulated and 2 down-regulated. Pathway analysis showed that they were significantly involved in the regulation of 100 different pathways. Among these, the most significant were: *Oncogene-induced senescence*, *SHC-related events triggered by IGF1R*, and *Cyclin D associated events in G1* pathways. The first involved CDK6, MDM2, MAPK1, and TNRC6A genes; the second IGF1R, KRAS, and MAPK1 genes; the third CCND1, CDK6, and CDKN1A genes. These are all involved both in longitudinal growth and cancer. Specifically, CDK6/CCND1 regulate chondrocyte maturation and cell cycle progression. MDM2 increases bone mineralization and is a negative regulator of p53. MAPK1 (ERK2) and TNRC6A are involved in many cancers. IGF1R is pivotal for growth but is overexpressed in many cancers. KRAS is an oncogene mutated in RASopathies which present both short stature and increased risk of cancer. CDKN1A (p21) regulates chondrocyte development but is also altered in malignancies. Eight miRNAs were selected for the validation step based on their target genes (hsa-miR-30c-5p, hsa-miR-140-5p, hsa-miR-340-5p, hsa-miR-199a-5p, hsa-miR-335-5p, hsa-miR-494-3p, hsa-miR-22-3p, hsa-miR-106a-5p). MiR-335-5p and miR-199a-5p were validated in 25 patients, and were found to be up-regulated after treatment with respect to baseline. Interestingly, both these miRNAs are predicted to target CRIM1, a protein which interacts with Bone Morphogenetic Proteins -4 and -7 contributing to bone formation.

**Conclusions**

MiR-335-5p and miR-199a-5p change in patients undergoing GH treatment and will be analysed to verify whether they predict response to treatment. Furthermore, the possible pro-oncogenic effect of these two miRNAs will be explored in *in vitro* models of osteosarcoma and osteoblasts.

***Rexhep Durmo***

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## PROGNOSTIC ROLE OF LESION DISSEMINATION FEATURE (Dmax) CALCULATED ON BASELINE PET/CT IN HODGKIN LYMPHOMA

**Background**

Hodgkin Lymphoma (HL) is one of the most curable cancers. However, relapse or refractory disease in a subset of patients who then requires salvage therapy and treatment-related toxicity still represents unsolved clinical problems. Identifying these relapsing or refractory patients is very important to improve risk stratification and individualize treatment. Recently, the largest distance between two lesions (Dmax), a simple imaging feature measured from fluorine-18-fluorodeoxyglucose positron emission tomography/computed tomography (FDG PET/CT) scans and reflecting lesions dissemination, has been identified as a new prognostic factor in diffuse large B cell lymphoma.

**Objectives**

The aim of this study was to investigate the prognostic value of Dmax in newly diagnosed HL patients and to define interaction of Dmax with other available prognostic factors.

**Methods**

We selected a retrospective cohort of patients treated at the Hematology unit of AUSL-IRCCS Hospital of Reggio Emilia between 2007 and 2020. Available baseline FDG PET/CT scan for review and clinical data were required for inclusion. From the baseline PET images all lesions were semiautomatically segmented. The centroid of each lesion was automatically obtained and considered as the lesion location. The distances between all pairs of lesions were calculated and Dmax was obtained for each patient. Dmax was dichotomized according to the median value within our cohort. Early metabolic response (iPET) was also reviewed when possible and reported according to the five-point Deauville scale (DS). iPET was judged as a complete metabolic response (iPET-) for Deauville scores (DS) 1–3 and not complete metabolic response (iPET+) for DS 4–5. Main study endpoint was Progression Free Survival (PFS).

**Results**

We identified a study population of 215 HL patients. Median age was 39 (15-88), 43% were younger than 45 years and 45% had stage III-IV. Dmax was calculated in 184/215 patients, median value was 20 cm (range 2.6-78). iPET was available in 187/215 patients and was positive in 34 cases (18%). 42% had a IPS score more than 2. Higher DMAX values were observed for males, for patients with low serum albumin, low LDH, elevated

ESR, and high metabolic tumor volume (MTV). Median follow up was 38 months and 5-year PFS was 77% (95% CI 70-82%). In univariate analysis, IPS>2 (HR 2.20 CI 1.26-3.87, p=0.006), iPET+ (HR 3.32 CI 1.73-6.39, p<0.001) and Dmax>20 (HR 2.42 CI 1.31-4.47, p=0.005): were associated with shorter PFS. Combining Dmax with iPET we were able to show a meaningful role of Dmax in the identification of patients at different risk of progression among iPET- cases. Using iPET- and DMAX<20cm as reference groups the iPET- and Dmax >20cm had a HR of 4.16 (95% CI 1.54-11.2), and iPET+ had a HR of 6.13 (95% IC 2.18-17.2).

**Conclusions**

Dmax, a PET feature reflecting the spread of the disease, is a promising prognostic factor in HL. Combining Dmax and iPET further improves risk stratification of patients with HL and might improve tailored therapy approach.

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## GENOME-WIDE IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF CHROMATIN REGIONS ORCHESTRATING MELANOMA METASTATIC PROGRESSION

**Background**

Cutaneous melanoma is the most threatening form of skin cancer. Its morbidity and mortality are mostly associated with metastatic disease. Despite the large amount of molecular and clinical research, the molecular determinants guiding melanoma metastasization are still poorly defined.

Recently, the ENCODE project showed that more than 80% of the non-coding genome has a biochemical function and cooperates to control gene expression. It has been postulated that sequence alterations in key regulatory elements may affect their regulatory function and cause aberrant gene expression programs.

Chromatin exists in multiple functional states that are defined by precise histone modification and that correlates with gene expression. This implies that variations in gene expression can be anticipated by changes on the nature and activation status of chromatin markers in non-coding regulatory elements.

All together these findings pave the way for the study of non-coding genomic elements activity and chromatin functional status as possible predictors of gene expression variations.

**Objectives**

The aims of the project are:

- Identification ENHs aberrantly activated during melanoma metastatic progression.
- Pointing out the upstream signals converging on the aberrant regulation of these ENHs and the downstream gene expression patterns affected by these regulatory regions.
- Definition of a gene-expression based model as prognostic tool to early predict metastatic progression and improve melanoma patients' risk-based stratification.
- Investigation of the presence and clinical relevance of genetic mutations in genomic regulatory regions functionally associated with melanoma metastatic progression.

**Methods**

ChIP-seq against H3K27Ac and RNA-seq analysis on a retrospective cohort of 20 primary and 20 metastatic melanomas from the Research Biobank of the AUSL-IRCCS of Reggio Emilia were performed. Given the limited sample size, the TCGA-SKCM expression and clinical datasets were downloaded through TCGABiolinks R package to further validate our data. Unsupervised clustering, differential gene expression and PCA analyses were performed on all three datasets. Diffbind and DESeq2 pipelines were used for differential analysis on

ChIP-seq and RNA-seq data respectively. The TCGA-SKCM expression data were preprocessed to filter out genes with low coverage (total reads count lower than 10 among all samples) and falling into the rRNA and misc_RNA categories. TCGA-SKCM patients were filtered based on clinical features: only Primary Tumor (PT) and Distant Metastasis (DM) were selected and patients that underwent neoadjuvant therapy were removed. Enrichment analysis on differentially expressed genes (DEGs) from all three datasets (ChIP-seq, RNAseq and TCGA-SKCM) was performed. The ROSE algorithm will be applied on ChIP-seq data to identify a list of enhancers (ENHs) and super-enhancers (SEs) putatively involved in melanoma progression. Transcription factors (TFs) binding sites enrichment on metastasis-associated ENHs and SEs will be predicted using ReMap database and the most significant TFs will be functionally validated in paired primary and metastatic melanoma cell lines using both molecular and cell biology approaches.

**Results**

Focusing on H3K27Ac distribution in primary and metastatic samples, we observed that more than 30% of the differentially enriched peaks were mapped as distal intergenic regions, while 24% were assigned to transcription starting sites. Unsupervised clustering analysis showed that activation status of non-coding elements by means of H3K27Ac sharply discriminates primary and metastatic melanomas. Two subgroups emerged within the class of primary melanomas differentiating the thin and less invasive primary lesions (Cluster 1) from the thicker and more invasive ones (Cluster 2). Further analyses were performed comparing metastatic samples with the primary lesions from cluster 2. Differential analysis on ChIP-seq data identified 1475 differentially activated regulatory elements assigned to 1159 target genes. In order to identify a core of genes deregulated during the metastatic progression, we performed the RNA-seq on the same patients' cohort. Differential analysis on RNA-seq data highlighted 430 deregulated genes, of which 43 were in common with ChIP-seq differential bound sites-associated genes. Given the limited sample size, we carried on the previously mentioned analyses on publicly available Skin Cutaneous Melanoma (SKCM) dataset from TCGA to extend our cohort and support our results. The dataset was downloaded and pre-processed to filter out confounders, namely lymph node metastasis and patients who underwent neoadjuvant therapy, and genes with low expression. Differential gene expression analysis of DM vs PT outlined 730 genes significantly altered in TCGA-SKCM dataset. Enrichment analysis performed on deregulated genes from all three datasets highlighted pathways related with skin development, melanocytes differentiation, cell-cell junction and extracellular matrix organization as downregulated in DM samples compared with PT.

**Conclusions**

Even if preliminary, our data suggest that malignant melanocytes undergo a dedifferentiation process during metastatic spreading.

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## CHEMICAL CHARACTERIZATION OF NON-PSYCHOACTIVE CANNABIS SATIVA L. EXTRACTS AND EVALUATION OF THEIR ANTIPROLIFERATIVE ACTIVITY

**Background**

Cannabis sativa L. is an annual cycle herbaceous plant belonging to the Cannabaceae family. Cannabinoids are mainly synthesized in glandular trichomes, which are more abundant in female inflorescences. Among them, the most representative compounds are Δ9-tetrahydrocannabinolic acid (Δ9-THCA), cannabidiolic acid (CBDA) and cannabigerolic acid (CBGA). These native acidic cannabinoids undergo a spontaneous decarboxylation under the action of light and heat, leading to the formation of their neutral counterparts, including Δ9-tetrahydrocannabinolol (Δ9-THC), cannabidiol (CBD), and cannabigerol (CBG). Fiber-type C. sativa (also known as hemp) is characterized by a high content of CBD and CBG, and a level of psychoactive Δ9-THC lower than 0.2-0.3%. CBD displays several biological activities related to the action on different targets. Recently, the interest in non-psychoactive C. sativa extracts is increased due to many biological activities related to cannabinoids and other compounds.

**Objectives**

As a prosecution of the study carried out during the first year of this PhD, the project of the second year was aimed at the detailed investigation of the possible role of non-psychoactive C. sativa extracts as antiproliferative agents. To do this, it was necessary to fully characterize the bioactive compounds present in different C. sativa extracts by means of high-performance liquid chromatography coupled with high-resolution mass spectrometry (HPLC-HRMS) and to evaluate their antiproliferative activity on different cancer cell lines. The possible synergic activity of conventional chemotherapeutic agents with either the crude extract or pure CBD was also assessed.

**Methods**

HPLC-HRMS was used for the qualitative analysis of the ethanolic extracts from three fiber-type Cannabis sativa L. varieties, having a different phytochemical composition (CBD-type, CBG-type and a hybrid variety). The compounds present in the extracts were identified through their m/z and their fragmentation patterns. The complete quantitative analysis of the main compounds was performed using HPLC-UV, following a previously validated method.

The K562 human chronic myelogenous leukemia cell line, the HT29 human colorectal adenocarcinoma cell line and the U87MG human glioblastoma cell line were used for the biological assays.

The colorimetric assay based on CCK-8 was used to evaluate the effect of the extracts on cell viability. Cytofluorimetry was used for the analysis of cell cycle distribution and to clarify the mechanism of cell death using the annexin V/PI assay. The expression and activity of the main apoptosis-related proteins was investigated through western blot.

The analysis of the mechanical properties of the membrane bilayer, using liposomes, was performed to see if the treatment was able to modify membrane fluctuation.

Finally, dose-response curves were built for the associations of the CBD-type extract at 5 µg/mL with anticancer drugs currently used in therapy, including vincristine, imatinib and doxorubicin at 48 h of treatment. The same was done for the association between these therapeutic agents and pure CBD at 5 µM for 48 h.

**Results**

The decarboxylated CBD-type hemp extract was the most active in inhibiting cell proliferation and the chronic myelogenous leukemia K562 was the most sensitive cell line. The cytofluorimetric analysis of K562 treated cells revealed that the antiproliferative effect was mainly due to the induction of apoptosis, as shown by an increase of cell population annexin V positive, although at present apo-necrosis or autophagic activity cannot be excluded. The analysis of cell cycle distribution revealed that K562 cell cycle was not affected by the treatment with the CBD-rich extract. The mechanism of cell death did not involve the main pro- and anti-apoptotic markers such as p53, Bcl-2 and Bcl-xl but an activation of caspase 3 and 7 was remarkable. Both the CBD-type extract and pure CBD were able to increase membrane fluctuation without altering the phase transition of phospholipids.

As for the synergistic activity assays, the dose-response curves did not show a significant decrease of the IC50 value for imatinib and doxorubicin in association with either the extract or with pure CBD. Vincristine associated with pure CBD did not change the trend of the curve as well. Differently, vincristine associated with the CBD-type extract showed a 10 times higher efficacy then the vincristine alone. This effect was found to be specific of the CBD-type extract.

**Conclusions**

The biological assays suggested that CBD can be involved in the antiproliferative activity of the CBD-type decarboxylated extract, even if the role of other minor compounds to be identified cannot be excluded, due to possible synergistic interactions. The mechanism of action of extracts and CBD is under investigation by using both in silico and proteomic analysis, and the results will be properly validated.

The project will allow us to define an ideal composition of hemp extract as a new effective product to be used possibly in combination with existing antileukemic treatments, with the ultimate aim to increase the efficiency of standard chemotherapy.

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## GHRELIN AS A BIOMARKER OF RESPONSE TO ANTIEPILEPTIC DRUGS

**Background**

Ghrelin and des-acyl ghrelin, neuroactive peptide hormones, were recently found in higher concentrations in plasma of individuals with epilepsy who responded positively to drug treatment, comparing with non-responders and healthy controls. Due to a possible anticonvulsive role seen in animal models, different plasmatic concentrations of ghrelin and des-acyl ghrelin may actually explain different responses to AEDs.

**Objectives**

Our objective is to confirm on a larger cohort the differences seen among plasmatic concentrations of ghrelin and des-acyl ghrelin of children with epilepsy showing a positive response to AEDs treatment, comparing with non-responders and with healthy controls.

**Methods**

This is a 24-months-lasting prospective study, conducted in four neuropediatric Italian centers (Modena, Verona, Rome, and Florence). Inclusion criteria for the group of interest were: i) subject with a suspicion of epilepsy; ii) subject between 0 and 16 years; iii) the obtaining of written informed consent by parents or caregivers. Exclusion criteria were: acute or chronic metabolic disorders with rare/sporadic seizures without epilepsy. At variance, control population was constituted of patients without any suspect of epilepsy and/or acute or chronic metabolic disorders with rare/sporadic seizures. In recruited patients, we measured ghrelin and des-acyl ghrelin by immunoassays in plasma samples obtained after overnight fast at two different times: before (T0) and beyond (T1) a month after initiation of drug treatment. Demographic data, clinical features, epilepsy diagnosis, glycemia and blood level of anti-epileptic drugs were obtained and included in a multivariate statistical analysis, comparing values of ghrelin and des-acyl ghrelin in the following groups: i) patients with positive response to drug treatment ("responders"); ii) patients with drug-resistant epilepsy ("non-responders"); iii) control population. Definition of drug resistant epilepsy was considered on the basis of Berg criteria (*Berg et al., 2006*) after a minimum of 18 months after treatment initiation.

**Results**

Sixty patients with suspicion of epilepsy were recruited. Among 60 recruited patients, 13 did not started any treatment because of the development of a benign form of epilepsy in which no treatment was required. No information about the pharmacological treatment was still available for other 10 patients. Thirty-seven out of 60 pediatric patients were treated with AEDs. Diagnosis of epilepsy was confirmed at follow-up. Particularly, 29 patients had epilepsy with focal seizures, 8 had epilepsy with generalized and focal seizures, 13 had epilepsy with generalized seizures. Etiology was determined in 33 out of 50 considered patients, and was genetic in 31 and structural in 2. Thirty-seven patients started one or more AEDs, namely: 32 received only 1 drug (13 Levetiracetam, 8 Carbamazepine, 8 Valproic Acid, 2 Ethosuximide, 1 Topiramate), 4 received 2 drugs (2 Valproic Acid and Ethosuximide, 1 Carbamazepine and 1 Clobazam, 1 Levetiracetam and Vigabatrin), and 1 received 3 drugs (ACTH, Vigabatrin and Topiramate). Twenty-four patients showed a good response to the first AED and were included in the "responders" group. Three continued to show seizures despite 2 or more ASMs and were included in the "non-responders" group. In the remaining 10 individuals, treatment was started for a period not sufficient to determine the kind of response. Preliminary data show not substantial differences of ghrelin and desacyl-ghrelin levels before and after treatment onset both in responders and non-responders. Conversely, moderate differences can be observed among two groups (the statistical analysis was not yet performed because of the limited number of non-responders and the lack of the control group): The plasma levels of ghrelin in responders were 441.59 ± 91.06 pg/mL at T0 and 397.97 ± 63.68 pg/mL at T1; whereas they were 264.87 ± 113.64 pg/mL at T0 and 259.93 ± 82.01 pg/mL at T1 in non-responders. The plasma levels of desacyl-ghrelin in responders were 91.58 ± 11.84 pg/mL at T0 and 80.46 ± 9.01 pg/mL at T1; whereas they were 71.35 ± 13.23 pg/mL at T0 and 76.97 ± 13.29 pg/mL at T1 in non-responders.

**Conclusion**

Our preliminary data seem to refute the hypothesis of an augmentation of ghrelin levels beyond (T1) one month after initiation of drug treatment in children with epilepsy showing a good response to AEDs. Conversely, it seems to endorse the hypothesis of pre-existing higher levels of ghrelin in the "responders" population.

Current data needs to be implemented by:

- recruiting new participants in order to expand the study cohort;
- extend the study to a second phase, with the aim to measure ghrelin and desacyl-ghrelin levels 1 year after treatment initiation, to exclude possible delayed increasing of levels.
- recruiting a cohort of non-epileptic controls to assess ghrelin and desacyl-ghrelin levels and evaluate possible differences with two study populations.

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## SAFETY AND USABILITY OF A SELF-TRIAGE CHATBOT DURING COVID-19 PANDEMIC: DATA FROM THE COVIDGUIDE EXPERIENCE

**Background**

The covid-19 pandemic and the need to find solutions to fight Sars-CoV-2 is having a disruptive effect on apps development and e-Health. Whilst many apps and programs for Covid-19 prevention and management were based on the contact tracing, few were based on symptom checking and triage, with the possibility to predict and manage clinical features of Covid-19.

COVID-Guide, a EC certified self-triage web-app, based on the broader SMASS/SMEd software developed in Swiss, represents one of the available options. Through the interaction with his artificial intelligence (AI) it is possible to evaluate through a chatbot the combination of symptoms reported by the patient and to refer the patient to the most appropriate health care solution.

**Objective**

The aim of this part of the project is to analyse the consultation outputs produced by the COVID-Guide web-app during an evaluation period carried out to test the specific Computerized Decision Support System (CDSS) developed for web-app and to evaluate its use, usability and its outputs.

**Methods**

Preliminary data from the consultations carried out in evaluation period (May-September 2020) of the COVID-Guide web-app were extracted from the overall dataset.

A total of 103,253 consultations results were available. Data came from Germany, Switzerland, Italy, Austria, Holland, France, Belgium, England, and Ireland. However, to work on a consistent database, only data coming from Germany, which was precociously involved in data collection and evaluation and provided the highest number of consultations (93% of the total number), were used. The preliminary data obtained referred to the outputs suggested by the web-app. They were analysed with Stata / SE 16.1 and organized in output frequency tables based on the time to treat and point of care output variables produced by the web-app.

**Results**

During the period under review, the total number of consultations carried out in Germany was 96,012. The output of 3,415 consultations (3.56%) indicated the need for immediate evaluation, by suggesting to call an

ambulance or the activation an emergency service for 1,942 (2.02%) or by advising the patient to go to hospital in 1,743 cases (1.54%).

Most consultations (73,015 – 76.04%) were referred by the COVID-Guide app to an immediate (11,856 - 12.35%), deferred (3,980 - 4.14%) or within 24 hours (57,179 – 59.55%) encounter with general practitioner. Self-monitoring with an invitation to reuse the app in case of changes in the clinical picture was the output of 3,120 consultations (3.25%). The web-app was not able to produce a clear output only for 3 consultations.

**Conclusions**

Preliminary data show a widespread and good usability of this web-app, at least in Germany, and its ability to produce timely outputs coherent with the patient inputs in almost all cases. However, as its use appeared to be not homogeneous across and insides countries, further investigations are needed - notably with a qualitative methodology - to understand barriers and facilitators to the use of a self-triage app.

The regular use of COVID-Guide could have different advantages. Firstly, it could be an efficient supporting tool able to help reduce the load on operators. Secondly, it could help collect easily and quickly available epidemiological data on the spread of (suspected) Covid-19 cases in all countries where the tool will be used and therefore it could become a useful tool for Telemedical Syndromic Surveillance in the next pandemic waves.

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## IMPLEMENTATION AND FIRST EVALUATION OF A CLINICAL ETHICS SUPPORT SERVICE

**Background**

This research program proposed the development and implementation of a multidisciplinary Clinical Ethics Support Service (CESSs) and its first evaluation since 1 year from its implementation.

A CESS is an ethical intervention which aims to promote a personalized care approach by reducing conflicting situations, promoting the ability of health care professionals (HPs) to recognize and manage difficult situations and supporting decision-making in ethical complex situations.

CESSs have been widely implemented among Europe and USA, with different forms and methodologies (Rasoal et al. 2017). In Italy, the implementation of CESSs is quite rare and represent local, spontaneous and unregulated experiences (De Panfilis et al. 2019). The ethical challenges raised by the Covid-19 pandemic reinforced the need to provide concrete support to help HPs in making difficult choices and conducting painful communications (De Panfilis L et al., 2020).

On July 2020, the Local Health Authority of Reggio Emilia – IRCCS deliberated the first regional Clinical Ethics Committee (CEC). The Bioethics Unit of the Local Health Authority of Reggio Emilia – IRCCS was responsible for CEC's composition, management, activities and will be responsible for its evaluation.

**Objectives**

The objectives of this part of the research projects are:

To manage the implementation process of the CEC;

To define and understand the intervention and the research context through a study context and the update of the literature review.

**Methods**

The Medical Research Council (MRC) framework for developing and evaluating complex interventions is the methodological framework of this project. Both quantitative and qualitative methods are integrated within the framework, in order to better appraise the effects of the (complex) intervention both as a whole and on its components.

**Preliminary Results**

<u>The service</u>

The CEC was firstly deliberated by the General Directorate on July,13, 2020. The implementation process was monitored and managed by the BU. The CEC's composition and task were delineated according with data from the scientific literature (Schildmann et al., 2019) and the Recommendations of the Italian Committee for Bioethics (Italian Committee for Bioethics, Opinion of 31 March 2017). Composition and Regulations were also deliberated by the General Directorate on November, 11, 2020.

At the moment, the CEC has been meting 8 times, once a month regularly. Each meeting lasted a mean of 96 min (range: 60 to 150 min). Meeting's topics deal with: specific request of ethics consultation; ethical aspect of care related to the pandemic emergency; organization of the formative course on ethics consultation and CEC. The CEC performed 3 ethics consultation and refused one request, due to the nature of the request, regarding both research and investigation. A specific dissemination process was developed to make the CEC known both inside and outside the Local Health Authority of Reggio Emilia – IRCCS: a dedicated website was created and the CEC's President presented the CEC during Department Meetings. The CEC developed 3 policy-documents on ethical aspect of care under Covid-19, namely: the triage pandemic; the ethics of vaccination during a pandemic; the ethical aspect of visitor policy (which is actually debated by the CEC members).

Phase 0-0.1 The study context

We performed a retrospective evaluation of the BU's activities in terms of research, ethical training programs and ethics consultation, integrating quantitative and qualitative analysis. Qualitative data analysis is still ongoing, while the quantitative one has been concluded.

Quantitative evaluation: A database was created to collect quantitative data regarding: the amount of research projects promoted and implemented by the BU, the number of ethics consultations provided, the amount of hours spent among provision of training course on ethics, ethics consultation for individual HPs and care teams. The evolution of such activities from April 2016 to December 2020 was also analysed, as well as the population involved and the topics considered. A descriptive statistical analysis was performed.

The quantitative results showed a general and constant increase of the BU's activities. Since its implementation, the BU developed a total of 41 research project (17 are concluded while 24 are actually ongoing). The BU is the main sponsor for 32%. Main collaboration are with Palliative care Unit (PCU), Qualitative Research Unit (QRU), Pyscho-oncology Unit (POU), Neurology (NU). 686 hours were spent among training (36%), ethics consultation (11%) and ethics supervision (53%) especially among adult palliative care teams.

Qualitative Evaluation: Data were collected through semi structured interview. We performed a purposive sampling based on the maximum variability of participants' characteristics. The final sample included 18 participants: 2 psychologists, 1 researcher in qualitative research, 1 biologist, 1 physiotherapist, 1 speech therapist, 6 nurses and 6 physicians. Interviews last a mean of 31 min... TRUNCATED ABSTRACT

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## SFLT-1 LEVELS IN COVID-19 PATIENTS: ASSOCIATION WITH OUTCOME AND THROMBOSIS

**Background**

Coronavirus disease 2019 (COVID-19) is a worldwide emergency. The mortality rate is high, and over 50% of severe cases die due to complications. Major thrombotic accidents represent one of the most severe and frequent complications. Early recognition of high-risk patients could facilitate appropriate supportive care and reduce the mortality rate. Therefore, it is crucial to identify reliable biomarkers associated with shortened survival and thrombosis. In this research, we decided to study the association between thrombosis and endothelial damage in COVID-19 patients using markers of vascular dysfunction. These markers, currently used in pre-eclampsia, sepsis, and acute pancreatitis, include the placental growth factor (PlGF), the soluble Fms-like tyrosine kinase-1 (sFlt-1), and the sFLT-1/PlGF ratio.

**Objectives**

1. Verify if the endothelial dysfunction in Covid-19 patients was associated with the same alterations documented in sepsis, acute pancreatitis, and pre-eclampsia.
2. Identify the best biomarker to predict severe cases among sFLT-1, PlGF, and sFLT-1/PlGF.
3. Evaluate if biomarkers level elevation was associated with shortened survival.
4. Analyze if major thrombotic accidents were associated with higher biomarkers levels.

**Methods**

The study population included 105 inpatients with Covid-19 diagnosis followed to death or until recovery. A peripheral blood sample has been collected 5 to 8 days after admission to the hospital. Standard methodologies have been used to assess the common laboratory values. IL-6, sFlt-1, and PlGF levels were analyzed on the Roche Cobas e411 analyzer, and the sFlt-1/PlGF ratio was calculated. The Mann-Whitney test has analyzed the differences between categories in the distribution of continuous variables. Patient groups with nominal variables have been compared using Fisher's exact test. The area under the receiver operating characteristic curve analysis has been used to determine the best threshold for continuous variables without standard values. A significant p-value has been considered less than 0.05.

**Results**

Univariate analysis showed significant differences in the distribution of eight variables between the survivors and the deceased. In multivariable analysis only three risk factors retained significance: age (P = .018), white blood cell count (P = .022), and sFlt-1 levels (P = .003). The best threshold of these predictors of shortened survival was determined with logistic regression: sFlt-1 > 165 pg/mL; Age > 65 years; WBC > 10 000 cells/µL. The predictive accuracy of the three new binomial categories combined was evaluated with the ROC curve: AUC 0.87. To better understand the endothelial damage role, we decided to rerun the analysis using only sFLT-1 > 165 pg/mL category: AUC = 0.75, OR = 11.61 (3.74-39.67). The same threshold was used to analyze the rate of major thrombotic events during hospitalization in the study population. The results showed that elevated sFlt-1 levels are significantly associated with thrombosis (P = .020).

**Conclusions**

The results highlighted that in Covid-19 patients:

1. The severe endothelial dysfunction is similar to the one documented in sepsis, acute pancreatitis, and preeclampsia-eclampsia.
2. sFlt-1 is the most reliable biomarker to predict severe Covid-19 cases.
3. Up-regulated sFlt-1 levels represent a valuable marker for predicting progression to death.
4. Major thrombotic accidents are associated with higher sFLT-1 levels.

In conclusion, sFlt-1 is a reliable tool to monitor endothelial dysfunction in Covid-19 patients.

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## ROLE OF INNATE IMMUNITY IN SEVERE COVID-19

**Background**

COVID-19 is a novel disease caused by SARS-CoV-2 infection, triggering a complex immune response that often worsens the course of the disease. It is important to understand the pathophysiological mechanisms that are triggered by all soluble molecules and immune cells, and their contribution to the heterogeneity of COVID-19 disease. Previous data from our group indicate that a dysregulated hyperactivation of innate immune cells, including monocytes and neutrophils, leads to a persistent inflammatory status that is involved in the immunopathogenesis of severe COVID-19.

**Objectives**

The main objective of my studies is the identification of the role of innate immune cells, like monocytes and neutrophils, in triggering or maintaining the inflammatory status in different pathologies. Starting from the analysis of samples from patients with SARS-CoV-2 infection, specific objectives are:

- To dissect metabolism, phenotype, and functions of peripheral blood monocytes and granulocytes from peripheral blood from severe COVID-19 patients;
- The identify plasma molecules that can act as biomarkers indicating the degree and quality of the activation of innate immunity, including among others, cytokines and chemokines.

**Methods**

Blood from 28 patients with severe COVID-19 pneumonia admitted at the University Hospital in Modena and from 27 healthy controls was collected and processed. Peripheral blood mononuclear cells (PBMC) were isolated according to standard procedures. Plasma was collected, centrifuged, and stored at -80°C until use. Monocytes were magnetically isolated from PBMCs, and identified by mAbs recognizing CD14, CD16, HLA-DR, CD64, CD13, PD1, TIM-3, CD15, CD11, CCR2, CD38, CXCR3, PD-L1 and polychromatic flow cytometry. Mitochondrial (mt) mass was analyzed by MitoTracker green and mt membrane potential by JC-1. Oxygen consumption rate (OCR), extracellular acidification rate (ECAR), and oxidative burst were quantified by using the Seahorse XFe96 Analyzer. To measure cytokine production, PBMC were stimulated with PMA (100 ng/ml) and Ionomycin (1 ug/ml) for 4h at 37°C, then stained with LIVE-DEAD Aqua and HLA-DR, CD14, CD16, IFN-γ and TNF mAbs. The ultrastructure of monocytes and quantification of mt size and shape were obtained by Transmission Electron Microscopy. Cytokine plasma levels (IL-6, TNF, IFN-γ, GM-CSF, PD-L1, IL-18, OPN, CCL2,

CCL11, CXCL10) was measured by a Luminex platform. Flow cytometry data were analyzed by using FlowJo software version X. Statistical analysis were performed by using Prism 8.0.

**Results**

**Defective oxidative and glycolytic metabolism in severe COVID-19 monocytes.** Monocytes from COVID-19 patients display reduced basal and maximal respiration, reduced proton-leak, and reduced spare respiratory capacity and diminished ECAR compared to healthy controls. Mt mass and the percentage of monocytes with depolarized mitochondria are increased in COVID-19 patients.

**Structural rearranged monocytes, especially in their mitochondrial counterpart.** Abundant endoplasmic reticulum, Golgi apparatus and small granules are present in the cytoplasm of infected monocytes. Chromatin in the nucleus is aggregated at the periphery and mitochondria display heterogenous size with higher area, perimeter and Feret's diameter.

**Dysfunctional monocytes in severe COVID-19 patients.** Dysfunctional monocytes express low levels of HLA-DR and show a reduced capacity to perform the oxidative burst as well as the immediate response. Despite their metabolic impairment, monocytes from COVID-19 patients maintain the capability to produce cytokines after stimulation with PMA/ionomycin. Surprisingly, a dominant TNF-driven inflammatory response over an IFN-γ response is detected.

**Different distribution of monocytes subsets and immune exhaustion.** Intermediate monocytes expressing $CD14^{+}$, $CD16^{++}$, $CD38^{+}$, or $CCR2^{+}$ are increased in severe COVID-19 subjects compared to controls. Intermediate monocytes are the main sources of pro-inflammatory cytokines such as IL-6, TNF, IL-1β, IL-18, which are increased in plasma from infected patients, contributing to the cytokine storm. In all subsets of monocytes (classical, intermediate, and non-classical) inhibitory checkpoints PD-1 and PD-L1 are over-expressed. Soluble PD-L1 is also present at high level concentration in plasma of infected patients, probably promoting immune exhaustion.

**Presence of immature monocytes in peripheral blood of severe COVID-19 patients.** In COVID-19 patients, plasma concentration of several mediators involved in monocyte regulation and migration is higher, including GM-CSF, suggesting the presence of emergency myelopoiesis in these patients. Indeed, immature monocytes ($HLA\text{-}DR^{+}CD14^{-}CD13^{-}CD64^{-}$) are significantly increased in peripheral blood from COVID-19 patients.

**Conclusions**

Monocytes from severe SARS-CoV-2 patients are heavily affected from a phenotypic, metabolic, and functional point of view. Cells show decreased respiration and glycolysis but are still capable to produce cytokines. Moreover, the upregulation of inhibitory checkpoints such as PD-1 and PD-L1 in monocytes, represents an interesting point that deserves further investigations. Indeed, blocking such checkpoints could transform monocytes in potential target of immunotherapy.

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## NANOMEDICINE ACROSS BARRIERS

**Background**

Nanomedicines (NMeds) are drug delivery systems capable of encapsulating a wide variety of sensitive, insoluble, or poorly bioavailable molecules such as proteins, peptides, enzymes etc. to protect them from degradation after administration. Moreover, NMeds can be surface engineered to target specific tissues which are usually difficult to reach, like the Central Nervous System and the retina. Nevertheless, once NMeds reach the desired target their ultimate fate is often unknown as they can undergo intracellular trafficking or also intercellular transposition via pathways such as transport via Tunneling Nanotubes (TNTs).

**Objectives**

The aim of this project is to investigate an innovative NMed for efficient encapsulation of neuroprotective agents to be selectively delivered to target diseased cells; a second aim is to understand if NMeds could eventually take advantage of the presence and exploit the formation rate of tunneling nanotubes in tissues.

**Methods**

Polymeric and hybrid NMeds were designed using FDA-approved components: poly-lactide-co-glycolide acid (PLGA), cationic lipids, and hyaluronic acid. The eyes of mice intravitreally injected with retinal targeted fluorescent NMeds were analyzed after 24 hours via confocal microscopy. Also, the impact of targeting ligands on NMed interactions with TNTs was studied using PLGA NMeds modified with diverse ligands, such as anti-cell surface vimentin antibody (GBM targeting) and g7 (BBB targeting), and tested in cell cultures.

**Results**

Among the formulations tested for retinal targeting after intravitreal injection, hybrid PLGA-cationic lipid NMeds coated with hyaluronic acid were the most promising. This type of NMed was abundantly found in the retina 24h after administration. At the same time, we were able to demonstrate selective uptake of PLGA nmeds decorated with anti-cell surface vimentin antibodies by GBM cell cultures, g7 engineered PLGA NMeds by neuronal cell cultures, and remarkably that they were trafficked by TNTs in both cell lines.

**Conclusions**

Data reported open the possibility to exploit TNT formation dynamics using engineered NMeds after successful targeting on difficult-to-reach tissues, which is one of the new challenges of nanomedicine.

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## MICROSURGICAL TREATMENT OF UPPER LIMB LYMPHEDEMA AFTER BREAST SURGERY AND RADIOTHERAPY FOR BREAST CANCER

**Background**

The project is taking place in the Breast Surgical Oncology and Plastic and Reconstructive Surgery Units of the University Hospital of Modena. Women diagnosed with non-metastatic breast cancer are treated with breast excision. Surgical staging of the axillary lymph nodes must be performed, to assess loco-regional lymphatic spread. When nodal involvement is found, axillary dissection and radiotherapy (n some cases) are performed. 15-20% of these women, develop upper limb lymphedema. When conservative treatment fails, women experience functional discomfort and a dramatic worsening in quality of life (QoL). At the state of the art, no instrument is available to predict risk factors for arm lymphedema, nor to tailor the best treatment. Microsurgical procedures are not widespread and the rate of success is frequently disappointing. At present, no dedicated programs are defined in the University Hospital of Modena. Some patients are referred to rehabilitation for a limited period of time. Neither continuative follow up nor surgical options are available for the most critical cases.

**Objectives**

1) To create a program inside the Breast Surgical Oncology Unit of Modena, for women with upper-limb lymphedema, defining a dedicated space and a "lymphedema team". 2) To elaborate a tool to predict a higher risk to develop lymphedema. 3) To elaborate a tool to help surgeons and radiotherapists to early recognize lymphedema onset. 4) To elaborate a tool to define the group of patients that may benefit from microsurgical treatment.

**Methods**

We started the recruitment with all female patients diagnosed with breast cancer that underwent axillary dissection in the Breast Surgical Oncology Unit of the University Hospital of Modena between 1st January 2015 and 31st December 2015. We started from this group to have a five-year follow up since the time of cancer diagnosis. First of all, we performed a retrospective analysis. For each patient, we collected multiple clusters of data: A) PATIENT-RELATED: biometrical parameters (age at diagnosis, BMI) and comorbidities, overall and disease free survival. B) TUMOR-RELATED: (immunohistochemistry, tumor and node staging at diagnosis). C) SURGERY-RELATED: (date and type of breast and axillary surgery, number of excised and metastatic lymph nodes, type and site of surgical incision, type of reconstruction) D) THERAPY-RELATED:

(indication to neoadjuvant or adjuvant chemotherapy, anti-hormonal therapy, radiotherapy with focus on treated fields). At the same time, a nine-point questionnaire was created to assess the grade of lymphedema that patients are experiencing and its impact on their QoL. We chose to investigate both signs and symptoms that patients can recognize (discomfort, pain, swelling, weakness, overcoming episodes of lymphangitis) and, also, we included an assessment on which treatments were performed to cure or prevent the complication (physiotherapy, manual drainage, pressotherapy, compressive sleeves, pharmacological treatment). A positive answer to each question accounted for 1 point on a total of 9, which meant that all signs and symptoms were experienced and that all possible conservative approaches had already been tried. All the patients were contacted (phone) and asked to voluntarily answer to the questionnaire. Data were collected in a dataset.

**Results**

130 patients underwent axillary dissection for breast cancer nodal metastasis from 1st January 2015 to 31st December 2015. 17 of them deceased and were thus excluded. The 92.9% of the remaining patients answered to the questionnaire (in 9.5% of cases through a caregiver). The threshold of 5 out of 9 points was arbitrarily chosen to consider the questionnaire as "positive" (surgical complication) or "negative" (no surgical complication). 31 patients (29,53% of the sample) answered with a score that was ≥5. A comparison between "positive" and "negative" group showed that a younger age is a risk factor in the development of lymphedema. No correlation was demonstrated with BMI and comorbidities. Regarding tumor-related data, neither the T and N staging nor the immunohistochemistry have an impact on the onset of lymphedema. From a surgical point of view, sentinel node biopsy and subsequent complete node dissection resulted as a risk factor when compared to direct dissection. Conversely, the type of breast surgery did not affect the development of the complication. The number of excised and metastatic lymph nodes did not differ significantly in the groups. Concerning the non-surgical therapeutical phase, only performing a neoadjuvant chemotherapic treatment weakly influenced the presence of arm lymphedema. Radiotherapy (different possible radiation fields were also considered as single independent variables) did not affect the onset of the complication.

**Conclusions**

Through the retrospective analysis, we could state which features are more and less frequent in patients that developed lymphedema. Moreover, through the questionnaire, we identified 31 patients whose QoL is highly affected by different grades of lymphedema or that needed to resort to all the available treatments to deal with it. At present, these patients have no evidence of tumor loco-regional relapse or distant metastasis (after a five-year follow up). This condition is mandatory to include them in a functional and rehabilitation program that may primarily influence their QoL.

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## NEUROIMAGING AND FLUIDS BIOMARKERS IN ADULTS PATIENTS WITH STATUS EPILEPTICUS

**Background**

Status Epilepticus (SE) is a common neurological emergency characterized by high short term morbidity and mortality. Non-Convulsive Status Epilepticus (NCSE) is characterized by a qualitative and/or quantitative alteration of consciousness without associated overt major motor phenomena and it represents often a diagnostic challenge. In these cases, the gold standard for the diagnosis is actually the EEG. Through the years, many different EEG criteria have been proposed, the latest of which named Salzburg Criteria for the diagnosis of Non-Convulsive Status Epilepticus (SCC) appeared in 2015. Nevertheless, there is still no consensus on them, especially among the more doubtful possible SE cases (P-NCSE). Thus, the evaluation of SE is rapidly moving toward a multimodal and comprehensive approach based either on the evaluation of neuroimaging correlates or the determination of fluids (serum and cerebro-spinal fluid, CSF) potential biomarkers of SE beside the EEG analysis. The principal aim of the project is to evaluate if a multimodal approach to the evaluation of SE in humans could improve SE management in clinical practice.

**Objectives**

This study aimed to:

1. Determine the cerebral CT perfusion (CTP) patterns correlated to SE and the definition of their role in supporting the diagnosis of NCSE.
2. Define the profile changes of serum and CSF neurosteroids, neuroinflammation and neuronal injury biomarkers and their potential role for the diagnosis, prognosis and as a therapeutic target in SE.
3. Define the usefulness of such a multimodal evaluation to improve SE treatment clinical practice.

**Methods**

This is a prospective monocentric collection of adult patients (≥14 years) with SE. A NCSE is suspected if patients present an acute-onset and enduring qualitative or quantitative disturb of consciousness not otherwise explained. In these cases, the patients enter the study if the clinical suspected diagnosis is supported by a standard 20-30 minutes EEG showing a Definite or Possible NCSE according to the SCC. Whenever possible, a cEEG (continuous EEG) monitoring either in Intensive Care Unit (ICU) or in Epilepsy Monitoring Unit (EMU) of our ward is applied. The cEEG allows us to better understand the dynamic and

evolving patterns in the so-called ictal-interictal continuum (IIC). Clinical information about the etiology, clinical semeiology, and therapeutic management of these patients are collected too.

Aim 1: to evaluate the role of CT Perfusion imaging patients undergo to CTP/CTA (Cerebral Tomography Angiography) study immediately after the neurological evaluation in emergency room. A CTP-EEG correlation analysis is then made. Aim 2: to define the profile changes of serum and CSF neurosteroids, and biomarkers of neuroinflammation and neuronal injury biofluids are collected in the acute phase of SE (within the first 48 hours from the diagnosis of SE). Neuroinflammation biomarkers (IL-8, IL-6, IL-1ß, TNF-α), neuronal injury biomarkers (neuron specific enolase, NSE and neurofilament light chain, NfL) and neurosteroids are measured. When available, CSF-serum albumin ratio and CSF TAU (t-TAU and p-TAU) as a markers of BBB breakdown and neuronal injury respectively are measured too. Aim 3: the results derived from neuroimaging and fluids biomarkers will be combined to define if this multimodal approach could provide improvement in SE management in clinical practice.

**Results**

Aim 1: at present we included 21 adult focal NCSE patients studied with CTP and EEG in the acute phase. Eighteen patients (86%) had focal hyperperfusion patterns (10 cortical only, 1 thalamic only and 7 cortical + thalamus) and 3 (14%) a normoperfusion patterns. Eighteen patients (86%) had an ictal EEG: 16 (89%) had a D-NCSE and 2 (11%) a P-NCSE; 12 had continuous EEG patterns (CP, 67%) and 6 had waxing and waning patterns (WWP, 33%); 6 had lateralized periodic discharges (LPD, 33%). In patients with hyperperfusion patterns there was a perfect (100%) concordance in spatial localization of focal multilobar cortical hyperperfusion and focal ictal activity. Either in hyperperfused or in normoperfused groups, we did not find statistically significant differences between D-NCSE and P-NCSE ($\chi^2$, p = 1), CP and WWP ($\chi^2$, p = 0.1) and between presence or absence of LPD ($\chi^2$, p = 0.1). Among the hyperperfused patients, all the 12 patients with CP showed cortical hyperperfusion while only 3 out of 6 (50%) with WWP had cortical hyperperfusion ($\chi^2$, p = 0.03). Aim 2: at present we included 30 patients with SE and we measured serum NfL levels (for 17 we measured NfL in CSF too). We compared them with serum NfL levels in 30 epileptic patients and 30 healthy subjects age and sex-matched ($\chi^2$, p = 0.147 and One-way ANOVA, p = 0.817 respectively). Serum NfL levels were higher in patients with SE (mean: 101.14 pg/ml) compared to epilepsy patients (mean: 8.54 pg/ml) and healthy controls (mean 13.14 pg/ml, One-way ANOVA, $p < 0.001$). In patients with SE serum NfL levels showed a high correlation with CSF NfL (N = 17, $\tau = 0.68$, $p < 0.001$) as well as CSF t-tau levels (N=17, $\tau = 0.627$, $p < 0.001$). Serum NfL levels were higher in SE lasting > 24 hours (Mann-Whitney U test, p = 0.013), in refractory SE (p = 0.004), and in patients who died within 30-days or who presented a worsening of clinical conditions (p = 0.001). Serum NfL values above 28.8 pg/ml resulted an independent predictor of 30-days clinical worsening or death (OR 7.78; 95% CI 1.26-48.22; p = 0.027).

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## EVALUATION OF IN VIVO INDUCED WOUND-HEALING BY MatriDerm® AND Integra® DERMAL MATRICES: A COMPARATIVE STUDY

**Background**

Scar-free healing requires minimal inflammation, scattered collagen deposition and regular distribution of capillaries, hair follicles and glands. Such kind of healing is the main goal of regenerative medicine and tissue engineering. Different techniques of skin reconstruction have been introduced in the last decades. Whenever possible, primary surgical closure remains the gold standard for skin wounds. In large and/or deep wounds, where primary wound closure is not feasible, coverage of the wound surface with both in vitro expanded epidermal sheets and autologous split-skin grafts are two valid therapeutic options both for chronic ulcers and surgical wounds. Despite those strategies are proven to enhance healing, they lack the dermal component, which is crucial to prevent wound contraction and to provide mechanical stability. Currently, the bilayer concept of wound coverage, where both epidermal and dermal analogues are used, is widely accepted. In particular, dermal elements have gained importance for their role in granulation tissue formation, remodeling and re-epithelization. Tissue engineering of artificial skin requires a biocompatible, non-immunogenic, degradable scaffold that can be assimilated into the body while new tissue is regenerated. When implanted, these constructs should attract progenitors that migrate into surrounding regions, where they undergo terminal differentiation, integrate, and contribute to regeneration of the lesioned areas. Dermal scaffolds may vary from temporary interfaces to permanently incorporated dermal elements. Natural scaffolding materials such as hyaluronic acid and purified collagen have been investigated as alternatives to synthetic scaffolds. MatriDerm® and Integra® are both acellular dermal substitutes widely used in the surgical setting, with similar characteristics in terms of healing time and clinical indication. Our preliminary data already demonstrated the efficacy of those constructs in enhancing collagen production by mesenchymal stromal cells (MSCs) in vitro and therefore promoting skin regeneration. It is important to compare their specific efficacy in the in vivo wound healing process and to evaluate potential differences in terms of clinical outcomes.

**Objectives**

The aim of our study is to demonstrate and compare the regenerative potential of MatriDerm® and Integra after oncological surgery in terms of clinical safety and efficacy. We also decided to assess the substitute ability to attract mesenchymal stem cells (MSCs) from peripheral blood, induce fibroblast migration from

wound edges and restoration of native extracellular matrix (ECM) architecture. Collagen secretion, production of other components of the ECM and neo- vascularization of the treated area are also considered as secondary endpoints of the present study.

**Methods**

The present monocentric interventional study was planned and conducted in accordance with the principles of Helsinki declaration and obtained approval from our institutional review board (Protocol n. CE890.2019). Twenty consecutive patients with medical indication to undergo demolitive dermatologic surgery and subsequent reconstruction were treated with a 2-step procedure composed of MatriDerm® and Integra acellular dermal substitute (ADS) positioning (1) and a split-thickness skin graft (2). Both the matrices were positioned, with half of the wound bed being covered with MatriDerm® and the other half with Integra®. In this way every subject acted both as a case and as a control to eliminate any potential confounding selection bias. Skin samples from the area treated with MatriDerm® and Integra® were then collected after 2 and 7 days from ADS positioning (t1 and t2): skin fragments were obtained during wound care standard procedures, such as cleaning and/or curettage of the surgical site. Moreover, during the second surgical intervention (t3, nearly after 3 weeks, according to our 2-step protocol), skin samples were obtained through curettage during surgical debridement or through a 4mm punch biopsy performed intraoperatively. Further instrumental analyses were performed on the skin samples obtained with the procedures illustrated above. These included: immunostaining for Stro1, CD90, vimentin, CD31; ELISA assays both for collagen and fibronectin; classic histology, with Hematoxylin Eosin, Masson Trichrome and Periodic Acid-Schiff -PAS.

**Results**

To date, we have collected preliminary data regarding the first 7 patients enrolled in the study. Most of the subjects were males (3F Vs 4M). Mean age was 86.4 years (range 80-94). One patient had a cerebral vascular accident and was lost to follow-up. Three out of 7 patients experienced infections of Integra® by Pseudomonas Aeruginosa at t1/2 while no infections of Matriderm® were ever detected. All the infections responded to oral antibiotic therapy. Cell colonization of the matrix was evident at t1-2 and seemed to be more prominent for Matriderm® rather than Integra®. Moreover, ADS integration in the neodermis was more evident for Matriderm® at t3, with Integra®-treated areas mainly still displaying histological evidence of persistence of the scaffolding material on the wound surface. More energic curettage of the wound bed was also required or Integra® before split-skin graft poisoning. No significant differences were detected after one month from t3 in the 3 patients that already completed the 2-step surgery and already entered clinical follow-up.

**Conclusions**

Despite the similarities existing for the two ADSs, probably Integra® and Matriderm® do not share exactly the same characteristics in the in vivo wound healing process.

***Robel Papotti***

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## CLINICAL IMPACT OF ON-TARGET AND OFF-TARGET ACTIVITY OF AID IN DIFFUSE LARGE B CELL LYMPHOMA: BCR INTRACLONAL DIVERSIFICATION AND TP53 MUTATIONS

**Background**

Diffuse Large B-cell Lymphoma (DLBCL) is the most frequent aggressive lymphoma, originating either from germinal-center (GCB group) or post-germinal cells (ABC group). After first-line therapy, primarily R-CHOP, approximately 30-40% of patient's progress or relapse. Currently, risk stratification includes the International Prognostic Score, PET-CT scans, Cell-Of-Origin (COO) and expression/translocation of BCL2/BCL6/MYC. Nevertheless, no molecular marker has been widely correlated with the risk of treatment failure. Genomic instability due to the Activation-induced cytidine deaminase (AID) enzyme and the TP53 gene could play a relevant role in DLBCL aggressiveness. The first can induce mutations by aberrant somatic hypermutation outside the Ig-loci, whereas TP53, possible target of AID "misfiring", is one of the most frequently and clonally persistent mutated genes in relapsed/refractory DLBCL.

**Objectives**

Here we propose to evaluate the clinical impact of AID activity in a large and well-characterized DLBCL cohort, both regarding its on-target activity, e.g. as a driver of B-cell receptor (BCR) intraclonal diversification, and in the context of its capability to operate off-target by promoting mutations of TP53. Ultimately, this could lead to the detection of DLBCL cases at higher risk of chemo-refractoriness and/or relapse eligible for novel clinical trials.

**Methods**

The project comprises two DLBCL well characterized cohorts, treated with R-CHOP.

- Cohort 1: 204 formalin-fixed paraffin embedded (FFPE) samples, all with clinical data of PFS and OS and PET scans (University-Hospital Polyclinic of Modena). COO has been evaluated both with immunohistochemistry (IHC - Hans algorithm) and with Nanostring platform (155/204 cases) through Lymph2Cx gene expression assay. Expression of BCL2 and c-MYC have been as well evaluated.
- Cohort 2: 62 DLBCL samples for validation of molecular studies (Centro di Riferimento Oncologico di Aviano). All cases underwent gene expression profiling (GEP) analysis both through Agilent Microarray system and Lymph2cx assay.

Comprehensive mutational analysis of both cohorts will be performed using a RNAseq strategy or through custom panel enrichment and sequenced on a NextSeq 550 sequencer (Illumina).

**Results**

Regarding Cohort 1: Hans algorithm identified 83 (40%) GCB and 124 (60%) ABC cases, whereas Lymph2Cx revealed 76 (49%) GCB, 58 (37%) ABC, and 21 (14%) Unclassified cases. We observed a good concordance according to Landis and Koch scale (0.719 k-statistic value) between IHC and Lymph2cx. GCB patients (Hans algorithm and Lymph2Cx) had a significantly longer PFS compared to ABC subset [Hans algorithm p=0.011, HR=1.98 (1.17-3.35); Lymph2cx p=0.027, HR=1.93 (1.08-3.47)]. Analysis of BCL2 expression identified 67 cases (33%) as BCL2- and 134 (67%) as BCL2+, while c-MYC expression showed 170 (85%) cases c-MYC- and 31 (15%) cases c-MYC+. Combining data of co-expression of BCL2 and c-MYC, 60 cases resulted BCL2-/c-MYC- (30%), 6 cases BCL2-/c-MYC+ (3%), 109 cases BCL+/c-MYC- (55%) and 25 cases BCL2+/c-MYC+ (12%), also known as Double Expressor. Cox regression analysis showed a 5-year PFS of 90% (78-96) for BCL2-/c-MYC-, 50% (39-60) for BCL2+/c-MYC- and of 52% (29-70) for BCL2+/c-MYC+, supporting the idea that BCL2 expression is an independent predictor of PFS irrespective of c-MYC expression (BCL2- vs BCL2+/c-MYC-, HR 7,8 CI 3-19, p<0.001; BCL2- vs BCL2+/c-MYC+, HR 8 CI 3-19, p<0.001).

Regarding Cohort 2: COO prediction based on Microarray GEP detected 30 cases ABC and 32 cases GCB. Utilizing the same cases with Lymph2cx assay, only 6 cases resulted differently classified: 6 GCB with Lymph2cx assay were identified as ABC by Microarray. Among 8 Unclassified cases according to Lymph2Cx, 7 were ABC and 1 GCB with GEP. Agilent Microarray analysis between GCB and ABC indicated 1295 differentially expressed probes (p <0.01, FC> 1.0), specifically 673 up-regulated and 622 down-regulated within the ABC cases. GEP data showed an AID overexpression in ABC (p=0.065, FC>2). On the contrary, TP53 mRNA levels showed no significant differences between COO groups.

**Conclusions**

We show that in the real-life context COO characterization truly proves to have a prognostic significance, suggesting a worse outcome for ABC subgroup compared to GCB. In addition, we show that the COO classification can be performed through IHC, Microarray and Lymph2cx assay with good correlation levels. Moreover, Double Expressor cases showed poor prognosis, probably due to the overexpression of BCL2, rather than c-MYC, since no PFS differences were observed between c-MYC+ or c-MYC- cases in the context of BCL2 positive cases.

First observation about AID suggested a major expression in the ABC subgroup, with a possible link to a significant activity in the contest of increased genomic instability. We will investigate whether this activity could be associated with an ongoing SHM and consequently to an intraclonal diversification or not. Detailed analysis of the Ig heterogeneity of DLBCL, as well as a deep evaluation of the genomic landscape, may provide novel useful information about the mechanisms responsible of treatment failure.

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## AGE-RELATED FUNCTIONAL CHANGES IN HUMAN BONE MARROW MESENCHYMAL STROMAL CELLS

**Background**

Mesenchymal stromal cells (MSC) are one of the main cellular components of the bone marrow microenvironment. An important feature of MSC is their immunomodulating capacity, partially mediated by secreted cytokines and extracellular vesicles (EVs). With host aging, MSC also undergo age-related changes, which play an important role in the pathogenesis of several diseases of the elderly, often related to a persistent low-grade systemic pro-inflammatory status defined "inflammaging". MSC present a promising therapeutic potential which rely on their genetic stability, poor immunogenicity, reparative and immunomodulatory abilities.

**Objectives**

The main aim of this study is to identify molecular and functional alterations of bone marrow (BM)-MSC derived from young and elderly donors. MSC were obtained, expanded and cryopreserved from more than 120 BM samples of healthy donors. Different characteristics will be evaluated:

-MSC morphology, phenotype and expansion;

-Senescence: β-galactosidase activation, CD264 and senescence-associated genes expression;

-Expression of genes implicated in cell proliferation, hematopoietic support, immunomodulation;

-MSC response to inflammatory priming;

-Immunomodulatory potential of MSC and EVs: effects on macrophage polarization;

-Establishment and comparison of the miRnome of MSC and derived EVs.

**Methods**

Mononuclear cells were isolated by Ficoll gradient centrifugation and then seeded in DMEM supplemented with FBS, L-glutamine and antibiotic/anti-mycotic solution. MSC were identified by analysis of cell-surface markers, according to the ISCT criteria. To evaluate response to inflammatory status, MSC were incubated for 24h in the presence of IL-1β (25 ng/ml), IFN-A (3000U/ml), IFN-ɣ (50 ng/ml) and TNF-α (15 ng/ml). Macrophages were obtained by differentiation of THP-1 cells. For polarization experiments, culture in DMEM media without FBS with the addition of 100 ng/ml LPS and 20 ng/ml IFN-ɣ (for M1 polarization, additional 24h exposure) or 20 ng/ml IL-4 and IL-13 (for M2 polarization, additional 72h exposure) was performed. MSCs

were plated at the bottom of the co-culture transwell system for all the time of cytokine exposure. EVs were isolated from supernatant obtained from MSC culture and ultra-centrifugation at 150.000 g-force for 1h. Total RNA from each cell culture was extracted in a single step using TriPure Isolation Reagent. Real-time PCR was performed on an ABI Prism 7900HT Sequence Detection System, using 25 ng of cDNA and SYBR Green PCR Master Mix. For miRNA expression we used the TaqMan microRNA quantitative PCR, using as endogenous control RNU48 gene. Cell surface markers were analyzed using specific florescence conjugated and non-conjugated antibodies.

**Results**

MSC derived from elderly donors are large, flat and granular and display increased β-galactosidase expression. Cell size and granularity increase with aging. We also observed a reduced proliferation of MSC from elderly donors as evidenced by population doubling and population doubling time. We reported an increased expression of pro-inflammatory genes (TGF-β, GAL1, IL-6, IL-8) in older patients in response to inflammatory priming. MSC activity show a significant impact on macrophage polarization towards M1 and M2 status. Particularly, MSC from young donors induced a reduction of typical M1 markers expression such as CCL2, IL-6, TNF-α, CD274, HLA-DR. Moreover, this effect is less marked when using replicative senescent MSC from the same donors. MSC enhance the switch to anti-inflammatory M2 status, as shown by increased levels of Il-10, TGM2, and CD206. The majority of EVs derived from MSC showed a size of 100-250 nm and were actively phagocyted from macrophages. The analysis of miRNA expression in EVs revealed a significant difference for miRNA known to be involved in macrophage polarization (miR-21, miR-27a and miR-125a-5p).

**Conclusions**

MSC show differences in size, granularity, population doubling and population doubling time according to donors' age. MSC gene expression profile in response to inflammatory priming seem to vary according to subjects' age and particularly in old donors seem to be characterized by an impaired immunomodulating activity, with a reduced inhibition of macrophage M1 status. Furthermore, macrophage polarization could be strongly influenced by EVs activity through miRNA production which seems to vary according to donors' age.

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## WHOLE-TRANSCRIPTOME PROFILING OF BIOPSIES FROM ADVANCED INTRAHEPATIC CHOLANGIOCARCINOMA (ICCA) REVEALS A PROGNOSTIC SIGNATURE WITH TREATMENT IMPLICATIONS

**Background and Objectives**

While transcriptomic data for resected CCA are widely available, scarce evidence is provided for advanced CCA with limited implications for therapeutic selection. We applied transcriptomics to a cohort of clinically-annotated advanced iCCA from patients receiving long-term benefit (Long Survivors, LS) or no-benefit (Rapid Progressor, RP) from first-line chemotherapy in order to 1) identify a prognostic signature of iCCA and 2) inform therapeutic strategies.

**Methods**

We used a pilot cohort of advanced iCCA patients representing the extreme spectrum of benefit from chemotherapy: RP≤6 months (N=7), LS≥23 months (N=6). Transcriptome profiling was performed with TempO-Seq targeted-sequencing on pretreatment liver biopsies. An RP-LS signature was developed from differentially expressed genes. This signature was evaluated in four cohorts of resected iCCA to define the representation of the advance-stage signature in early stage disease and explore its clinical value in association to survival, signaling pathways, immune functionality. Cell type-specific transcriptome analysis was performed by digital cytometry.

**Results**

RP and LS groups were well-balanced regarding clinico-pathologic features. A 504 gene differential expression signature was identified, including 310 genes categorized as LS-high (over-represented in Hedgehog signaling and mismatch repair), and 194 genes categorized as RP-high (over-represented in NOTCH, IL-17, TNF pathways). Virtual microdissection identified differences in cell reprogramming in tumour cells and microenvironment cells (T cells, macrophages, fibroblasts). RP cases showed higher inflammatory clinic-pathological scores, and lower TIDE scores, suggesting a likelihood of response to immune checkpoint inhibitors. The RP-LS signature was found to be a stage- and genomic alteration-independent prognostic feature in resected iCCAs (n=401 cases). Further, this signature was significantly associated with microsatellite instability, decreased M2 polarization in macrophages and IFN-gamma-dominant immune subtypes.

**Conclusions**

We identified a transcriptomic-signature capable of predicting prognosis and treatment benefit. Based on our data, we speculate that patients rapidly progressing on chemotherapy may benefit from immune checkpoint inhibitors, and that the myeloid component contributes to chemoresistance and poor prognosis.

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## EGOFET-BASED BIOSENSORS FOR DETECTION OF MULTIPLE SCLEROSIS BIOMARKERS IN PLASMA

**Background**

Organic electronics-based immunosensors, such as electrolyte-gated organic transistors (EGOTs), are receiving increasing attention as an alternative strategy for ultrasensitive and label-free detection of biological analytes. EGOTs are classified as Organic Electrochemical Transistors (OECTs) and Electrolyte-Gated Organic Field-Effect Transistors (EGOFETs), depending on the permeability of the active layer to the electrolyte ions. Both EGOFETs and OECTs are three-electrode devices, where the current flowing within the organic (semi)conductor bridging source and drain electrodes is controlled by the potential applied to the gate electrode.

Multiple sclerosis (MS) is a chronic and inflammatory disorder of the central nervous system characterized by progressive neurodegeneration. The accurate detection and quantification of MS biomarkers is an urgent need to correctly assess the diagnosis and management of the disease. Therefore, highly sensitive methods, able to detect very low concentrations of the biomarker and discriminate MS patients from healthy individuals are required.

**Objectives**

The accurate detection and quantification of biomarkers of neural degeneration is an urgent need to correctly assess the diagnosis of MS and the management of the disease. Therefore, highly sensitive methods, able to detect very low neurofilament light (NF-L) protein concentrations and discriminate MS patients from healthy individuals are required. In the present work, we focused our attention on the development of an EGOFET-based biosensor for the selective detection and quantification NF-L.

**Methods**

EGOFET biosensors were fabricated using a quartz substrate with interdigitated gold source and drain electrodes (W/L=2000), and the semiconductor material TIPS-pentacene was spin-coated on top. A polycrystalline Au wire, immersed in a droplet (55 µL) of the electrolyte (50 mM PBS, pH 7.4), was used as gate electrode. The specific recognition of the biomarker was ensured by immobilizing anti-NF-L antibodies on the gate electrode, with a controlled and uniform orientation. Transfer characteristics were recorded in the -0.1 to -0.6 V range in PBS solutions containing increasing concentrations of NF-L, from 1 pM to 10 nM.

**Results**

A concentration-dependent change in the output current was observed as a consequence of the binding events occurring at the gate surface. The current change showed aa monotonic increase with increasing concentrations of NF-L, with a trend that could be approximated with a linear dependence on the logarithm of the target concentration.

This current decrease caused by NF-L recognition might be ascribed to the concomitant decrease of transconductance gm and shift of the threshold voltage Vth towards more negative values. Therefore, both observables were monitored during the experiments, and a monotonic trend that followed increasing concentrations of the target analyte was observed for both parameters.

Finally, control experiments demonstrated the absence of a non-specific response.

**Conclusions**

The successful detection of NF-L with an EGOFET-based biosensor was demonstrated in a wide dynamic range of concentrations, even in the presence of a potentially interfering protein. Although we did not explore [NF-L] values in the fM range, the results obtained let us infer that the biosensor might be able to detect neurofilaments even in a wider dynamic range, indicating that it could be safely implemented at the point-of-care for real-time monitoring of the disease.

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## OBSERVATIONAL STUDY FOR THE EVALUATION OF THE EPIDEMIOLOGICAL AND EVOLUTIVE FEATURES OF INTERSTITIAL LUNG DISEASE IN PATIENTS AFFECTED BY SJÖGREN'S SYNDROME (EMERGE STUDY)

**Background**

Interstitial lung disease (ILD) represents the most frequent and serious pulmonary complication in primary Sjögren's syndrome (pSS), with a prevalence ranging from 6 to 70% of patients, significant morbidity and mortality. However, few studies have investigated the frequency of ILD in pSS, generally referred to retrospective studies with small series of patients and numerous biases. Non-fibrosing patterns, primarily non-specific interstitial pneumonia (NSIP), as the most common ILD subtype, and organizing pneumonia (OP), are described as the most frequent in pSS-ILD, in opposition to fibrotic ones, such as fibrotic NSPI, fibrotic OP and usual interstitial pneumonia (UIP). Lymphocytic interstitial pneumonia is highly typical for pSS but it occurs only in a few cases.

This complication is often underrated and, nowadays, there are no randomized controlled clinical trials to support therapeutic guidelines.

Therefore, there is an unmet need of prospective studies to clarify some crucial points such as the incidence and prevalence of ILD, its clinical features (modality of onset and clinical evolution), radiological characteristics and the possible predictive factors.

**Objectives**

Aim of this study is to evaluate prevalence and incidence of ILD in patients with pSS, to assess radiological features, predictive factors for the development of ILD, evolutive characteristics and prognosis.

**Methods**

The present study is a multicentre observational prospective study involving 9 rheumatologic centres.

All consecutive pSS patients will be screened for signs or symptoms suggesting pulmonary involvement (dry cough and/or progressive dyspnea, Velcro crackles traditionally and digitally detected, etc.). An ILD will be suspected on the basis of clinical and auscultatory parameters and subjects with suspicion of pulmonary disease will undergo a high-resolution computed tomography (HRCT), the gold standard for the diagnosis of ILD. Patients will undergo HRCT even in presence of suspected ILD in a chest x-ray.

As regard the assessment of prevalence, all subjects will be evaluated cross-sectionally while, as regards the assessment of incidence, patients without signs or symptoms suggesting pulmonary involvement will be re-

screened in case of appearance of respiratory symptoms or every 6 months. All subjects with newly diagnosed pSS will screened prospectively every 6 months for a period of 5 years to evaluate predictive factors and the onset of ILD with respect to the natural history of pSS.

Prevalence and incidence will be provided along with their 95% confidence intervals.

The HRCT images will be re-evaluated by an expert radiologist, in order to confirm the presence of ILD and to classify it according to the current radiological classifications, in particular discriminating between fibrotic and non-fibrotic radiological patterns.

Finally, a review summarising the most recent literature on pSS treatment strategies was performed.

**Results**

During the last year, we have enrolled 32 pSS patients, increasing the population number by 17,02% and reaching a total of 220 subjects involved up to now (201 females and 19 males). Among them, 40 showed ILD (18.18%). Six subjects were males and 34 females. Eleven patients are awaiting a CT-scan after the detection of Velcro-crackles.

Despite previous observations, our data suggest a high prevalence of fibrosing ILD pattern in pSS patients.

In comparison to current prevalence data, our study could evidence an even more high prevalence of clinically significant ILD. HRCTs performed during the last year are still under evaluation by the expert radiologist; preliminary data suggest a possible different distribution in ILD subsets, with a high prevalence of fibrosing ILD pattern in pSS patients.

The optimal therapeutic regimen of pSS-ILD has not been yet determined. In asymptomatic patients, with mild or non-progressive ILD and without significant abnormalities on lung function tests, a “see and wait” strategy could be acceptable, while glucocorticoids, alone or in combination with immunosuppressive drugs (cyclophosphamide, mycophenolate mofetil, azathioprine, rituximab), usually represent the first-line therapy in patients with progressive or severe disease.

Based on the INBUILD® trial results, antifibrotic therapies, such as nintedanib, may have beneficial effect also in patients with progressive fibrosing ILD, including those associated to pSS. Moreover, considering the variable degree of inflammatory and fibrotic aspects in pSS lung involvement, an association between antifibrotic and traditional immunosuppressive agents could be suggested.

Conservative therapy, including pulmonary rehabilitation, psychological and educational support, can be associated to the pharmacological treatment or may be recommended for patients with mild and non-progressive disease or contraindications to immunosuppressive drugs. Oxygen supplementation can be a major palliative therapy to improve quality of life in patients with severe lung disease. Lung transplantation may be an option in end-stage ILD, but there are few studies evaluating post-transplant outcome in CTD-ILD.

# XXXVI cycle

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## CORRELATION BETWEEN AUTOFLUORESCENCE INTENSITY AND HISTOPATHOLOGICAL FEATURES IN NON-MELANOMA SKIN CANCER: AN EX VIVO STUDY

**Background**

Non-melanoma skin cancer (NMSC) is the most common malignant tumor affecting fair-skinned people, with an increasing of incidence worldwide. The management of these tumors represents a very important and tricky challenge for dermatologist. Surgical excision with clear margins, represents the best approach to reduce the rate of recurrence.

Autofluorescence (AF) is the property of some tissue to absorb and re-emit light with specific wave-length. This phenomenon is due to the presence of particular molecules called fluorophores. Spectrophotometric devices can read autofluorescence emission spectra of the skin. AF spectroscopy can be used as an optical biopsy tool, for the early detection of NMSC and for the better preoperative evaluation of the margins of excision. The study of the differences in spectral characteristics between healthy and neoplastic tissue and understanding the correlation between histological features and AF intensity can be very useful to improve dermatologic diagnostic process.

**Objectives**

- The primary objective of the present pre-clinical ex vivo study is to investigate the correlation between the intensity of cutaneous AF and the histopathological features of NMSC.
- To validate autofluorescence as useful techniques for the optical biopsy in the NMSC

**Methods**

This is a prospective mono-centric ex -vivo study. Thirty-four NMSCs (26 basal-cell carcinomas - BCCs, 8 squamous-cell carcinomas – SCCs) were surgically excised. After removal, in every specimen we put on some landmarks with suture stitches, at least one of them being on the neoplastic tissue and one on the perilesional healthy skin. After that, we illuminated by a violet light (400-430nm) the specimen and the emission spectra were collected by a probe and processed by spectrophotometer linked by mini pc. Comparison between the emission spectra of healthy perilesional point and tumor tissue at the 500 nm wavelength (maximum skin emission intensity) were done. Differences in emission AF spectra intensity (AF $\Delta$) between cases and controls were calculated. For every single lesion a histologic diagnosis was done; every single landmark was evaluated

to establish the presence of any of the histological variables (hyperkeratosis, epithelial thickening, fibrosis, elastosis, neovascularization and cellular atypia).

Histopathological findings were compared with the characteristics of the spectrum in order to investigate any correlation between specific histopathological pattern and spectrum characteristics.

**Expected results**

Spectrometric evaluation was performed on 83 reference points.

The preliminary data are in line with the previous our study, which shown the prevalence of hypo fluorescent aspect of the autofluorescence spectra in NMSC compared to healthy controls. Therefore, it can be hypothesized that such tissue alterations are among the possible biophysical and biochemical bases of difference in emission AF intensity between neoplastic and healthy tissue. The statistical correlation between every single histopathological features and AF will be performed once completed the enrollment. Our preliminary results confirm the usefulness of autofluorescence in highlighting NMSC in a non-invasive, real-time way.

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## ENVIRONMENTAL AND LIFE-STYLE RISK FACTORS OF MILD COGNITIVE IMPAIRMENT ONSET AND PROGRESSION TO DEMENTIA IN A SELECTED MODENA AND REGGIO EMILIA COHORT

**Background**

Mild cognitive impairment (MCI) may be a prodromal condition of clinically overt dementia. MCI can be classified into two subtypes: amnestic or non-amnestic. Amnestic MCI is characterized by memory loss, whereas non-amnestic MCI features include deficits in language, executive function, or visuospatial ability, in both cases without fulfilling clinical criteria for dementia diagnosis [1]. MCI patients can progress into different forms of dementia. Alzheimer's disease is the most common one, contributing to 50-75 % of cases [2]. Other major forms include frontotemporal dementia, vascular dementia, and dementia with Lewy bodies [3].

The incidence of dementia increases with age, which is regarded as the strongest risk factor for its onset and progression. Several other environmental and lifestyle factors have been investigated for their possible role in dementia etiology, while little evidence for a role of genetic factors in dementia onset and progression has been provided [4]. Suspected environmental risk factors of dementia include exposure to air pollutants, pesticides, and electric and magnetic fields [5]. In addition, recent findings highlight the possible role of nutrition, since some dietary patterns, such as the Mediterranean-type diet, could be protective against dementia onset [6]. On the contrary, dietary intake of metals and metalloids such as aluminum, silicon [7, 8] and selenium, especially in its inorganic forms [9], might be risk factors of dementia.

**Objectives**

The aim of the project is to assess the modifiable determinants, both environmental and lifestyle-related, which may be classified as risk factors for dementia onset and progression in patients with MCI.

**Methods**

Patients with newly-diagnosed MCI in the 2019-2022 period are being recruited from Neurology Clinic of Policlinico University Hospital (formerly Sant'Agostino-Estense) and Neurology Clinic of AUSL-IRCCS of Reggio Emilia (formerly Santa Maria Nuova Hospital). Detailed anamnestic history, residential history, lifestyle habits, as well as neuropsychological assessment data are collected. Usual diet is evaluated through a validated semi-quantitative food frequency questionnaire, specifically developed for the Central-Northern Italy population. Dietary intake of foods, nutrients and contaminants will be assessed with a previously

developed procedure [10]. A subset of patients will undergo magnetic resonance imaging and cerebrospinal fluid collection according to current clinical practice. In patients with CSF collection levels of amyloid ratio (β-amyloid1-42 / β-amyloid1-40) and tau protein will be assessed. Furthermore, the CSF content of several trace elements will be analyzed. Follow-up visits will be performed after 18-24 months from baseline to assess patients' cognitive status.

All data will be collected in a database and analyzed using Stata software (Stata Corp., v. 17 College Station, TX).

**Expected results**

During the follow-up and up to the end of the enrollment phase (2022), around 200 MCI patients will be recruited in this cohort study. According to local epidemiologic data [11], an approximated number of 40-60 newly-diagnosed dementia cases are expected to occur in these patients during the study follow-up.


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## INVESTIGATION ON THE ORIGIN OF THE $\Delta^9$-TETRAHYDROCANNABINOL IMPURITY IN CANNABIDIOL SAMPLES

**Background**

Cannabidiol (CBD), the nonintoxicating constituent of cannabis, is largely employed for pharmaceutical and cosmetic purposes. CBD can be extracted from the plant or chemically synthesized. Extracted CBD is a scheduled substance due to the potential contamination by the psychotropic component of cannabis $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC).

**Objectives**

This study aims to shed light on the current distinction between synthetic and extracted CBD by studying their impurity profile.

**Methods**

Synthetic and extracted CBD samples were analyzed by high performance liquid chromatography coupled to high-resolution mass spectrometry (HPLC-HRMS) after an accelerated stability test, which involved the following conditions: 10 samples placed in open vials in oven set at 50 °C and 75% of RH; 10 samples placed in open vials in oven set at 50 °C and 55% of relative humidity (RH); 10 samples placed in sealed vials under $N_2$ atmosphere at 50 °C; 10 samples placed in sealed vials under $CO_2$ atmosphere at 50 °C; 10 samples placed in open vials at 60 °C and 25% RH. All samples were stored for 35 days and two samples were analyzed by HPLC-HRMS at 7-day intervals. Chromatographic separation was performed using a Poroshell 120 EC-C18 column (3.0 × 100 mm, 2.7 µm) and eluting 0.1% aqueous formic acid (A) and 0.1% formic acid in ACN (B) as mobile phase. Method: Isocratic elution at 70% B, 15 min; Isocratic elution at 95% B, 13 min; drastic re-equilibration to the initial conditions (70% B). Total run time: 30 min. Flow rate 0.5 mL/min. Injection volume 10 µL. A stock solution of internal standard (IS, $\Delta^9$-THC-$d_3$ 100 ng/mL) was prepared diluting (1:1000) a solution of THC-$d_3$ (100 µg/mL) in ACN. Samples were prepared by diluting a 10 mg/mL stock solution of CBD with IS to a final concentration of 100 µg/mL.

**Expected results**

No trace of THC species was detected in either synthetic or extracted CBD. In the contrast, THC impurities can be formed in both under particular storage conditions (percentage detected < 0.1%, which is the limit for

an impurity to be identified according to ICH guidelines). Experiments carried out under inert atmosphere in the absence of humidity or carbon dioxide led to no trace of THC over time even at high temperature. The results suggested that the co-presence of carbon dioxide and water from the air could be the key for creating the acidic environment responsible for the cyclization of CBD. These findings suggest that it might be appropriate to review the storage conditions indicated on the label of commercially available CBD.

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## RUNX2 and RAIN: coding and non-coding interplay in cancer progression

**Background**

Transcription Factors (TFs) involved in embryonic development are often deregulated in cancers. RUNX2, master regulator of osteoblasts' differentiation, was found to be aberrantly expressed in Breast (BC) and Thyroid (TC) carcinomas. Several lines of evidence linked RUNX2 to cancer aggressiveness both in BC and TC. Our Laboratory recently described the RUNX2 Associated Intergenic long-noncoding RNA (RAIN), a new long-non coding RNA (lncRNA) which is transcribed starting from one of RUNX2 active enhancers in cancer. We demonstrated that RAIN, beside promoting RUNX2 expression, has additional transcriptionally related but RUNX2-independent oncogenic properties in both BC and TC.

**Objectives**

Aim1. To unveil the gene expression program and the genomic dynamics governed by RUNX2 in sustaining BC and TC progression.

Aim2. To evaluate the RUNX2 common and lineage-specific functions in BC and TC.

Aim3. To understand how RAIN impacts on cancer biology in a RUNX2-independent manner.

**Methods**

Primary and metastatic BC and TC cell lines were chosen as models for our analyses. ChIP-seq will be conducted to obtain the genomic profile of RUNX2, RNA-polII, H3K27ac, H3K4me3 and H3K4me1 in order to functionally characterize the RUNX2-binding elements and to predict RUNX2 potential transcriptional cooperators. Merge between RUNX2 and H3K27ac ChIP-seq will be also used to identify the RUNX2-associated Super-Enhancers using the ROSE26 algorithm and predict their target genes.

RUNX2 and RAIN will be knocked-down using an RNA-guided CRISPR-interference (CRISPRi) approach. RNA-seq analyses will be performed to get insight the gene expression program controlled by RUNX2/RAIN. Merging the RNA-seq with the ChIP-seq data will provide a list of target genes directly regulated by RUNX2, together with the information of which kind of their regulatory regions are bound by the TF. The top scoring genes will be functionally validated by in-vitro analyses.

ChIRP-seq and RNA-pool down followed by mass spectrometry will be performed to explore the RAIN genomic distribution and its interactome.

**Expected results**

This work is expected to explain how RUNX2 interprets the genomic information in promoting BC and TC progression. Indeed, we expected to define: 1.the gene expression program promoted by the TF; 2.the non-coding regulatory elements preferentially bound; 3.the network of RUNX2 cooperators. Comparative analyses allow us to identify the common and lineage-specific function of this TF.

We also expect to clarify the role of RAIN in cancer biology and to define its RUNX2-independent functions. In particular, we expect to disclose: 1.the RAIN exclusive target genes; 2. the genomics distribution of RAIN; 3.the RAIN's protein interactome; 4.the effect of RAIN silencing on the chromatin functional state.

***Dr. Adriana Romanzi***

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## AGGRESSIVE HEPATOCELLULAR CARCINOMA AND CHOLANGIOCARCINOMA SHARE CARCINOGENIC PATHWAYS AND NOVEL THERAPEUTIC TARGETS

**Background**

Hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (iCCA) are the main primary tumors affecting the liver. Although traditionally considered very different, they share several similarities. Within the HCC group, we identified a subgroup of patients expressing a 5-gene neoangiogenic signature, Transcriptomic signature (TS). They are characterized by scarce response to any type of therapy and extremely low survival. Of note, 50% of the iCCA we tested for the neoangiogenic signature were found to be TS+, suggesting the existence of iCCA subgroup characterizable by the TS-like HCC. Beside the severe clinical behaviour, also the imaging features in TS+ HCC and iCCA are similar. This relationship has not been explored so far as HCCs are usually examined as a homogenous group, failing to identify similarities with other tumors.

**Objectives**

This project aims to clarify the fundamental mechanisms underlying the development of more aggressive HCC and to understand whether these are closer to those characterizing iCCA development. In particular, we will evaluate the effects of specific angiogenic growth factor (Angiopoietin-2 and/or VEGF) in tumor progression and assess the role of hypoxia in HCC and iCCA tumoral transformation.

**Methods**

HCC and iCCA immortalized cell lines, respectively HepG2 and HuCCT-1, were used to build up 3D cultures models to test the effect of angiogenic factor treatment. We used also EGI-1 cell lines (derived from extrahepatic cholangiocarcinoma - eCCA) as negative control. All these tumoral cells were seeded in low attach 6 wells plate and filled with expansion medium for 3/5 days. Following the expansion of the cell culture, HCC, iCCA and eCCA-derived spheroids were stimulated with 100 ng/ml or 200 ng/ml of recombinant Human Angiopoietin-2 alone or in combination with 100 ng/ml or 200 ng/ml of recombinant Human VEGF protein for 48h. At 0, 24 and 48h, we visualized the cells and we captured three images/well, in order to measure the size of spheroids and analyze the migration of peripheral cells, counting the number of migrating cells.

In order to determine the expression pattern of marker involved in EMT, spheroids, treated as above described, were grown on coverslip and fixed in formalin 4% for immunofluorescence staining with E-

cadherin, B-catenin and Twist antibodies. In parallel experiments, HCC, iCCA and eCCA cell-derived spheroids were pelleted following treatment for protein quantification by Western Blotting.

**Expected results**

As main goal, we will expect to define whether aggressive HCCs have a close relationship with iCCAs.

Preliminary results showed that Angiopoietin-2 (alone or in combination with VEGF) seems to be able to boost HepG2 spheres growth.

Furthermore, we will expect to identify an important role of hypoxia as trigger event for tumoral transformation, common to both epithelial cancers.

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## GENDER-RELATED RESPONSE TO TYROSINE KINASE-INHIBITOR DRUGS IN HEPATOCELLULAR CARCINOMA

**Background**

Hepatocellular carcinoma (HCC) is the fifth most common cancer in men and the seventh in women. It is the second most common cause of death for cancer worldwide in males and the sixth in females.

This type of tumor is highly malignant and characterized by rapid progression and a poor prognosis.

One type of target therapies available about HCC are tyrosine kinases inhibitors drugs (TKI). These drugs target tyrosine kinases because their overexpression can increase cell growth and proliferation, angiogenesis, metastasis and antiapoptotic effects.

Unfortunately, in patients with risk factors such as microvascular invasion, recurrences are very frequent even after liver transplantation (LT). This high rate of recurrence in HCC after LT greatly decreases survival. This project is therefore focused on the possibility of identifying genes and proteins related to post-LT development of HCC and whether there is a different gender-response on their expression in patients treated with TKI drugs.

**Objectives**

The aim of this study was to analyze the biological, histopathological and clinical characteristics of patients with HCC in order to understand the development of HCC after LT, with particular attention to the expression in the explanted liver of a series of proteins linked with neo-angiogenesis.
The results obtained in both groups of patients treated with TKI drugs were compared to identify possible differences and correlations with post-treatment recurrence and to detect whether there was a different expression of these proteins correlated with a gender response.

**Methods**

Formalin-fixed paraffin-embedded samples from explanted liver tissue from recruited patients were subjected immunohistochemical analysis for Angiopoietin-2, Angiopoietin-1, Tie2, Podoplanin and Clec-2. Briefly, after deparaffinization and rehydration, antigen unmasking was performed with 1 mM EDTA buffer, pH 8, at 98°C for 15 minutes. The sections were then incubated in methanol 5% and $H_2O_2$ 1% for 5 minutes for blocking endogenous peroxidases; nonspecific sites were blocked using a blocking solution reagent with

bovine serum albumin 3% for 30 minutes at room temperature. Sections were then incubated with goat anti-ANGPT2 (AF623; R&D Systems) primary antibody at working dilution of 1:50, or with goat anti-ANGPT1 (AF923; R&D Systems) primary antibody at working dilution of 1:150, or with goat anti-Tie2 (AF313; R&D Systems) primary antibody at working dilution of 1:80, or with rabbit anti-Clec1b/Clec-2 (LS-B12627; LSBio) primary antibody at working dilution of 1:80, each at 4°C overnight in humidity chamber, or with prediluted podoplanin D2-40 PAb (Cell Marque, Roche diagnostics) primary antibodies. Sections were then incubated with prediluted OmniMap anti-goat (for Angiopoietin-1, Angiopoietin-2 and Tie-2), anti-rabbit (for Clec1b/Clec-2) and anti-mouse (for Podoplanin D2-40 PAb) horseradish peroxidase conjugated secondary antibody (Ventana Medical Systems, Tucson, AZ) for 20 minutes in humidity chamber and then with detection kit reagents (ultra-view universal horseradish peroxidase multimer and diaminobenzidine [DAB] chromogen, Ventana Medical Systems) following the manufacturer's instructions. The sections were then counterstained with hematoxylin, dehydrated, and permanently mounted for microscopic examination. Images of stained liver tissue were processed with ImageJ software (http://rsbweb.nih.gov/) to obtain the intensity value of DAB signal.

**Expected results**

In this part of the study, we expect to find a significant difference between the protein expression's level in recurrent and non-recurrent HCC. This could help finding predictive factors of recurrence. We also expect to find different levels of expression according to gender and to response in patients treated with TKI.

The statistical difference between the individual group was analyzed by applying the student t-test.

In our preliminary data, the intensity of ANGPT2 signal is higher on the endothelium of vessel in recurrent tissue (OD=0.387±0.007) than in non-recurrent tissue (OD=0.315±0.005), the increase is significant: p=0.0012.

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## THE ROLE OF PERIPHERAL STEROIDS AND NEUROSTEROIDS IN PATHOPHYSIOLOGY OF THE CENTRAL NERVOUS SYSTEM

**Background**

Steroids produced by the nervous tissue, also known as neurosteroids, together with peripherally born neuroactive steroids play major roles as regulators of neuronal functions and they are hypothesized to be involved in neurologic disorders. Some of them, such as allopregnanolone and pregnanolone, are positive modulators of inhibitory currents generated by γ–aminobutyric acid receptor A and, for this reason, they act as anticonvulsants. Others, instead, display the opposite property, as in the case of pregnenolone sulfate. 3β-hydroxysteroid dehydrogenase (3β-HSD) converts pregnenolone to progesterone, or dehydroepiandrosterone to androstenedione. Trilostane is a synthetic steroid that selectively inhibits the enzyme 3β-HSD and acts by blocking the conversion of pregnenolone to progesterone in the adrenal cortex. Previously, it was demonstrated that the injection of trilostane 16 and 2 hours before the killing can increase levels of pregnenolone, progesterone, 5α-dihydroprogesterone, and allopregnanolone in both the hippocampus and neocortex. Furthermore, the aforementioned pretreatment with trilostane before the administration of kainic acid (KA) resulted in remarkable effects on the dynamics of status epilepticus (SE).

**Objectives**

To challenge the hypothesis that changes in the level of neurosteroids could modify the seizure onset in an animal model of temporal lobe epilepsy (TLE), we will assess the effects of a subcutaneous administration of trilostane before and after the development of spontaneous recurrent seizures.

**Methods**

In the first experiment, animals will receive a single injection of trilostane (50 mg/kg), 10 minutes after the intraperitoneal administration of KA (15mg/kg) to induce SE. In the second experiment, trilostane will be injected once a day for six consecutive days, after the development of SE. Then, levels of different neurosteroids will be analyzed by tandem mass spectrometry and changes in the pathophysiology of the epilepsy will be determined by immunohistochemistry and/or molecular biology techniques.

**Expected results**

By changing brain levels of neurosteroids, we expect that trilostane could modify the development of epileptogenesis and the epilepsy characteristics. Thus, trilostane could display anticonvulsant and/or antiepileptogenic properties in an animal model of TLE.

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## CLINICAL EFFECTIVENESSE STUDY ON FIRST TRIMESTER SCREENING FOR PRE-ECLAMPSIA

**Background**

Pre-eclampsia (PE) is a common pregnancy specific disease, that presents with hypertension and a variety of organ failures, including malfunction of kidneys, liver and lungs. It complicates 2–8% of all pregnancies and is a major cause of mortality and morbidity for the mother and perinatal death and impairment for the baby, especially when it occurs preterm. Recent evidence suggests that the use of 150 mg/die of Aspirin from 11-14 weeks to 36 weeks can reduce the incidence of preterm PE by 62%. At present, several national guidelines (i.e. NICE or ACOG) offer Aspirin in the presence of risk factors based on maternal characteristics or history. However, a recent multicentre study compared the NICE risk-based versus 1st trimester Fetal Medicine Foundation (FMF) multifactorial algorithm-based screening programmes for PE and showed its superiority with both a significant reduction in screen-positive rate and an increase in detection for PE. Despite so, Italian guidelines for PE are not yet recommending any screening test for PE but considering it as an opportunity to improve health care when implementation of the screening is feasible in a routine setting.

**Objectives**

To provide cost effective prevention of preterm PE and reduce the burden of maternal and neonatal morbidity and mortality in women receiving maternity care at AUSL of Reggio Emilia with the adoption of the FMF-algorithm based screening for PE and aspirin prophylaxis.

**Methods**

All women attending for a 11-13 weeks scan at AUSL Reggio Emilia will be screened for PE with FMF algorithm using maternal factors, mean arterial pressure, Uterine Artery Doppler and PAPP-A with no additional costs. Women resulting at high-risk (cut off 1:100, expecting a high-risk positive rate of 10%) will receive Aspirin (150 mg/die) prophylaxis till 36 weeks and will be intensively monitored with an additional growth scan at 28 and 36 weeks. Moreover, the induction of labour will be proposed at 40 weeks in women without any other additional risk factor developed in pregnancy and requiring it before 40 weeks.

Placental dysfunction incidence (preterm, term, and all PE, incidence of placental abruption, fetal growth restriction/small for gestational age babies and intrauterine fetal death) will be compared between women screened vs. women not screened for PE for any reason and delivering in our hospital in the same time frame.

**Expected results**

We expect to show that the FMF multifactorial algorithm-based screening programme is feasible and can be added to the first trimester screening without adding costs in a public healthcare setting. Moreover, we expect to show that it is effective in reducing the incidence of placental dysfunctions of at least 25% among screened women compared to controls.

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## MOLECULAR, CELLULAR GONADOTROPINS' ACTION ON TARGET CELLS, AND PHARMACOLOGICAL IMPLICATION IN MALE FERTILITY DYSFUNCTION

**Background**

The number of couples seeking consultation for infertility problems has steadily increased over the past decade. It is estimated that infertility affects 10%−15% of the sexually active population. Male factor contributes to approximately 50% of all infertility causes and it is generally diagnosed through an abnormal spermatogenesis. Interestingly, more than 40% such cases are still classified as idiopathic conditions.

Spermatogenesis is follicle stimulating hormone (FSH)- and luteinizing hormone (LH)-dependent. Although LH and choriogonadotropin (hCG) act through the same receptor, several recent studies showed that LH and hCG activate different signal transduction pathways at molecular level on granulosa cells and no data is yet available using human Leydig cells. The FSHR is expressed in the non-steroidogenic Sertoli cells, in the testes. Thus, FSH-mediated steroidogenesis does not occur in human males.

Fertility in males may be restored by specific treatments, such as pulsatile gonadotropin releasing hormone (GnRH) or hCG alone or in combination with FSH. Less is known about the efficacy of FSH alone or in association with LH. Some studies have been conducted to assess efficacy and safety of FSH and hCG co-treatment or FSH alone in restoring fertility in male, unfortunately with contradictory results. Furthermore, personalized treatment for male infertility, based on patients' profile, was never considered as potential valid clinical alternative to improve efficacy of infertility treatment.

A limitation of the available studies is the lack of objectively assessable clinical endpoint/outcomes. Semen analysis, in association with patients' hormonal profile, are the clinical parameters to diagnose infertility and to assess treatment efficacy in restoring fertility. Semen analysis, despite a WHO standard method is available, is quite subjective and is not an effective clinical endpoint due to the inter/intra-operator variability. More recently, assessment of sperm DNA fragmentation (sDF) has been proposed to discriminate fertile from infertile men and to predict response to gonadotropin treatment. In a recent meta-analysis of 28 studies showed that sDF levels were significantly higher in infertile men, independently of the sDF method applied. Thus, sDF can be considered a potentially valid diagnosis and prognosis biomarker and a suitable endpoint for male infertility treatment efficacy.

**Objectives**

Based on available evidence, different scientific questions should be still explored to support efficient strategy for male infertility treatment. Assess mode of actions of different gonadotropins on target cell; validation of objective clinical endpoint to assess clinical efficacy of gonadotropins; evidence of clinical efficacy of available treatments for different patients' profile. In particular, this project is aimed at:

- Investigate LH and hCG differential steroidogenesis pathway on target Leydig cells.
- Assess pharmacodynamics and safety of rec-hLH (Investigational treatment) and hCG (standard treatment) in hypogonadotropic hypogonadal men. The general clinical question is whether LH supplementation could be more efficient than hCG in those cases in which LH-activity is required, such as men with HH or male infertility
- Evaluate the biological/clinical correlation between sDF, hormonal profile and semen parameters

**Methods**

To investigate LH and hCG differential steroidogenesis pathway, murine and human Leydig tumor cells lines will be used. Cells will be stimulated for different time interval with equipotent concentrations of hCG and LH. Steroids levels will be measured by LC-MS.

To Assess pharmacodynamics and safety of rec-hLH (Investigational treatment) and hCG (standard treatment) in acquired hypogonadotropic hypogonadal men, a multicentre longitudinal, interventional, randomized, open-label, phase II, clinical trial has been designed and approved (EudraCT 2019-004677-12). The statistical hypothesis is non-inferiority of the highest LH dose employed compared to hCG. Primary endpoint: serum testosterone levels evaluated by liquid-chromatography, tandem mass spectrometry (LC-MS/MS). Secondary endpoints: Safety and tolerability will be evaluated. A total of 32 of subjects will be enrolled

To evaluate the biological/clinical correlation between sDF, hormonal profile and semen parameters a post doc analysis using original raw data from three independent existing RCTs will be performed (Individual Patient Data post-hoc analysis).

**Expected results**

The present PhD program will address the above-mentioned open questions with the ultimate goal of identifying strategies for personalized therapy in male infertility. The three projects, part of the PhD plan, have been approved by Modena and Regio Emilia University as well as from Merck K.G.a.A and are ongoing, no data yet available.

***Dr. Beatrice Melli***

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## UPDATE AND IMPROVEMENT OF THE GENETIC TEST FOR FAMILIAL MELANOMA IN USE AT THE AUSL-IRCCS OF REGGIO EMILIA

**Background**

With an increasing incidence worldwide, cutaneous melanoma is one of the most aggressive human cancers. Approximately 10% of all cutaneous melanoma occurs in families whose members have multiple primary melanomas (MPM). Different susceptibility genes have been identified among these families, and several mutations in these genes were observed in MPM. The pattern of heritability is consistent with an autosomal dominant inheritance that presents an incomplete penetrance where several environmental factors, such as exposure to UV rays, can contribute to the development of lesions.

CDKN2A is currently the most clinically relevant melanoma-susceptibility gene, accounting for about 20–40% of hereditary melanoma. Germline susceptibility has also been associated with mutations in CDK4, BAP1, TERT, POT1, ACD, TERF2IP genes, and other with variants in intermediate-risk genes, MC1R and MITF and activating mutations in two genes, GNAQ and GNA11, both associated with uveal melanoma.

Moreover, genes such as PTEN, BRCA1-2, TP53, have been linked to cutaneous melanoma. However, they are known to confer a primary predisposition to other neoplasms (i.e. breast, ovary, pancreas) and are preferably included in the screening panels for these pathologies.
For those reasons, since 2013, AUSL-IRCCS RE proposes for familial melanoma cases the use of the genetic test “Sanger” for the sequencing of the CDKN2A (exons 1a, 1b, 2, 3) and CDK4 (exon 2) mutational hotspots.

**Objectives**

This project aims to test an updated list of high and medium risk genes for primary susceptibility to familial melanoma through next-generation sequencing (NGS), and to verify their diagnostic yield compared to the currently used method (Sanger sequencing). Moreover, we want to compare the incidence of these variants to the rates reported in the literature in order to possibly identify genetic clusters among patients born in the province of Reggio Emilia as a pilot study for a future study in Emilia Romagna.

**Methods**

The study is in collaboration with Medical Genetics, Skin Cancer Unit (SCU) and the Molecular Biology laboratory. The 76 individuals already tested with the Sanger test and still in follow-up at the SCU will be re-evaluated. Only adult individuals with a previous diagnosis of familial melanoma and consenting to participate in the study will be included. The libraries for the NGS will be prepared from the patient's genomic DNA using a panel for familiar melanoma with probes for the coding regions and splice junctions of the CDKN2A, CDK4, BAP1, MITF, POT1, ACD, TERF2IP, MC1R, TERT (plus promoter), GNAQ and GNA11 genes. The NGS analysis will be performed on the MySeq tool (Illumina), with an average coverage of the regions of interest> 200x. The data will be analyzed according to Illumina's pipelines for calling and filtering variants. Deletions and insertions also will be detected at the exonic level with suitable algorithms (XCAVATOR). The variants frequency identified in each gene of the panel will be compared with the literature data, by a $\chi$-square test or by multivariate analysis that considers covariables in the different cohorts.

The currently used Sanger test (CDKN2A/CDK4) will also be performed in parallel for the newly enrolled patients.

**Expected results**

We will verify whether the NGS test can confirm the data obtained by the Sanger test and identify other variations/mutations thanks to greater sensitivity and search for variants in susceptibility genes not previously examined. We expect to identify susceptibility variants already known in the literature, new variants classifiable as probably predisposing on the basis of bioinformatics evaluation and other variants of uncertain significance.

***Dr. Federica Capitani***

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## EFFECT OF ENZYME REPLACEMENT THERAPY (ERT) AND GENISTEIN TREATMENT ON THE STRUCTURAL CHARACTERIZATION OF GALACTOSAMINOGLYCANS IN AN ANIMAL MODEL OF HURLER SYNDROME (MUCOPOLYSACCHARIDOSIS TYPE I)

**Background**

Mucopolysaccharidosis type I (MPS I) is a rare autosomal inherited disease caused by a deficiency of the lysosomal enzyme α-L-Iduronidase. The lack of the enzyme leads to the accumulation of GAGs, dermatan sulfate (DS), and heparan sulfate (HS), in tissues and organs.

Therapeutical approach is based on treatment and prevention of complications. Hematopoietic stem cell transplantation (HSCT) is considered the standard care for children with severe MPS I (involving CNS). Enzyme replacement therapy (ERT) is the standard treatment for non-involving-CNS MPS I, it is a lifelong therapy.

Genistein has been proposed as a therapy for MPS patients. This isoflavone can impair the synthesis of GAGs by regulating the activity of specific transcription factors. In murine models genistein can cross the blood-brain barrier, but further investigations are needed on human models.

**Objectives**

The focus of the present project is the qualitative and quantitative characterization of galactosaminoglycans in tissues and organs of a murine model affected by MPS I. Firstly, the comparison of GAGs concentration between MPS I mice and wild-type subjects will give us the possibility to validate the MPS I murine model. Then, we will analyze and compare GAGs profile in non-treated MPS I model, and treated with ERT or genistein, to better understand the effects of these two treatments.

**Methods**

Biological fluids and organs samples were collected and provided by the University of Padova to our laboratory. Samples were collected (0, 2, 4, 6, 12, 18, 24 weeks after the beginning of the treatment) from: wild-type mice, MPS I non-treated mice, MPS I mice treated with ERT, wild-type mice treated with genistein and MPS I mice treated with genistein.

Each sample will be analyzed according to a standardized protocol for GAGs extraction. After extraction GAGs will be digested with enzyme to obtain disaccharides that will be tagged with a fluorescent molecule. Tagged disaccharides will be separated and quantified by capillary electrophoresis.

**Expected results**

Characterization of galactosaminoglycans in a wild-type mouse versus a MPS I murine model will be useful to validate this animal model of Hurler Syndrome. Furthermore, the results will help us in evaluating the effectiveness of ERT and genistein treatment in reducing the accumulation of GAGs.

***Dr. Giacomo Santandrea***

*CEM Curriculum: Translational Medicine*
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## EX VIVO FLUORESCENCE CONFOCAL MICROSCOPY TECHNIQUE FOR A FAST INTRAOPERATIVE EVALUATION IN DIFFERENT ONCOLOGICAL SETTINGS: MOHS SURGERY FOR BASAL CELL CARCINOMA OF THE SKIN, BREAST SURGERY AND GASTRO-INTESTINAL ENDOSCOPY

**Background**

In the current era, surgical oncology aims to ensure a precise intraoperative diagnosis and the complete removal of the disease while sparing non-cancerous tissue as much as possible. This is evident for skin Basal Cell Carcinoma (BCC) in sun-exposed sites, for functional/aesthetic reasons, such as ears, lips and eyelid and for breast cancer surgery, increasingly based on oncoplastic principles.

For BCC Mohs micrographic surgery (MMS) represents the gold standard: the tumor is excised, mapped, and processed with frozen sections (FS) for a real time histologic evaluation. In case of positive margins with residual tumor, additional cutting is performed, allowing for complete tumor removal while sparing normal tissue

For Breast Carcinoma (BC) the goal is a tumor excision with a real-time control of surgical margins, performing a re-excision only when deemed necessary, thus improving cosmetic outcomes. Intraoperative evaluation of the specimen performed by the pathologist is based either on gross examination or FS or both.

To date, the intraoperative diagnosis is based on frozen section analysis, that has limited feasibility due to high costs, human resources and time-consumption.

Furthermore, in gastrointestinal (GI) endoscopy, a real-time microscopic diagnosis could improve patient's management. Frozen Sections in this setting are not advised as hampered by a high tissue consumption. Endoscopic en bloc or piecemeal resection is the procedure of choice for the removal of GI lesions. In case of suspicious superficial carcinoma, some issues - such as tumor differentiation, the degree of sub-mucosa involvement, the distance of the tumor from the surgical margin – should be histologically addressed in a real-time to enhance the disease management.

Ex vivo fluorescence confocal microscopy (FCM) is a next-generation digital microscopy tool providing fast microscopic digital imaging of unfixed tissue specimens. Another advantage of FCM is the total preservation of organic material for further conventional Hematoxylin Eosin (HE) histological exam and molecular tests.

A committee of the College of American Pathologist has recently described the potential advantages of FCM over conventional histopathology proposing its application in the intraoperative setting.

To date, no studies have tried to integrate this technology in the routine oncological diagnostic workflow, except for skin basal cell carcinoma and prostate cancer.

**Objectives**

1. *Skin Cancer* Setting: To define the effectiveness of the intraoperative use of FCM to reduce the rate of positive surgical margins when compared to a standard approach (histological final diagnosis).
2. *Breast cancer setting*: To test FCM diagnostic accuracy in detecting cancer tissue persisting on surgical margins after breast surgery. FCM diagnosis will be correlated with intraoperative and histological final diagnosis (gold standard).
3. *GI endoscopy setting:* To test FCM diagnostic accuracy on biopsies from GI endoscopic procedures to assess the presence of cancer, grade of differentiation and depth of infiltration.

**Methods**

Patients' enrollment will be at the Azienda USL – IRCCS of Reggio Emilia. We will test two devices: a Histolog Scanner (HS) (SamanTree Medical SA, Switzerland) for Breast Cancer Setting and a VivaScope 2500 (VS) (Caliber Imaging and Diagnostics Inc, Rochester, New York) in the Skin and GI endoscopy settings. These are the only 2 commercially available confocal scanning microscopes that are designed specifically for ex vivo imaging of fresh biologic tissue specimens. Statistical analysis will be performed. Results will be collected.

1. Skin Cancer Setting: the study will be prospective, interventional. Enrolled patients will meet the criteria for conservative skin surgery. FCM images will be collected and read by a dermatologist and pathologist in a double-blind fashion, for a rapid diagnosis and margin assessment. In case of discordance or concordant diagnosis of "positive" margin, a further surgical sectioning will be performed. In case of concordant diagnosis of "negative" margin, no further actions will be performed. The specimen will be formalin-fixed and paraffin-embedded to obtain HE stained tissue slides. Final histopathological diagnoses will be rendered in a blinded fashion with respect to digital images diagnosis.

2. Breast Cancer Setting: the study will be prospective, observational. Enrolled patients will meet the eligibility criteria for conservative breast surgery. In the surgery room, the breast surgeon will remove the specimen, scan the tissue with the FCM device and acquire digital images to analyze the surface of all margins. After that he will transfer the digital images to a dedicated pathologist, via telepathology. The specimen will then undergo the routine intraoperative examination in the pathology lab (macroscopic analysis with or without FSs depending on pathology judgment). Finally, the specimen will be formalin-fixed and standard grossing sampling followed by paraffin-embedding process will be performed to obtain HE stained tissue slides. Intraoperative and final histopathological diagnoses will be rendered in a blinded fashion with respect to digital images diagnosis... TRUNCATED ABSTRACT

***Dr. Pierluigi Di Vinci***

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## THE ROLE OF ENDOGENOUS INOSITOL IN PLACENTA VILLOUS TISSUE IN OBESE PREGNANT WOMEN

**Background**

Metabolic syndrome (MS) is a combination of conditions, which increase the risk of developing cardiovascular disease and diabetes. Maternal obesity and MS creates an altered fetal programming in utero, leading the development of cardiovascular diseases and metabolic disorders later in adult life. Placental villous tissue via trophoblast cells, has a fundamental role in providing oxygen and nutrients to the developing fetus. Recent studies demonstrate beneficial effects of diet supplemented with Inositols (INOs) during early stages of pregnancy to control glucose homeostasis, correlated with insulin pathway.

**Objectives**

The aim of the project is to study the role of INOs in primary culture of trophoblast cells, isolated from placenta in obese women or healthy controls. We aim to investigate the changes in protein levels and in mRNA expression of targets involved in cellular INOs metabolism.

**Methods**

Trophoblast cells were isolated from placental villous tissue after cesarean section and immediately processed. Isolation protocol included three steps of 20 min enzymatic digestions with Trypsin10X and DNAse I. Thus, supernatant was separated by Percoll density gradient. To define trophoblast cell population, MoAb staining procedure for FACS analysis occurred. Trophoblast cells were positive for Cytokeratin7. The markers studied are GLUT4, IRS1, GSK3, SMIT1, HMIT and MIOX. Protein level and enzyme activity were analyzed by Western Blotting. mRNA levels were quantified by RT-PCR. Moreover, in vitro culture cells were then stimulated with INOs stereoisomers.

**Expected results**

Once optimized isolation protocol to culture high number of trophoblast cells (30-40 10^6 cells for 40g of villous tissue) was performed, we expect to observe significant changes in protein level and RNA expression of markers previously mentioned. We aim to demonstrate alterations in enzymes and receptors responsible for the cellular INOs metabolism and glucose uptake, in obese condition compared to healthy controls. In vitro, we expect to detect beneficial improvements in glucose uptake and metabolism verifying the role of

INOs in regulating expression and activity of targets. Deeply knowing the metabolic profile of the placenta, allow us to find pathways to improve intrauterine environment, thus reducing metabolic disorders of mother and fetus in obesity.

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# CLINICAL MANAGEMENT, COMORBIDITIES, AND ASSOCIATED ADVERSE OUTCOMES IN EUROPEAN PATIENTS WITH ATRIAL FIBRILLATION: ANALYSES FROM THE ESC-EHRA EORP-AF GENERAL LONG-TERM REGISTRY

**Background**

Atrial fibrillation (AF) is the most common sustained arrhythmia encountered in clinical practice, and its incidence and prevalence are increasing worldwide. AF is associated with an approximately fivefold increase in the risk for stroke and a twofold increase in the risk for all-cause mortality. Management of AF may be challenging in clinical practice. Given the complexity of AF patients and the existing differences across European countries between epidemiology, health-care and socio-economic systems, there is a need for programmes aimed at collecting and analyzing "real-world clinical practice" data regarding epidemiology, comorbidities, diagnostic/therapeutic processes and assessing adherence to the guidelines. In 2009 the European Society of Cardiology (ESC) decided to start an innovative programme, the EURObservational Research Programme (EORP), based on observational data collected with strong scientific methodology and independent from direct contribution from industry. The EORP includes general registries aimed at assessing the management of major cardiovascular diseases and the impact of new therapeutic or diagnostic procedures. In 2012 the EORP launched one of the largest registries on AF, the ESC-EORP AF General Long-Term Registry, an international, large-scale, multicenter registry which collected data from 250 centres of 27 ESC countries and enrolled more than 11,000 AF patients from October 2013 to September 2016.

**Objectives**

The general aim of this project is to investigate the epidemiology and clinical management of AF in the contemporary cohort of European patients enrolled in the ESC- EORP AF General Long-Term Registry.

In detail, the aims of our project are:

- To verify real-world applicability and evaluate the impact of guidelines recommendations on the diagnosis, treatment and prevention of AF;
- To investigate the outcome of different management strategies in AF patients and the associated major adverse events over a long-term follow-up period;
- To investigate the applicability of the results of recent clinical trials in everyday clinical practice;

- To investigate the role of clinical biomarkers in AF (e.g. NT-pro-BNP, cardiac troponins, RDW) in daily clinical practice as predictors of adverse events;
- To analyze the outcome of different comorbidities and cardiovascular (CV) risk factors associated with AF.

**Methods**

The EORP-AF Long-Term General Registry is a prospective, observational, large-scale multicentre registry on AF patients in current cardiology practice held by the ESC and endorsed by the European Heart Rhythm Association (EHRA). The registry enrolled AF consecutive patients in 250 centres from 27 participating ESC Countries. Both in- and outpatients were consecutively enrolled when presenting with AF as a primary or secondary diagnosis from October 2013 to September 2016. All patients were ≥ 18 years old and provided written informed consent form. The qualifying AF event had to be recorded by a 12-lead ECG, 24h ECG Holter, or other electrocardiographic documentation within the 12 months before enrolment. All follow-up was performed at 1 and 2 years after enrolment. All the following incident major adverse clinical events were recorded: (i) all cause death; (ii) any haemorrhagic events (i.e. intracranial bleeding, major bleeding or clinically relevant non-major bleeding); (iii) any thromboembolism (TE) (including stroke, transient ischaemic attack, and any peripheral embolism); (iv) any ACS; (v) CV-death and (vi) any myocardial revascularization (including percutaneous coronary intervention and coronary artery bypass grafting). All data about hospital admissions (any admission, AF-related, CV-related and non CV-related) were also recorded. All-cause death, Major Adverse Cardiovascular Events (MACE, as the composite of any TE/ACS/CV death), any hemorrhagic events will be the primary endpoints of our analyses.

**Expected results**

We expect that our analysis will contribute to investigate the pathophysiology, epidemiology, and clinical management of European AF patients in daily clinical practice. The analyses derived from this European registry will help to adapt or strengthen European practice guidelines recommendations in “real-world” AF patients identifying where care is not guideline adherent and where treatment gaps exist.

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## STUDY OF ADAPTIVE RESPONSE IN PATIENTS WITH COVID-19 INFECTION

**Background**

SARS-CoV-2, the virus causing COVID-19 infection, is the pathogen responsible for the actual pandemic we are all facing. Due to its capacity of spreading and to the important clinical manifestations it causes, understanding its immunopathology has an outmost relevance. After almost one year and a half from the first reported cases of COVID-19 the problem of the length of long-term immunity is still under the magnifying lens.

Neutralizing antibody responses are essentially related to the activity of lymphocytes. Studies reported that during the infection the capacity of T cells response is directly correlated with mild disease, indicating the importance of CD4 and CD8 T cells in the control and resolution of the infection. It has been shown that lymphopenia characterizes severe COVID-19 patients, and that those with a faster recover in lymphocyte count produce more antibodies and display a neutralizing responses. Studies are needed to understand the complex interaction(s) between cells that produce antibodies and those that help this function. Accordingly, in COVID-19 patients the activity of specific T cells needs to be carefully investigated, along with the correlation with different degrees of infection severity.

**Objectives**

The aim of the project is to investigate the adaptive response in individuals both during the infection and convalescent phase of COVID-19, always with an eye on the impact of comorbidities, age, sex and on the stratification of patients.

The principal aims of this project are:

- The identification of the subsets of T cells responsive to SARS-CoV-2 specific stimulation and the definition of their role in terms of cytokines production.
- The isolation of responsive T cells from previously stratified patients, to perform transcriptomic analysis.
- The investigation of T follicular helper (Tfh) cells role and their cooperation with B cells.

**Methods**

Blood samples from patients admitted at the University Hospital in Modena will be collected. Peripheral Blood Mononuclear cells (PBMCs) will be isolated and frozen. Thawed PBMCs will be stimulated *in vitro* with SARS-CoV-2 specific peptides and the characterization of the phenotypic markers expression (CD3, CD3, CD8, CD137, CD69, OX40, CD25) and cytokines production (IFN-, TNF, IL-2, IL-4, IL-17, CD107, GRZB) will be performed by flow-cytometry. Isolation of antigen specific T cells will be performed by cell sorting, followed by RNA extraction and RNA-sequencing.

Tfh and B cells will also be characterized by flow cytometry and their interaction will be investigated by *in vitro* co-culture experiments.

Supervised and unsupervised analysis will be performed, subdividing patients for disease severity as well as age, sex and comorbidities.

**Expected results**

This project will describe the different T cells activity and characteristics in patients affected by COVID-19 finding the possible features that diversify the long-term response among differentially stratified patients. Moreover, it will add insights in the interaction between Tfh and B cells and the process that lead to the humoral immunity.

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## IN VITRO EFFICACY AND BIOCOMPATIBILITY OF A WEARABLE MEDICAL DEVICE FOR PERITONEAL DIALYSIS

**Background**

Patients in end stage kidney disease (ESKD) are treated either by hemodialysis (HD) or peritoneal dialysis (PD) to replace kidney function. Dialysis is a lifesaving procedure that can substitute the kidney in blood purification functions, such as the remotion of toxic nitrogenous products from blood. Peritoneal dialysis is a blood-free dialysis technique that removes waste solutes from blood by diffusion in the abdominal cavity across peritoneal membrane of dialysis fluid, thanks to an osmotic gradient. The dialysis fluid is manually replaced by patient 3-4 times/day. This technique provides a continuous and gradual depuration, resulting in stable toxin concentrations. Nevertheless, technique failure rate is high, due to recurrent peritonitis or functional deterioration of the peritoneal membrane caused by exposure to high glucose concentrations.

In this contest a wearable artificial kidney (WEAKID) was developed by Nanodialysis (The Netherlands) with the aim to decrease the number of disconnection procedure and to continue regenerate the effluent maintaining a stable osmotic gradient with low levels of glucose. In WEAKID device, the dialysate is continuously purified by a sorbent unit and reused. Waste solutes and toxins move by diffusion and convection across a high flux dialyzer into a purification circuit, including a sorbent unit with nanostructured sorbents and an electrocatalytic oxidation technology combined with activated carbon for removal of organic waste solutes. Electro-oxidation (EO) is a smart system to convert urea in nitrogen gas, carbon dioxide and water. Direct oxidation of urea through the application of an electrical voltage to the dialysate solution is cheaper and could be miniaturized.

The new device is very promising in terms of uremic waste depuration, portability and offers lower costs than conventional procedures.

**Objectives**

WEAKID is a European project that involves four partners: UMC University of Utrecht (The Netherlands), Nanodialysis (The Netherlands), University Hospital La Paz (Spain) and Unimore (Italy).

The aims of the project are to evaluate the safety of EO technology, the in vitro efficacy and biocompatibility of the WEAKID system.

**Methods**

The efficacy of the EO system will be studied in vitro to assess its ability in urea removing as well as to investigate the effect on glucose molecule. Cyclic Voltammetry will allow to study redox properties of the compounds, which will be evaluated by Gas Chromatography. A targeted metabolomic assay will be performed to a toxicological evaluation on EO technology and a degradation products' toxicity will be defined.

In vitro efficacy and biocompatibility of the whole device will be assessed using a patient PD waste recirculated through the WEAKID device with and without EO. Samples will be taken hourly to verify the removal of urea, creatinine, beta2-microglobulin and electrolytes.

The device biocompatibility, genotoxicity and cytotoxicity will be evaluated, following ISO 10993 ('Biological evaluation of medical devices'). Cytotoxicity will be performed using MeT-5A cells from ATCC (ATCC® CRL9444™), a virus-transformed human peritoneal mesothelial cell culture; immunotoxicity will be assessed on peripheral blood mononuclear cells (PBMCs) from healthy donors.

**Expected results**

A good efficacy in removing uremic waste product and a complete safety and biocompatibility of the device are the main end-points to be verified and validated. Further on a prototyped device for a first in human clinical trial will be designed.

***Dr. Marta Starnoni***

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## THERAPEUTIC MICROSURGICAL SOLUTIONS FOR BREAST-CANCER RELATED UPPER LIMB LYMPHEDEMA

**Background**

Breast cancer-related lymphedema (BCRL) represents a global healthcare issue affecting the emotional and life quality of breast cancer survivors significantly. The clinical presentation is characterized by swelling of the affected upper limb, that may be accompanied by atrophic skin findings, pain and recurrent cellulitis. Cardinal principles of lymphedema management are the use of complex decongestive therapy and patient education. Recently, new microsurgery procedures have been reported with interesting results, bringing in a new opportunity to care postmastectomy lymphedema. Lympho-lymphatic bypass, vascularized lymph node transfer, vascularized lymph node transfer (VLNT) and lymphovenous anastomosis (LVA) are the most innovative options that microsurgery offers.

**Objectives**

The aim of the project is to set up a Lymphedema Unit in the Policlinic of Modena. Chronic and debilitating lymphedema is mainly caused by lymphadenectomies or radiotherapy for oncologic surgery of the extremities. The institution of a Lymphedema Unit can be very challenging because many aspects have to be taken into consideration including clinical examination, imaging techniques, patient selection and proper treatments. Actually, only a limited number of surgical treatment options are possible. It is mandatory a close collaboration among different specialists in order to offer the best treatment solution according to the patient clinical condition.

The project includes:

- The recruitment of a multidisciplinary team (plastic surgeon, breast surgeon, physiatrist, physiotherapist, and radiologist) to provide a coordinated support for the patient;
- Patient selection to be treated with microsurgical solutions;
- Training of the plastic surgeon in super-microsurgical and innovative imaging techniques;
- Creation of a schedule to easily report lymphedema severity, clinical examination, treatments, results and follow-up of the patient.

**Methods**

In order to gain awareness on recent updates on diagnosis, severity and available treatments for breast cancer-related lymphedema, a review of the literature has to be performed according to the scientific method. Based on data and results, a multidisciplinary team can be recruited. The role of each specialist has to be clearly defined from diagnosis to patient selection, classification, and treatment. A schedule to easily report lymphedema severity, clinical examination, available treatments, results and follow-up of the patient can be created based on the current literature. Training of the plastic surgeon includes improvement of super-microsurgical technical skills throughout the execution of micro-anastomosis on vessels < 0.5mm of diameter using 11-0 and 12-0 nylon suture under the microscope for three times a week and every session lasting four hours. Training on the use of innovative imaging techniques including the indocyanine green lymphography and high-magnification microscope Zeiss Pentero 800 has to performed throughout a personalized manufacturer company course (minimum 20 hours) and the use of these technologies during the routinely traditional surgeries of our Unit (minimum six microsurgical reconstructions per month). Superfine microsurgical instruments have to be collected and used during traditional microsurgical reconstruction (minimum six microsurgical reconstructions per month).

**Expected results**

An effective Lymphedema Unit is expected to be created with a proper Diagnostic Therapeutic and Assistance Pathways (PDTA). The Unit will include different specialists with clearly defined roles. The plastic surgeon is expected to have a good learning curve in order to offer the proper surgical solution to the patient. All patients in our Lymphedema Unit will be treated with an individualized program starting from the diagnosis, disease severity, selection of appropriate treatment (surgical vs non-surgical) and finally the postoperative management. The final expectation is an improvement of the quality of life of the patients affected by extremity lymphedema with reduction of the use of compression garment, reduce swelling without any episode of cellulitis.

***Dr. Giovanni Neri***

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## DEVELOPING ANTI-GD2 CAR-T CELLS TO TREAT SMALL CELL LUNG CANCER

**Background**

Small-cell lung cancer (SCLC) is a high-grade neuroendocrine carcinoma accounting for the 15% of all lung cancers and is predominant in current or former smokers. To date, it is distinguished by a dismal prognosis that reflects the lack of appropriate therapeutic strategies.

Chimeric Antigen Receptor (CAR) T cells is a novel approach that has been adopted initially to treat blood malignancies such as Acute Lymphoblastic Leukemia (ALL). It consists of patient's lymphocytes genetically engineered *ex-vivo* to recognize a specific tumor-associated antigen (TAA) via an engineered receptor (CAR) which is composed mainly by an extracellular antibody ScFv binding moiety and an intracellular co-stimulatory domain. The binding of CAR to TAA triggers the immune response leading to CAR-T secretion of cytotoxic molecules such as granzyme B, TRAIL, INF-γ□□TNFα□etc. The advantage of CAR-T cells is not only represented by the intrinsic plethora of cytotoxic substances they secrete, but also by their homing ability and by their possibility to expand and persist *in vivo*. Considered the treatment efficiency on blood tumors, CAR-T cells have been recently translated to solid tumors.

According to a study published by Furukawa and colleagues, SCLC retains high expression levels of the disialoganglioside GD2 on its surface, while it is known it has a highly restricted expression in healthy tissues. Hence, engineering CAR-T cells to target GD2 antigen could represent a novel and a promising approach to contrast SCLC either as a stand-alone treatment or in combinatorial regimens.

**Objectives**

The three-year-long research project aims to:

- Transduce with a retroviral vector the anti-GD2 CAR cassette into T cell populations
- Evaluate GD2 expression on SCLC cell lines (antigen-positive and antigen-negative cell lines)
- Evaluate and develop different protocols to increase the expansion of specific T cell subpopulations such as NK, NKT and □□ T cells to be transduced with GD2 CAR receptor.
- Characterize the cellular product via flowcytometry in order to define memory, cytotoxic, regulatory and exhaustion profile of fresh CAR-T cells. *In vitro* co-cultures will be used to assess whether the contact with tumor leads to changes in the immunophenotypic profile.
- Assess *in vitro* cytotoxicity of CAR T cells over different time points in both 2D and 3D tumor models and *in vitro* imaging via EVOS cell imaging system.

- Evaluate cytokine release in multiplex assays.
- Assess CAR-T antitumor activity in *in vivo* tumor models, either as a stand-alone treatment or in combination with check-point inhibitors (CI) such as Nivolumab or Pembrolizumab.

**Methods**

Cell culture techniques: isolation of PBMC, collection of lymphocytes, retroviral transduction and culture of lymphocytes. Culture of SCLC cell lines. Culture of FLYRD18 packaging cell line. 3D cell modelling using spheroids or VITVO™ platform. *In vitro* cytotoxicity studies via Promega Glomax Discover Multimode Microplate Reader, immunophenotyping using BD FACS ARIA III.

Molecular techniques: Cytokine release assay using Luminex technology with custom made kit by ThermoFisher.

Animal models: Establishment of tumor xenograft models in immune deficient mice. Monitoring of the *in vivo* studies with IVIS spectrum In Vivo imaging system by PerkinElmer to assess tumor growth and treatment efficacy of CAR T cells.

**Expected results for the first and second year**

During the first and second year of research, we expect to validate a CAR-T system already developed in our Laboratory to target GD2 antigen in SCLC preclinical models. In particular, the first tasks to be accomplished along the next months will be to transduce SCLC cell lines with luciferase and/or DSred, to select and expand T cell sub-populations (e.g. $\gamma\delta$ T cells) to be transduced with the chimeric antigen receptor by retroviral transduction. To date, we have characterized the immunophenotype profile of CAR-T cells and we are now focusing on experiments to assess whether the exhaustion profile of our product changes according to the tumor priming. We expect to find higher levels of exhaustion markers within primed lymphocytes compared to naïve ones. Secondly, we will carry out *in vitro* cytotoxicity studies with different Effector:Target (E:T) ratios to evaluate the anti-tumor activity overtime of our cellular product.

***Dr. Sara Grisanti***

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## CLINICAL-INSTRUMENTAL PHENOTYPING AND BIOMARKERS ANALYSIS IN THE EVOLUTION OF PATIENTS AFFECTED BY PARKINSON'S DISEASE

**Background**

Parkinson's Disease (PD) represents the second most common neurodegenerative disease after Alzheimer disease, heating till 1% people older than 60. The cause of this pathology is the degeneration of dopaminergic neurons in substantia nigra pars compacta (SNc), nucleus located at mesencephalic level, associated also with the presence of pathological proteins aggregated called Lewy bodies. The main motor symptoms of PD are bradykinesia, rigidity, rest tremor and gait/axial alterations, in combination with different non-motor symptoms like hyposmia, depression and constipation. Nowadays there is not any therapy capable to stop the progression of PD or even improve it. PD is a heterogeneous disease from genetical point of view, with familiar and sporadic cases. At present 23 genes or loci have been identified. 15% of PD patients has a familiar history of the pathology and additional 10% a monogenic cause. The diagnosis of PD is mainly based on clinical data supported by bioimaging like brain MRI and single photon emission computed tomography (SPECT) with Ioflupane I123 injection (DaTscan™). These instrumental approaches are becoming more and more precise and integrated by quantitative methods to identify predictive data on disease progression. An example of it consists on DatQuant software, capable of extrapolating volumetric quantitative data from the DATSCAN exam. The recent technological evolvements in terms of quantitative and heterogeneous data about PD patients have brought to new approaches of investigation based on clustering to identify subgroups and predict different disease progression pathways.

**Objectives**

The objective of this prospective observational project is to perform a deep phenotypic clinic-instrumental characterization of the genetic cohort of PD patients (conducted at the Movement Disorders Center of the AUSL-IRCCS of Reggio Emilia, Italy), comparing them with a control group of idiopathic, genetically negative, PD patients in order to detect the presence of significant differences between the two groups.

The characterization will be based on several clinical and instrumental data:

- Clinical data: analysis of the differences in clinical official scales (UPDRS; Hoehn and Yahr, MOCA, Schwab and England, PDQ-39) between the two groups

- DATSCAN data: analysis of the differences in terms of quantitative parameters between the two groups using the software DatQUANT.
- Brain MRI: analysis of the differences in terms of qualitative and quantitative data between the two groups applying the software QBIM.
- Parenchymal ultrasonography: analysis of the differences in terms of qualitative and quantitative data between the two groups

**Methods**

The clinical research project has been structured in this way:

- Definition of the clinical and instrumental data to collect
- Definition of the inclusion and exclusion criteria to recruit patients of the two groups
- Database preparation collecting clinical and instrumental data from baseline to last follow-up
- Statistical analyses, mainly based on covariance and regression
- Interpretation of the results to define the key significant biomarkers which discriminate the two clinical phenotypes

The clinical departments involved, in addition to the Movement Disorders Center of the AUSL-IRCCS of Reggio Emilia, are: Nuclear Medicine Unit, Neuroradiology Unit and Clinical Studies and Statistics Service, all from the AUSL-IRCCS of Reggio Emilia.

**Expected results**

The expected finding of this research project is the definition of the main clinical parameters and biomarkers which significantly characterize the genetic PD group from the idiopathic PD group and their distinct pathology evolution.

***Dr. Ilaria Martinelli***

*CEM Curriculum: Translational Medicine*
*Tutor: Prof. Jessica Mandrioli*

## FOCUS ON CATASTROPHIC AMYOTROPHIC LATERAL SCLEROSIS: A BIOMARKER STUDY

**Background**

Amyotrophic Lateral Sclerosis (ALS) is characterized by a wide range of clinical presentation, heterogeneous progression and variable survival, with some patients (called "catastrophic", rated as < 10%) having an extremely deleterious progression leading to death or tracheostomy within one year from symptom onset. This population is extremely fragile, and represent a great challenge even for experienced clinicians, as their conditions relentlessly worsen before support procedures are carried out. We hypothesize that emerging modifiers of ALS progression including systemic and neurological inflammation, microbiota composition and metabolic profile, may aberrantly interact to precipitate ALS course especially in this specific population.

**Objectives**

The aim of the project is to deeply characterize with clinical, neurophysiological, immunological and metabolic signatures a cohort of "catastrophic" ALS patients with respect to "classical" ALS patients.
For this purpose, our broad objectives are as follows:

- To compare immune and metabolic profiles of "catastrophic" and "classical" ALS patient
- To decipher optimal immunological-microbiota intersection to predict ALS progression as biomarkers for the diagnosis and prognosis in ALS.

**Methods**

We conduct a prospective study at a single tertiary MND Center set in Modena, Northern Italy. According to our prevalent population, we estimate to enrol 60 patients, with a 1:3 ratio (15 catastrophic vs 45 classic ALS patients). A "catastrophic" evolution has been defined as based on survival <12 months from onset or on progression rate as measured by ALSFRS-R slope of ≥3 points/month. ALS patients will be deeply clinically and neurophysiologically phenotyped, applying techniques as EMG, MEPs, as well as 3T brain MRI and genetic analysis by NGS whenever collected as part of the diagnostic process. Systemic inflammation biomarkers (including neutrophils/lymphocytes ratio, along with an extensive set of cytokines and chemokines), microglial (as serpinA1 and CHI3L1) and neuronal injury biomarkers (NSE and NF) are measured on sera and, whenever collected per clinical practice on Cerebrospinal Fluid (CSF). Metabolic changes are examined by dosing cholesterol metabolites, amino acids and energy intermediates. Intestinal inflammatory response and

gut microbiota composition are evaluated in fecal samples. Comparisons between the two groups will be performed by logistic regression and survival models.

**Expected results**

Based on literature evidence, we expect to find that in catastrophic ALS the dysregulation of the inflammatory response, talking with metabolic background and microbiota composition, would be more impactful compared to patients with a slower disease. We aim to establish a set of immunological and metabolic features allowing an early identification of this subset of patients, for accurate stratification for clinical trials and for timely management of patients need. Finally, we hope to individuate vulnerable processes that could be targeted to modulate disease progression.

***Dr. Arianna Rinaldi***

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## INNOVATIVE NANOTECHNOLOGICAL APPROACHES FOR GLIOBLASTOMA TARGETING

In the context of International Mobility Program "VINCI2020", the following 36-month PhD Project is based on a joint supervision between University of Modena and Reggio Emilia (Italy, months 1-18) and University of Angers (France, months 18-36).

**Background**

Glioblastoma multiforme (GBM) is the most aggressive and common malignant brain tumour, characterized by a life expectancy of only 12–18 months after diagnosis. Due to high invasiveness of GBM stem cells, drug resistance, high infiltration into surrounding brain tissues, as well as the presence of the blood brain barrier (BBB), current therapies are ineffective, and patients often develop tumour recurrences.

Numerous nanomedicines (NMed) have been developed for the treatment of GBM using different drugs. Among them, Temozolomide (TMZ) is a DNA alkylating agent used as standard chemotherapeutic agent for GBM. The encapsulation of TMZ into nanocarriers allows to increase its bioavailability, enhance its accumulation in tumor site and decrease its toxicity. However, novel nanotechnological approaches are urgently required to achieve TMZ crossing of the Blood Brain Barrier and effectively target GBM cells.

Furthermore, one the major problem limiting TMZ clinical efficacy is drug resistance, which is mainly ascribed to the activation of DNA repair mechanisms by cancer cells or many other altered signalling pathways. In this regard, small interfering RNA (siRNA) oligonucleotides are emerging as promising therapeutic agents for GBM treatment. Many siRNA-loaded Nanomedicines have recently shown the ability to downregulate over-expressed oncogenes responsible for resistance to Temozolomide and to reinforce its cytotoxicity, when the two therapeutics are combined.

**Objective**

- Design, characterize and test novel nanomedicines (NMed), either for systemic or loco-regional administration, intended for targeting GBM cells that remain after surgical resection and are mainly responsible for cancer recurrences.

- The systemic approach (carried out at *University of Modena and Reggio Emilia*, ITALY) is based on designing injectable, tailored and surface-engineered nanocarriers that specifically deliver anti-cancer molecules across the blood brain barrier and to cancer cells. More specifically, the idea is to project two different nanomedicines: the first one is a Temozolomide-loaded NMed (TMZ-NMed), while the second

one is a siRNA-loaded NMed (siRNA-NMed). A proper siRNA will be selected by virtue of its potential ability to block the expression of a gene responsible for TMZ resistance.

- For the loco-regional approach (carried out at *University of Angers*, FRANCE), the objective is to develop and test proper loco-regional formulations of the selected NMed.

- The most promising formulations of TMZ-NMed and siRNA-NMed, in terms of pharmaceutical and biopharmaceutical properties, will be submitted to *in vitro* and then to *in vivo* studies for both the systemic and the loco-regional approach. siRNA-NMed and TMZ-NMed will be tested separately and, in a second phase, in combination by co-administration to test the ability of siRNA NMed to enhance the anti-tumor activity of the TMZ-loaded one.

**Methods**

As a first step of the systemic approach, hybrid nanoparticles composed by poly-(D,L-lactide-co-glycolide) polymer (PLGA) and Cholesterol (Chol), named MIX-NPs, were investigated as potential platforms for the encapsulation of TMZ. Temozolomide-loaded MIX-NPs were formulated by using nanoprecipitation method. With the aim of optimizing TMZ loading into Mix-NPs, the effects of varying formulation parameters (such as ratio between PLGA and Chol, surfactant type and concentration, aqueous/organic phase ratio, etc.) on size, polydispersity index, zeta potential, drug encapsulation efficiency and *loading content of NPs are currently under investigation.*

**Expected results**

For the optimized formulation, Temozolomide-loaded MIX-NPs will be further characterized and functionalized onto their surface with novel ligands selected to drive the tailored nanomedicine in the Central Nervous System (CNS) and selectively towards GBM cells.

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## CONTROVERSIAL FEATURES OF *ROS1* REARRANGEMENTS DETECTION IN NON-SMALL CELL LUNG CANCER AND THEIR CLINICAL IMPLICATIONS

**Background**

Non-small cell lung cancer (NSCLC) currently represents up to 80-90% of lung cancer. *ROS1* rearrangements have been identified in about 1-2% of non-squamous NSCLC and their identification has become fundamental after crizotinib demonstrated robust antitumor activity as first-line treatment for advanced disease. Different methods are available for detection of *ROS1* rearrangements and all have peculiar advantages or limits, but consensus about the method of choice is lacking. Immunohistochemistry (IHC) is considered as an effective screening tool, while "break-apart" fluorescence in situ hybridization (FISH) has been "historically" considered as the gold standard for detection of *ROS1* rearrangements. In recent years, next generation sequencing (NGS) has been used to sequence thousands of genomic alterations with one assay, and it is usually characterized by high sensitivity and specificity, but data about the role of NGS in the assessment of *ROS1* rearrangements are limited. Moreover, the use of high sensitivity and multiplexed methodologies has led to the increase detection of concomitant mutations within the same tumor. This subset of multiple-mutated NSCLC currently represents a therapeutic issue, since it is still not clarified if concomitant mutations may affect tumor microenvironment, and it may be difficult for physicians to interpret molecular results and choose the best treatment upfront.

**Objectives**

Aims of the current study are to compare different techniques used in the detection of *ROS1* rearrangement assessing their sensitivity, specificity and concordance, and to relate diagnostic results with treatment outcome. Secondary aim is to assess the occurrence of concomitant mutations and to evaluate whether they affect response to treatment.

**Methods**

This is an observational single Institution study. Patients with *ROS1* rearrangements will be selected from a pool of consecutive patients with diagnosis of locally advanced/metastatic NSCLC whose samples underwent predictive IHC assays and molecular profiling, as for standard local practice. Therefore, all patients are expected to have *ROS1* rearrangements evaluated by IHC, FISH and NGS allowing to compare concordance

between different assays. Clinical data will be retrieved by the electronic medical documentation system of the University Hospital of Modena and will be collected in an anonymized dataset.

**Expected results**

This study faces a current and urgent issue in clinical practice, that is which is the best assay in diagnosis of *ROS1*-rearrangements and the need to comprehend whether the co-existence of molecular drivers may influence prognosis of patients and their outcome upon use of targeted agents. We expect our analysis to represent a useful tool for clinicians and pathologists to improve interpretation of molecular and pathological data and to effectively translate them in clinical practice, to improve NSCLC patients' selection and consequently their clinical outcome with target therapies.

***Dr. Sara D'Alessandro***

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## CROSS-INTERACTION BETWEEN PITUITARY GLYCOPROTEIN HORMONES AND THEIR RECEPTORS IN THYROID AND OVARIAN CELLS

**Background**

Thyroid is an endocrine gland responsible for the secretion of hormones triiodothyronine (T3) and thyroxine (T4) whose release is stimulated by pituitary thyroid-stimulating hormone (TSH). The increase of thyroid cancer prevalence occurs in females around the age of 20 and peaks near menopause at the age of 50 years, while around 70 years in men. A large number of studies have shown that the incidence of thyroid cancer in women is 4-fold higher than in men, suggesting that estrogens, such as the estradiol (E2), are involved in the pathogenesis. Given structural similarities, we hypothesize that the TSH receptor (TSHR) may form heteromers with the G protein-coupled estrogen receptor (GPER), modulating proliferative signals in thyroid cells. Similar findings were achieved by our Research Group, which demonstrated the formation of GPER-follicle and -lutenizing stimulating hormone receptor (FSHR and LHCGR) heteromers.

**Objectives**

The aim of this project is to define whether there is an influence of estrogen in modulating TSH-like, proliferative signals in papillary and follicular thyroid cancer cells. The cross-interaction between TSH, E2 and choriogonadotropin (hCG), as a placental hormone binding TSHR, will be investigated.

**Methods**

Experiments are performed using papillary thyroid carcinoma (K1) and follicular epithelial (Nthy-ori 3-1) thyroid cell lines. Transfected COS7 and HEK293 will be used as control cell lines to evaluate the activation of specific transduction pathways. Ovarian derived cell lines obtained by primary granulosa cells (hGL5 and KGN) and luteinized primary granulosa are used as well, since expressing TSHR-like receptors and producing steroids.

Cells were transfected with plasmids encoding pituitary glycoprotein hormone receptors (FSHR, LHCGR and TSHR) and treated with their specific ligand, in the presence or in the absence of other sexual steroid or thyroid hormones to evaluate the intracellular increase of cyclic guanosine and adenosine monophosphate (cGMP and cAMP) and Inositol-1-phosphate (IP1), through dose-response and time-course experiments using BRET/HTRF and/or ELISA. The activation of this molecule are associated with apoptosis by evaluation of

caspase 3 cleavage, using a specific BRET biosensor, or cell proliferation. Cell viability and cell death are investigated with MTT assay and propidium iodide staining, and nuclear fragmentation evaluated by DAPI staining. Gene expression analysis will be performed to study which pathway leads to cell death.

**Preliminary and expected results**

Preliminary BRET and HTRF results demonstrated that, in COS7 cells, TSHR and GPER do not form heteromers. However, the co-expression of these receptors leads to differential modulation of the TSH- and E2-induced proliferative cAMP an IP1 pathways. cAMP increased in cells expressing TSHR and in those co-expressing GPER and TSHR. However, IP1 production is increased in TSH-treated cells expressing TSHR, while the hormone failed in inducing IP1 production under GPER/TSHR co-expression. E2 resulted to not influence both cAMP and IP1 production.

The close proximity between TSHR and GPER on the cell membrane is investigated by Proximity Ligation Assay (ongoing experiment), to investigate whether the lack of heteromer formation is a false negative result due to BRET biosensors incompatibility. Further experiments will be performed to better understand the role of estrogens and its receptors on thyroid cancer development and how glycoprotein hormones can cross-interact with receptors structurally similar to their own to regulate the activation of cell viability and death signals.

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## BRONCHODILATOR THERAPY IN PATIENTS WITH COMBINED PULMONARY FIBROSIS AND EMPHYSEMA (CPFE): A PILOT STUDY

**Background**

Combined Pulmonary Fibrosis and Emphysema (CPFE) is a smoking-related interstitial lung disease characterized by the coexistence of both defects and accounts for the 35% of total patients with Idiopathic Pulmonary Fibrosis (IPF). The frequent association with pulmonary hypertension negatively affects the natural course of CPFE, whose prognosis is even worse as compared with IPF without emphysema. Emphysema and fibrosis cause different physiologic effects. While emphysema reduces lung elastance and negatively affects maximal expiratory flows causing an obstructive deficit, fibrosis reduces lung compliance with a restrictive pattern at the pulmonary function tests (PFTs). Although CPFE patients presents a respiratory functional profile with relatively spared lung volumes and flows (given the balance between lung restriction and obstruction), the diffusing capacity of lung for carbon monoxide (DLCO) results dramatically reduced. Furthermore, CPFE patients suffer from symptoms associated with both conditions and, due to the lack of any approved therapy, present an extremely impaired quality of life. As such, inhaled long-acting beta-agonist or anti-muscarinic (LABA or LAMA) bronchodilator therapy for emphysema and chronic obstructive pulmonary disease has proved so far efficacy in reducing symptoms and improving quality of life. However, data are lacking on the effect of these medications in patients with CPFE.

**Objectives**

The aim of this pilot study is to assess the impact on quality of life including self-reported symptoms, respiratory function and functional performance following bronchodilator therapy in a cohort of patients with CPFE.

**Methods**

As a preliminary investigation we did not perform an *a priori* sample size calculation.

All patients with established diagnosis of CPFE referred to our Center for Rare Lung Disease of the University Hospital of Modena will be consecutively enrolled. Patients showing obstructive defect at the PFTs and aged < 18 years will be excluded.

After enrollment, patients will receive inhaled bronchodilator therapy over 6 consecutive months. Assessment of symptoms and quality of life (by the Saint George Respiratory Questionnaire-SGRQ), pulmonary function tests (by full spirometry), and functional performance (by the 6-minute walking test-6MWD) will take place before and 6-month after bronchodilation therapy.

Change of SGRQ over time will be considered as the primary study goal, whereas changes in either respiratory function and functional performance will be explored as secondary outcomes. Student's t test and Fisher exact test or Chi square test (as appropriate) will be used for analysis. To assess the impact of bronchodilator therapy on the primary and secondary outcome multivariable regression analysis will be used. Statistical significance will be set at 0.05.

**Expected results**

We estimate a total number of 30 patients to run a preliminary analysis.

In this pilot study a significant improvement in quality of life (i.e., reduction of respiratory symptoms such as shortness of breath, cough and sputum production) is expected. If confirmed, data will be used for further studies to explore the potential benefit of bronchodilator therapy in patients with CPFE. Results will be therefore used to set-up a randomized cross-over trial.

***Dr. Adriana Scamporlino***

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## A NEW METHOD TO ESTIMATE PNEUMOTHORAX SIZE ON DIGITAL CHEST RADIOGRAPHS

**Background**

Determining the size of a pneumothorax is an important factor to guide its treatment, namely whether active drainage is required. Objective methods to measure pneumothorax are advisable to better guide clinical practice. Five different methods to estimate the pneumothorax size on chest-x-ray (CXR) have been described in the literature. The methods of Light and Collins are currently considered the most reliable. Nonetheless, they all have drawbacks and issues that have limited their routinely applications in clinical practice.

**Objectives**

The aim of the study is to present a novel method to estimate the size of a pneumothorax that is simple, rapid and suitable to be applied on digital CXR. The first part of the study evaluated the consistency of the proposed method; the second part compared it with the two currently most accepted methods (*i.e.* Light and Collins methods).

**Methods**

The study was conducted on patients with primary spontaneous pneumothorax treated at the Thoracic Surgery Unit of the University Hospital of Modena between 2010 and 2019. Digital CXR displaying the conditions of pneumothorax were retrospectively reviewed. The proposed method to estimate pneumothorax size, expressed as percentage (%PNX), consisted in calculating the ratio between the area of free-air ($A_{pnx}$) within the hemithorax and the total area of the hemithorax itself ($A_{ht}$). Both areas were manually drawn following anatomical landmarks and they were computed automatically by the software in $mm^2$. Intrarater and interrater reliability analysis were used to express consistency of measures obtained with our method. Linear regression was used to analyze the correlation between the proposed method and those of Light and Collins.

**Expected results**

The study included 200 patients and their corresponding 200 CXR. A very high level of agreement between measures taken multiple times by the same rater (intrarater reliability) and by three different raters (interrater reliability) was found. Linear regression showed a very high correlation between pneumothorax size measured by our methods and by both Light and Collins ones.

***Dr, Filippo Monelli***

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**QUANTITATIVE IMAGING TO PREDICT BIOLOGICAL AND GENETIC CHARACTERISTICS INFLUENCING TREATMENT RESPONSE IN ONCOLOGIC SETTING.**

**Background**

The project will explore the applicability of quantitative imaging in the prediction of treatment response. The first part of this project will focus on liver metastasis of colorectal cancer (CRC). CRC is the third most incident cancer worldwide in western countries and 25% of patients with CRC present with unresecable liver metastases at initial diagnosis. The management of metastatic CRC (mCRC) patients was improved by the introduction of target therapies. For example, the addition of cetuximab to first line regimen significantly improves response rate, progression free survival and overall survival in patients with wild type KRAS and RAS. Quantitative imaging is changing the role of radiology, allowing extraction of more information from medical images acquired in the clinical setting. While radiologist's eyes can perceive a limited amount of data, the analysis, through dedicated software, of medical images can extract hidden information which can be related to biological and genetic characteristics of the studied tissue. Liquid biopsies (LB) are a non-invasive method to obtain information on mutation status of genes involved in treatment response from circulating cancerous DNA.

**Objectives**

Project's purpose is to evaluate the feasibility of a process built to analyze routine medical images to extract useful information related to biological and genetic characteristics of the tumor and to correlate them to therapy response. In the mCRC study we will explore the presence of a correlation between quantitative features extracted from contrast enhanced CTs (CECT), cancer biological and genetic characteristics and treatment response. A predictive algorithm, including different quantitative features, will be produced both with a radiomic and a deep learning approach. The secondary aim is to compare the quantitative models to standard radiologists' evaluation through RECIST1.1 and morphological criteria, to evaluate the added value of radiomics in the evaluation of tumor characteristics and response to therapy.

**Methods**

First, a systematic review is being performed to evaluate current literature on the topic, both on the main project and on mCRC. The mCRC part of the project is an ancillary study from the ERMES trial on mCRC wild type KRAS, NRAS and BRAF patients treated with FOLFIRI plus cetuximab. Every patient received a CECT in portal venous phase at baseline, after 8 weeks of treatment, at disease progression (DP) and at 3 months after DP. At the same timepoints, a LB was collected from every patient to assess KRAS, NRAS and BRAF mutation status. A blinded radiologist will perform RECIST 1.1 and morphological evaluation of every CECT

segmenting every index lesion as volume of interest (VOI). VOIs will be extracted and associated with clinical, biological and genetic characteristics of the patient. For the radiomic approach methods, CECTs and VOIs will be processed in accordance with image biomarker standardization initiative and current literature on the topic using PyRadiomics derived software to obtain quantitative features. Those features will be analyzed using a machine learning method, based on the production of an algorithm to predict mutation statuses and response to therapy. As regards the deep learning approach, a deep learning system will be designed to process imaging and clinical data with the objective of reducing the complexity extracting useful information to obtain the prediction. Reported results will derive by the application of the produced algorithm to a validation cohort. Those results will be then compared to standard RECIST1.1 and morphological criteria.

**Expected results**

Expected results are to gather significant information from routine CECT exams on biological and genetic characteristics of the tumor which may predict mutation status, response to therapy and prognosis. In the future, this quantitative approach to medical images could lead to a better therapy selection pursuing precision medicine and personalized cancer care.

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