University of Modena and Reggio Emilia

PhD COURSE OF CLINICAL AND EXPERIMENTAL MEDICINE

PhD DAY 2020

Abstracts

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

This year back cover is dedicated to the efforts made by our PhD students in coping with the Coronavirus (COVID-19) emergency. An interview to Ivana and Roberto was reported by the magazine *Time* at the following link: https://time.com/5808933/italy-doctors-couple/

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

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

From 2018 (cycle XXXIV) 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

XXXIII cycle

Dr. Aida Meto

CEM Curriculum: Public Health
Tutor: Prof. Elisabetta Blasi
CoTutor: Prof. Eva Pericolini

INHIBITION OF *PSEUDOMONAS AERUGINOSA* GROWTH, BIOFILM FORMATION, PHENAZINE PRODUCTION AND eDNA RELEASE BY ALBANIAN PROPOLIS

Background

Pseudomonas aeruginosa (*P. aeruginosa*), an opportunistic pathogen responsible for wide spectrum infections especially in hospitalized individuals, has many virulence factors, including the ability to form biofilm onto both biotic and abiotic surfaces. Such event, known to be clinically crucial because often associated with negative outcomes, is regulated by complex mechanisms, involving tight adhesion, production of extracellular polysaccharide matrix and release of multi-functional molecules such as quorum sensing signals, phenazine, eDNA. Clinical and experimental studies provide growing evidence on the relevance of propolis, as a natural product with interesting anti-inflammatory and anti-microbial properties.

Objectives

By an engineered bioluminescent (BLI)-strain chosen as a ductile *in vitro* prototype, we assessed the susceptibility of *P. aeruginosa* to propolis; in particular, we focused on the effects of three different propolis extracts on microbial growth, biofilm formation, phenazine production and eDNA release.

Methods

Initially, an Albanian propolis was processed using dynamic maceration with three solvents [absolute ethanol (EtOH), propylene glycol (PG) and polyethylene glycol 400 (PEG 400)]; then, polyphenolic compounds present in the extracts were identified through High Performance Liquid Chromatography–Mass Spectrometric (HPLC-MS) analysis. Then, the three propolis extracts (EtOH-propolis, PG-propolis, PEG 400-propolis) were tested for anti-*P. aeruginosa* activity by Minimal Inhibitory Concentration (MIC) assay, while their effects on *P. aeruginosa* growth and biofilm formation were kinetically evaluated by the BLI-based assay. The phenazine production and eDNA release, in cell-free supernatants from *P. aeruginosa* exposed or not to propolis extracts were measured by LC-MS and fluorescence assay, respectively.

Results

The MIC values obtained for both propolis EtOH and PG extracts were 15.6 µg/mL. Differently, the MIC obtained for propolis PEG 400 extract was as high as 62.5 µg/mL. Moreover, the EtOH-propolis extract significantly inhibited *Pseudomonas* growth and biofilm formation as well; interestingly, this phenomenon was accompanied by a consistent reduction in phenazine production and eDNA release in the presence of

EtOH-propolis. Differently, the PG-propolis, PEG 400-propolis had partial or inconsistent effects, likely because of the toxicity of the solvent *per se*. Overall, we provide initial insights on the events related to the anti-*P. aeruginosa* activity of propolis.

Conclusions

Our results strengthen the importance of propolis as a natural antimicrobial product, active also against a clinically relevant pathogen such as *P. aeruginosa*. By a highly sensitive luminescence-based model, we provide the first evidence that propolis impairs *Pseudomonas* growth ability; also the production of biofilm and the release of molecules, such as phenazines and eDNA, are relevantly affected. In perspective, we may envisage propolis as a natural source of precious compounds, useful for the development of new therapeutics, particularly against biofilm-related infections.

Dr. Silvia Tanzi

CEM Curriculum: Translational Medicine
Tutor: Prof. S. Luminari

EARLY PALLIATIVE CARE IN HEMATOLOGIC ADVANCED PATIENTS

Background

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

Objectives

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

Methods

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

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

Results

The research protocol was published in BMC Palliative Care (https://doi.org/10.1186/s12904-020-00561-w). The Systematic review was under review in BMJ supportive and Palliative Care. A total of 315 studies titles were revised. Eight articles were included in the synthesis of the results, two controlled studies provided data on the comparative effectiveness of PC interventions, and six one-arm studies were included. Since data pooling and meta-analysis were not possible, only a narrative synthesis of the study results was performed. The quality of the two included comparative studies was low overall. The 6 one-arm studies provided information on the population, setting and intervention characteristics without the possibility of linking the observed results to the implemented interventions.

Patients' recruitment started in November 2018 and 13 patients were enrolled out of 60 expected. It is not possible to have preliminary data due to this low accrual. To improve patients' enrollment, we will open the trial as a multicenter study for 2020-2021.

Conclusions

Studies on early palliative care and hematologic cancer patients are scarce and have not been prospectively designed. The main investigated population is the post-transplanted population for whom the only RCT was performed, and another study is ongoing. Expected results of my controlled trial will be the description of new feasible integration between palliative and hematological care for advanced patients. Important preliminary results on the efficacy in QoL's dimensions will be collected, such as qualitative data on patients, involved caregivers and professionals.

Dr. Michele De Maria

CEM Curriculum: Translational Medicine
Tutor: Dr. Luigi Fontana
CoTutor: Dr. Domenico Merlo

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

Background

Despite the continuous development and the high level of safety of modern phacoemulsification, the proper management of intraocular inflammation is still a topic of debate. Uncontrolled inflammation after eye surgery increases the risk of post-operative complications, such as cystoid macular edema (CME). The majority of the studies analyzed the anti-inflammatory effect of ophthalmic steroids and non-steroidal anti-inflammatory drugs (NSAIDs) for preventing or treating of CME, principally measuring macular changes after surgery, more than quantitatively assessing the AC inflammatory response.

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

Objectives

The main objectives of this project are:

1. To compare the effectiveness of single therapy with NSAIDs versus corticosteroids in controlling intraocular inflammation after uncomplicated cataract surgery (Bromfenac vs Dexamethasone Study - *BVD Study)*;
2. to adopt the laser flare photometry (LFP) to obtain a quantitative and non-invasive assessment of the anterior chamber inflammation (*BVD Study*);
3. to review the literature focused on the results of studies that evaluated AC inflammation after uneventful cataract surgery, aiming to identify evidence of a correlation between quantitative measurements of AC inflammation and the risk of CME development.

Methods

Objective 1 and 2. The *BVD study* was a phase IV, single center, randomized, active-control, parallel design, open-label trial comparing Bromfenac 0.09% solution and Dexamethasone 0.1% suspension in patients that underwent uncomplicated cataract surgery. Patients with senile cataract and no other ocular comorbidities who underwent uneventful phacoemulsification were randomized 1:1 to receive dexamethasone 0.1% ophthalmic suspension or bromfenac ophthalmic solution 0.09% for two weeks. All patients underwent a complete ophthalmological evaluation on the day before surgery and postoperatively at day 1, 3, 7, 9, 11,

14, and 30. The LFP was used to objectively quantify anterior chamber inflammation and optical coherence tomography (OCT) to measure the macular thickness.

The follow-up was extended at 3 and 6 months from surgery. All patients were recalled to perform the LFP and the OCT to analyze the anterior chamber inflammation and the macular thickness in a long-term follow-up.

Objective 3. We searched the PubMed database (1949-2019) and Ovid Medline (1946-2018) for peer-reviewed publications relevant to the topic of AC inflammation after cataract surgery starting from 1989. Keywords included: cataract surgery, cystoid macular edema, AC inflammation, laser flare and cells photometry, anterior segment optical coherence tomography (AS-OCT) and aqueous sample. Only prospective studies on uneventful cataract surgery were selected. Only papers in which one of the primary or secondary outcomes were the assessment of AC inflammation were included.

Results

Objective 1 and 2. Seventy-six patients (37 in group 1 - Dexamethasone and 39 in group 2 - Bromfenac) with senile cataract and no other ocular comorbidities have been enrolled. Dexamethasone was not superior to bromfenac to reduce inflammation to the preoperative level. In both treatment groups, the mean LFP value increased the day after surgery and progressively decreased after starting the topical therapies, with no return at baseline at day 30. There was no statistically significant difference at all time points between dexamethasone and bromfenac groups in terms of mean laser flare. Visual acuity improved steadily after surgery in both groups. The mean macular thickness was higher in the dexamethasone group one month after surgery (293.51 ± 61.55 vs 274.82 ± 28.17 microns; $p = 0.0467$). The proportion of patients with central macular thickness >300 microns at day 30 did not differ between treatment groups.

In the long term follow-up, when compared to preoperative values, laser flare photometry demonstrated persistent ocular inflammation at post-operative day 90 and 180 in group 1, but not in group 2. Laser flare values showed a significant reduction in group 2 compared to group 1 throughout all the follow-up ($p<0.001$). The increase in mean CMT at day 90 and 180 respect to baseline was statistically significant in group 1 but not in group 2, where it decreased to levels similar to preoperative value. Dexamethasone group showed a higher increase in mean CMT compared to Bromfenac group throughout all the follow-up ($p<0.001$). The proportion of patients that developed pseudophakic CME was 14% (n=5) and 0% (n=0) in the Dexamethasone group and Bromfenac group, respectively ($p=0.02$). The bivariate analysis demonstrated a positive correlation among laser flare and CMT values in group 1 but not in group 2.

Objective 3. One hundred eighty-seven prospective trials investigating AC inflammation after uncomplicated cataract surgery were identified. Methods of analysis of AC inflammation and the frequency of macular changes were recorded. In the majority (51%) of the studies, inflammation was assessed by clinical grading, followed by laser flare and cell photometry (LFCP) (42%) and aqueous humour sample (4%). Few studies (4%) adopted a combined LFCP and aqueous sample or clinical grading analysis. Sixteen (9%) studies investigated

AC inflammation and macular changes by OCT (7%) or fluorescein angiography (2%). Correlation between the amount of post-operative AC inflammation and frequency of CME was documented in 7 studies, not confirmed in 2 studies, and not examined in the other 7.

Conclusion

Objective 1 and 2. Ophthalmic bromfenac and dexamethasone appear equally effective in reducing anterior chamber inflammation measured by laser flare after uncomplicated phacoemulsification.

Evaluation of operated eyes using the Laser Flare Photometer revealed subtle aspects of ocular inflammation that could not be detected by simple clinical observation. Inflammation measured as mean laser flare values increased after surgery and significantly decreased after starting topical treatment in both groups, but not all the patients returned to their preoperative laser flare values one month after cataract surgery. It would be useful to individuate beforehand the eyes that will need prolonged anti-inflammatory therapy after cataract surgery.

Long-term LFP analysis revealed that anterior chamber inflammation persists for more than 30 days in a significant proportion of patients after uncomplicated cataract surgery up to 3 months from surgery. This long-lasting inflammation might explain the occurrence of pseudophakic macular edema several weeks after uneventful cataract surgery in eyes that might have had prolonged subclinical inflammation but appeared unremarkable at slit-lamp examination.

Objective 3. LFP, more than the other methods of analysis, correlated with the frequency of CME postoperatively. Investigation of the relationship between AC inflammation and the risk of CME changes requires the adoption of quantitative methods of analysis of the inflammatory response after surgery. For this purpose, due to the low level of inflammation in the AC after uncomplicated cataract surgery, LFP, more than subjective clinical grading, seems a more sensitive and reproducible method of measurement.

The inflammation assessment after cataract surgery has a potential role in predicting the risk of CME development and may help to titrate the duration and intensity of treatment in relation to the surgical inflammatory response.

Dr. Francesco Venturelli

CEM Curriculum: Public Health
Tutor: Dr. Paolo Giorgi Rossi
CoTutor: Prof. Annalisa Bargellini

NEW TOOLS FOR HPV-BASED CERVICAL CANCER SCREENING

Background

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

Objectives

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

Methods

Reggio Emilia is coordinating the follow up and the analyses of the New Technologies in Cervical Cancer 2 (NTCC2) double testing accuracy trial. NTCC2 aims to measure the accuracy of mRNA and p16/Ki67 and their negative predictive value for CIN2 or more severe lesions (CIN2+). The project recruited women who were invited for a new screening round based on HPV-DNA test within the screening programs (i.e. aged 25-59). HPV-DNA positive women were tested for cytology, E6/E7 mRNA and p16/Ki67. Women with ASC-US or more severe (ASC-US+) cytology were referred to colposcopy whereas women with negative cytology were randomized to immediate colposcopy or to 1-year follow up by HPV-DNA test. All women referred to 1-year control were tested for HPV-DNA, mRNA and p16. Women negative to all tests were referred to 4-year follow up. Regardless of mRNA and p16 results, HPV-DNA positive women were referred to colposcopy. The final endpoint of the study is the confirmed CIN2+. All the lesions found during the 5.5-year follow up will be

included in the endpoint. The data collection on the 12 months follow up from all the five recruiting centres is complete.

The cross-sectional accuracy and the role of mRNA and p16/ki67 as predictors of CIN2+ and infection persistence were assessed.

Regarding the third objective, the first recommendation on the HPV vaccination of women treated for CIN2 or more severe lesions will be submitted to the National Institute of Health (SNLG) by the end of July 2020.

Results

To date, the recruitment phase of NTCC2 and the 12-month follow up has been completed and 40,509 women including 3147 (7.8%) HVP-DNA positive were included in the final analysis. Cumulatively, 174 CIN2+ (including 95 CIN3 and 1 Adenocarcinoma *in situ*) were found considering the first colposcopy performed at baseline or 12 months follow up.

Cross-sectional accuracy

The adjusted sensitivity for CIN2+ was 61.0% (95%CI 53.6-68.0), 94.4% (95%CI 89.1-97.3), and 75.2% (95%CI 68.1-81.6) for cytology, mRNA, and p16/ki67 respectively.

The immediate referral (test positivity) was 25.6% for cytology, 67.4% for mRNA, and 29.0% for p16/ki67, while overall referral (i.e. including 1-year referral) was 65.3%, 78.6%, and 63.7%, respectively. The Positive Predictive Value (PPV) was 9.5% for cytology. 8.3% for mRNA, and 10.1% for p16/ki67.

Prognostic value for regression of CIN2+ lesions and clearance of HPV DNA.

Of the 2308 HPV-positive cytology-negative women, relative detection in those randomized at 1-year retesting versus immediate colposcopy suggests a -28% CIN2+ regression; (95 % CI from a regression of -57% to an increase of 20%). Regression was almost null in women who were biomarker-positive at baseline, while in those biomarker-negatives was [-76% (95%CI from -97% to more than 100%) and -39% (95%CI from -72% to 33%) for E6/E7 mRNA and p16/ki67, respectively]. However, the test for interaction was not statistically significant in either case.

Moreover, among HPV DNA-positive/cytology-negative women, the clearance of HPV DNA after 12 months in mRNA-negative women was 1.9 times (95% CI 1.7, 2.2) that in mRNA-positive women and clearance in p16/ki67-negative women was 1.9 times (95% CI 1.5, 2.5) that in p16/ki67-positive women.

Conclusions

The most promising test as triage for HPV DNA-positive women was P16/ki67. mRNA E6/E7 overexpression showed too high referral rate among HPV DNA-positive women to be efficient as triage test, but when negative it showed a good prognostic value to identify infections at high probability of clearance and regressive CIN2+. The performance of combinations of tests in triage of HPV positive women and the performance of mRNA E6/E7 overexpression as primary test for cervical cancer screening will be analysed.

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

The first recommendation of the updated GISCi guidelines is expected to be published by the end of 2020.

Dr. Mariarosa Maiorana

CEM Curriculum: Translational Medicine
Tutor: Dr. Francesco Iannuzzella

ROCK STUDY: RESEARCH STUDY OF CANCER-ASSOCIATED KIDNEY DISEASE

Background

The improvement in the survival rates of cancer patients due to the new oncological and biological agents has led to an increase in those who develop kidney diseases.

It is now well known that chronic kidney disease (CKD), acute kidney injury (AKI) and cancer are connected in several ways. However, the overall incidence and prevalence of CKD and AKI in cancer patients are still uncertain.

Objectives

ROCK study had as main objectives to estimate the prevalence and incidence of CKD and the incidence rate of AKI in patients included in the Cancer Registry of the province of Reggio Emilia in the first 24 months after cancer diagnosis; to evaluate the effect of AKI on clinical outcomes of mortality and progression of nephropathy. Moreover, to evaluate the prevalence of CKD for various types of primary cancer; identify critical issues for a specialist nephrological counseling course for cancer patients at high risk of AKI and progression to CKD.

Methods

We performed a monocentric, observational, retrospective cohort study on data relating to patients included in the Cancer Registry of the province of Reggio Emilia, from January 1, 2016 to December 31, 2016. Moreover, a case-control study was performed. Inclusion criteria were: patients included in the Cancer Registry of the province of Reggio Emilia with a cancer diagnosis occurred in 2016; age > 18 years. Patients with age < 18 years, chronic myeloproliferative diseases and myelodysplastic syndromes and/or non-melanomatous skin cancer were excluded. Different data sources were used (Cancer Registry, Laboratory Results Database, Diabetes and Mortality Registry, Hospital discharge records archive).

For each patient, we collected data about gender, age, ethnicity, weight, serum creatinine and estimated glomerular filtration rate, type of tumor, possible presence of metastasis and prescribed anticancer drugs (type of drug used, number of cycles of therapy).

Results

The study population consisted of 4254 patients with cancer diagnosis; 202 patients were excluded (data not available); 505 patients (12%) had a pre-existing CKD (eGFR < 60 ml/min); 807 patients (19.9%) had an eGFR < 60 ml/min at the moment of cancer diagnosis. Of 505 patients with CKD, 53% were male, 65% aged 65-84 and 37% had diabetes mellitus. The most frequent types of cancer were lungs (11%) and colorectal tumor (8.5%), followed by kidney and urinary tract cancer (7.7%). Further data are still ongoing.

Conclusions

Expected results of ROCK Study are the application of measures to reduce the risk of AKI and its progression to CKD; improvement of clinical outcomes high risk hospitalized cancer patients; creating a predictive score to identify high risk patients for developing AKI (confirmed by prospective studies).

Dr. Maurizio Zizzo

CEM Curriculum: Translational Medicine
Tutor: Dr. Filippo Lococo

ACUTE RADIATION COLITIS AFTER PREOPERATIVE SHORT-COURSE RADIOTHERAPY FOR RECTAL CANCER: A MORPHOLOGICAL, IMMUNOHISTOCHEMICAL AND GENETIC STUDY

Background

Rectal cancer is one of the most common malignant tumors in Western Countries. Surgery is the treatment of choice in rectal cancer and the use of surgical procedures such as total mesorectal excision (TME) has significantly improved the local control of the disease. Currently, radiotherapy is considered the standard treatment for patients with locally advanced rectal cancer with a high risk of local recurrence, if treated with surgery alone. Preoperative radiotherapy is often preferred to postoperative radiation because of better tolerability. Preoperative radiotherapy with or without chemotherapy has been demonstrated of value in reducing local recurrence rates and improving overall survival.

Based on the National Comprehensive Cancer Network (NCCN) Clinical Practical Guidelines in Oncology, there are two neoadjuvant regimens accepted for rectal cancer patients: preoperative long-course radiotherapy (PLRT) - 45-50 Gy in 4-6 weeks, followed by surgery 4 weeks later - with concomitant chemotherapy and preoperative short-course radiotherapy (PSRT) - 25 Gy administered in 5 consecutive days, followed by surgery a few days after. PSRT is not recommended for low-lying tumors, <5 cm from anal verge. PLRT is preferred for either low-seated or bulky, unresectable tumors which should benefit from radiation-induced down-staging.

Preoperative radiation may produce morphological modifications in terms of tumor regression by replacing neoplastic glands with fibrosis and inflammation. Tumor regression is mainly seen in PLRT. PSRT is not associated with significant tumor regression, as the interval from the end of radiotherapy to surgery is too short to allow tumor down-staging.

Very few studies analyzed in details the histopathological features of radiation damage on normal colonic mucosa. Depending on the type of preoperative radiotherapy whether short-course or long-course, different modifications may occur in normal colonic mucosa either in terms of inflammation or of glandular distortion and nuclear/cytoplasmic atypia. Of note are the glandular disarray and cytological atypia associated with PSRT.

Objectives

Intrigued by the initial observation that PSRT-associated morphological abnormalities may closely mimic dysplasia, causing possible diagnostic misinterpretation with serious implications, we designed the current clinicopathological and molecular study comparing two groups of rectal cancer patients treated either with PSRT or PLRT.

In particular, we performed pathological examinations of surgical specimens and DNA mutation analysis of both tumor and mucosa with atypical features, on a subset of PSRT cases in order to further confirm that the morphological "dysplastic-like" features were not true dysplasia.

As immunohistochemical p53 staining is commonly considered a surrogate for mutational analysis, we performed p53 immunohistochemistry (IHC) in the same subset of PSRT cases evaluated by next generation sequencing (NGS) analysis. Additionally, an increase in endocrine cells has been previously reported in irradiated carcinoma of rectum, we expanded that observation assessing this phenomenon in both the benign and malignant tissue of both PSRT and PLRT patients.

Methods

A retrospective analysis of clinical, pathological, surgical and follow-up data were collected using a dedicate anonymous database. All consecutive patients treated with radical surgery and preoperative radiotherapy for locally advanced rectal adenocarcinoma at the Azienda Unità Sanitaria Locale-IRCCS di Reggio Emilia - Presidio Ospedaliero Arcispedale Santa Maria Nuova di Reggio Emilia) between 2000 and 2017 were enrolled in the present study.

The patients were divided into two groups according to the preoperative radiation protocol.

We collected surgical specimens of primary site (rectal cancer) at the Pathology Unit of Promoting Center (Azienda Unità Sanitaria Locale-IRCCS di Reggio Emilia). All hematoxylin and eosin-stained slides were reviewed independently by two pathologists. Non-neoplastic mucosa and tumor sections were assessed to detect radiation-induced changes and neuroendocrine cells presence and morphology. Representative tumor sections were selected for grading of tumor regression according to Dworak system. The degree of radiation damage was assessed in the non-neoplastic mucosa, including the surgical resection margins. The following histological parameters of radiation colitis were evaluated: inflammation in the lamina propria, architectural crypt distortion, nuclear and cytoplasmic atypia of glandular epithelium and apoptotic bodies. All these features were scored as absent, mild, moderate and severe.

Using an automated immunostainer (Benchmark, Ventana, Tucson, AZ) a subset of samples was stained with chromogranin A (LK 2H10, monoclonal antibody, Ventana), synaptophysin (SP11, monoclonal antibody, Ventana) and p53 (DO-7 monoclonal antibody, Ventana).

Finally, genomic DNA, from both tumor and "dysplastic-like" mucosa samples of a subset of 24 PSRT patients was isolated by Maxwell DNA FFPE Kit (Promega, USA), according to the manufacturer instructions. DNA concentration was determined using Qubit dsDNA HS assay kit (Invitrogene). 22 samples were eligible for

sequencing analysis. For the Next Generation Sequencing analysis, DNA libraries were prepared using Myriapod NGS-IL 56G Onco Panel for Illumina (Diatech Pharmacogenetics), that allows the identification of main mutations in 56 oncogenes, following the manufacturer instructions. Libraries quality and quantity were assessed by Agilent Bioanalyzer High Sensitivity kit (Agilent Technologies) and Qubit dsDNA HS assay kit (Invitrogene) respectively. Sequencing run on Illumina MiSeq V2 (2x151) cartridge. Sequencing data analysis was conducted by Myriapod NGS Analysis software ad further analysis were performed using R software (v 3.5.1).

Results

Tumor regression

According to the tumor regression system by Dworak *et al*, in the PSRT group, 31 cases were classified as not regressed (grade 0) and 14 cases as grade 1. In the PLRT group the tumors were classified as follows: 12 cases: grade 0; 15 cases: grade 1; 20 cases: grade 2; 2 cases: grade 3 and only 1 case: grade 4 with a complete pathological response and no residual tumor.

Radiation-induced morphological features

The histopathological features of radiation colitis were noted in the non-neoplastic mucosa in samples taken close to the tumor and in samples taken up to 4 cm from the tumor, but within the irradiated volume. Radiation-induced features were identified even in the resection margins, if within the irradiated volume. Two types of parameters were analyzed: the inflammatory component and the glandular ("dysplastic-like") abnormalities.

Inflammatory component

In PSRT cases, the non-neoplastic mucosa within the irradiated volume showed a moderate to marked inflammation within the lamina propria. The inflammation consisted of histiocytes, lymphocytes, plasma cells and often numerous granulocytes, mainly eosinophils. The eosinophils themselves were identified in the lamina propria either scattered or in small aggregates and within the glandular epithelium. In PLRT samples the inflammation went from absent to a mild chronic inflammatory infiltrate consisting of histiocytes, lymphocytes and plasma cells. Of note, eosinophils were scarcely represented.

"Dysplastic-like" features

In PSRT cases, there was a moderate to marked degree of glandular disarray and distortion as well as nuclear and cytoplasmic atypia. The crypts were often decreased in number and appeared often dilated or with a slit-like lumen. The crypt epithelium was either flattened or pseudostratified showing a variable degree of nuclear pleomorphism, sometimes with rather bizarre nuclei. The cytoplasm of the crypt epithelium was eosinophilic or vacuolated. Apoptotic bodies were often identified. All these features were named "dysplastic-like" for simplicity.

In contrast to the short-course group, in PLRT cases the dysplastic-like features (i.e. glandular architectural abnormalities, cytological atypia, and apoptotic bodies) were either absent or only occasionally identified.

Endocrine features

The presence of chromogranin- and synaptophysin-positive endocrine cells showed differences according to the type of radiation protocol. In PSRT cases, the radiation-damaged mucosa with “dysplastic-like” features showed an increase in endocrine cells either with isolated cells (5) or micronests (19), whereas absence of endocrine cells was seen only in one case. Compared to PSRT cases, in the PLRT group, the non-neoplastic mucosa within the irradiated volume showed either absence of endocrine cells (20) or only a mild increase with isolated cells (5). No relevant differences were seen in terms of endocrine differentiation in tumor samples of both protocols. In PSRT tumor samples the endocrine cells were either absent (23), isolated (1) or in micronests (1). In PLRT tumor samples the endocrine cells were absent.

p53 immunohistochemical results

In a subset of 22 PSRT cases, different p53 staining patterns were identified in tumors. A strong and diffuse p53 expression (“positive-pattern”) was present in 15/22 tumors; a complete lack of expression (“negative-pattern”) was seen in 5/22 tumors; scattered p53-positive cells (“reactive-pattern”) were present in 2/22 tumors.

In all samples examined, the mucosa with acute radiation-damage showed a positive p53 staining limited to the deep portion of the glandular epithelium which represents the proliferative compartment of the glands.

Genetic alterations in tumors and in tissue samples with “dysplastic-like" features

We evaluated quality of 48 tissue DNA from both tumor and “dysplastic-like” mucosa of 24 PSRT patients included in the study. Only in 22 patients we obtained, from both components, DNA eligible for NGS analysis. On these samples, we performed a deep sequencing analysis on a commercial panel of 56 genes frequently mutated in cancer, detecting 958 alterations. Subsequently, the analysis was restricted to coding regions variants, excluding intronic, 5’-3’ UTR and downstream gene variants. Two hundred sixty-six mutations were found, of which 146 (54.9%) were synonymous, 103 (38.7%) missense and a small percentage was composed by stop gained and frameshift alterations (4.1% and 2.3% respectively). Based on literature, ExAc frequency and variant frequency 230/266 alterations (86.5%) were classified as germinal and 36/266 (13.5%) as somatic. In each patient, dysplastic-like mucosa and tumor, shared the same germline alterations confirming the constitutiveness of these variants and the validity of our analysis. By contrast, somatic mutations were present only in tumors, suggesting that “dysplastic-like” tissues are not genetically transformed. Between tumor-associated somatic mutations 52.8% were missense, 30.6% were stop gained and 16.6% were frameshift.

Somatic mutations were detected in 7/56 genes and gene alterations frequencies were in line with TCGA data on colorectal cancer. The most frequently mutated genes were APC and TP53 detected in 47.8% of patients. 66.7% of the described somatic alterations were annotated in these genes, mutations in APC were in particular stop gained and frameshift variants while TP53 presented a majority of missense mutations. Moreover, 26.1% of analyzed tumors presented missense mutations in KRAS (6/22) and 13% in PIK3CA. Finally,

only one tumor presented a missense mutation in FBXW7 and a stop mutation in SMAD4 and one had a missense mutation in EGFR.

Comparison between p53 phenotype and TP53 genotype in tumor and mucosa with "dysplastic-like" features

Mutant TP53 was associated with diffuse and intense p53 immunostaining ("positive-pattern") in 9/22 tumors. This "positive-pattern" was also present in 6/22 tumors with wild-type TP53. Of 5/22 completely p53-negative ("negative-pattern") tumors, 2 cases had a mutation of TP53 and 3 cases were wild-type TP53. Two tumors with wild-type TP53 showed only rare, scattered p53-positive cells ("reactive-pattern").

All 22 samples of mucosa with "dysplastic-like" features were TP53 wild-type and showed p53 immunostaining only in the deep, proliferative portion of the glandular epithelium.

Conclusions

The data of the present study are a comprehensive and detailed morphological description of radiation-induced abnormalities identified after radiotherapy for rectal cancer. The use of NGS analysis further validated the morphological concept that radiation-induced abnormalities do not represent pre-neoplastic lesions. Pathologists need to be aware of the rather challenging morphological features induced by radiation, in order to avoid serious diagnostic problems.

Dr. Giulia Besutti

CEM Curriculum: Translational Medicine
Tutor: Dr. Paolo Giorgi Rossi
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EFFICIENCY AND APPLICABILITY OF INTERNATIONAL GUIDELINES FOR NON-ALCOHOLIC FATTY LIVER DISEASE ASSESSMENT IN HIGH RISK PATIENTS AND ACCURACY OF IMAGING TESTS IN THE DIAGNOSIS OF NON-ALCOHOLIC STEATOHEPATITIS AND FIBROSIS

Background

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

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

Objectives

AIM 1: The primary objective is to evaluate guideline efficiency in diagnosing NASH/fibrosis among high-risk patients, by calculating the positive predictive value of specialist referral (PPV1) and liver biopsy (PPV2). Secondarily, we will evaluate: 1) PPV1-PPV2 in subgroups with different referral criteria; 2) burden of generated clinical exams; 3) number of subjects excluded for other conditions; 4) adherence to referrals.

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

Methods

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

AIM 2: We included in the systematic review cross-sectional/cohort studies of NAFLD patients comparing imaging with LB searched on MEDLINE, Scopus, EMBASE and Cochrane databases, screened on title/abstract and assessed for eligibility on full-text. Risk of bias was assessed using QUADAS-2. In an ongoing prospective study, NAFLD patients referred for LB undergo: 1) US obtaining US-FLI and Shear Wave Elastography (SWE) stiffness, 2) multiparametric liver non-contrast-enhanced MR, obtaining Proton Density Fat Fraction (PDFF), T2*, T1, spectroscopy-derived fat peaks, and IVIM diffusion parameters (D, D*, F). Sensitivity and specificity of different imaging tests for NASH and fibrosis diagnosis will be calculated, using LB as the reference.

Results

AIM 1: Between January 2019 and January 2020, guidelines have been applied to 272 T2DM patients (40,4% females, 60±15.5 y/o) at the first access to the Reggio Emilia diabetology clinic. Of them, 30 were aged >75 y/o, 31 had other chronic liver disease/causes of steatosis, and 41 had incomplete serum markers to calculate risk and will be re-evaluated. Of the remaining 170, 35 needed no further referrals, 135 were referred to liver US (60 for elevated liver enzymes and 75 for high scores). Of the 125 patients who underwent US, 108 were referred to the hepatologist, and 30/72 patients evaluated by the hepatologist were referred for LB. The 14 biopsies performed by now resulted in 12 NASH, only 1 of them with significant fibrosis.

AIM 2: Among techniques included in the systematic review on imaging diagnosis of NASH (641 records screened, 61 included in a scoping review, 30 with accuracy results included in data synthesis), US and MR imaging (elastography and non-elastographic methods), have shown promising accuracy (AUROCs 0.82-1.00). However, the studies were heterogeneous, conducted in limited series of patients, and lacking independent validation. Between March 2019 and February 2020, 23 NAFLD patients with indication for LB performed US and MR. Of the 18/23 patients with already available LB results, 4 had significant fibrosis, and 15 had NASH (activity=2 in 9, >2 in 6 patients). US resulted in poor accuracy for NASH diagnosis (7 TP, 1 TN, 3 FP, 7 FN; sensitivity 50%, specificity 25% for US-FLI≥4), but ruled out significant fibrosis (4 TP, 8 TN, 6 FP, for SWE stiffness >6 Kpa). MR-T1 <325 msec resulted in 67% sensitivity and 79% specificity in the diagnosis of significant fibrosis (11 TN, 2 TP, 1 FN, 3 FP), and 38% sensitivity and 80% specificity in NASH diagnosis (5 TP, 4 TN, 8 FN, 1 FP); an algorithm enabling T2* correction is under development to improve MR-T1 performance.

Conclusions and future perspectives

The application of recommendations to a selected cohort of high-risk patients is generating a high burden of hepatologist evaluations and LBs. Future results (AIM 1) will help assessing the feasibility of recommendations and will allow the evaluation of different scenarios according to changes in the referral criteria. Our systematic review demonstrated that there is currently insufficient evidence to support the use of imaging to diagnose NASH. With our study (AIM 2) we expect to contribute to test whether any the combination of imaging tests could be accurate enough to potentially replace LB.

Dr. Jovana Milic

CEM Curriculum: Translational Medicine
Tutor: Prof. Giovanni Guaraldi

LIVER STEATOSIS AND NON-ALCOHOLIC FATTY LIVER DISEASE WITH FIBROSIS ARE PREDICTORS OF FRAILTY IN PEOPLE LIVING WITH HIV

Background

Non-alcoholic fatty liver disease (NAFLD) has become an emerging condition in general aging population and the most common cause of chronic liver disease. Recent studies confirm the increasing burden of NAFLD in people living with HIV (PLWH), as viral hepatitis prevalence and associated mortality decline. Nevertheless, NAFLD should not be considered as a liver condition only, but rather as a multisystemic state involving other organs. Accumulation of non-infectious co-morbidities described by multimorbidity, is a typical feature in people reaching geriatric age, nevertheless this construct fails to describe the clinical complexity of aging. So far, it has been shown that frailty represents a more accurate measure of an individual's biological age. Frailty is defined as a condition characterized by the reduction of homeostatic reserves exposing the individual to a greater risk of negative outcomes such as multimorbidity, falls, disability, nursing home placement and death. We hypothesized that NAFLD could be a significant determinant of frailty, in the context of a multisystemic nature of both these conditions.

Objectives

The objective of the study was to investigate the correlation between liver steatosis and significant fibrosis alone and in association (NAFLD with fibrosis) and frailty, as a measure of biological age, in PLWH.

Methods

This was a cross-sectional study that included consecutive PLWH attending Modena HIV Metabolic Clinic (MHMC) from June 2018 to May 2019. We included ART-experienced PLWH who were evaluated for liver steatosis and fibrosis by transient elastography (TE) at MHMC. Patients with hepatitis B (HBV), hepatitis C (HCV) co-infection and hazardous alcohol intake were excluded from the study. HBV and HCV co-infection diagnosis were based on serology, while the alcohol intake was evaluated through an AUDIT questionnaire. Liver stiffness measurement (LSM) and associated controlled attenuation parameter (CAP) were evaluated using TE with M probe. Liver steatosis was diagnosed by CAP as follows: S0 (no steatosis; CAP<248 dB/m), S1 (mild steatosis; 248≥ CAP<268), S2 (moderate steatosis; 269≥ CAP<280), S3 (severe steatosis; CAP≥ 280) dB/m. All measurements >248 dB/m were considered as NAFLD. Liver fibrosis was diagnosed by LSM as follows: stage F0-F1 (mild fibrosis, LSM<7.1 kPa), F2-F3 (significant fibrosis, 7.1≥LSM<13), F4 (cirrhosis,

LSM≥13 kPa). NAFLD with fibrosis was defined as the contemporary presence of liver steatosis (CAP≥248) and significant liver fibrosis or cirrhosis (stage ≥F2). Frailty was determined using 36-Item frailty index (FI). Each variable included in the FI was coded with a value of 1 when a deficit was present, and 0 when it was absent. We categorized PLWH according to FI score as fit (<0.25), frail (0.25-0.4), most frail (>0.4). Logistic regression models were built to explore the contribution of liver steatosis and significant fibrosis alone and in association (NAFLD with fibrosis) to frailty along with age, gender, diabetes mellitus and multi-morbidity.

Results

We analyzed 707 PLWH. Mean age was 53.5 years, 76.2% were males, mean BMI was 24.6 (±4.2), 18.3% had T2DM, median CD4 was 700 µL (IQR=540-889), HIV RNA viral load was undetectable in 98.7% of cases. NAFLD with fibrosis was present in 10.2%, frail and most-frail in 18.9% and 3.9%, respectively. Frailty was present in 41.2% and 52.3% in PLWH with mild/moderate and severe liver steatosis respectively. In PLWH with NAFLD with fibrosis, frailty was identified in 69%.

NAFLD with fibrosis group showed a higher prevalence of obesity (36% vs. 6%) (p<0.001). With regards to HIV variables, PLWH with NAFLD with fibrosis presented a longer mean HIV duration and lower CD4 nadir. The use of NNRTI was observed in 24% of PLWH with more than two times higher use in people without NAFLD with fibrosis. Univariate analysis demonstrated that neurocognitive impairment (OR=5.08, 1.61-14.96), Vitamin D insufficiency (OR=1.94, 1.18-3.24), obesity (OR=8.06, 4.44-14.55), T2DM (OR=3.24, 1.91-5.59) and osteoporosis (OR=0.37, 0.16-0.76) were associated with NAFLD with fibrosis.

To better explore this association a multivariate logistic model was built. Independent positive predictors for FI were steatosis (OR=2.12, 1.3-3.45) and fibrosis (OR=1.95, 1.03-3.66) alone and in association – NAFLD with fibrosis (OR=9.19, 5.17-16.79), T2DM (OR=1.65, 1-2.74) and MM (OR=2.46, 1.53-4.01).

Conclusions

Liver steatosis and fibrosis alone and in association increased the risk of frailty. NAFLD with fibrosis exceeded multimorbidity in the prediction of frailty, suggesting the former as an indicator of metabolic age in PLWH.

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MODELS OF MESIAL TEMPORAL LOBE EPILEPSY IN VIVO: HYPERTROPHY IN AMYGDALOPETAL CIRCUITRIES AND INNOVATIVE THERAPEUTICAL APPROACHES

Background

The mesial temporal lobe epilepsy (MTLE) is the most common (about ¾ of adult people with epilepsy) form of structural epilepsy (that is, related to a well-defined histopathologic lesion). About a half to ⅔ of people with MTLE do not achieve satisfying control of seizures with the standard clinical treatment, that, at least regarding the odds ratio of SUDEP, is considered ≤1 generalized seizure per year. Also, people with epilepsy present an incidence of cognitive and psychiatric comorbidities significantly higher than age- and sex-matched non-epileptic people. This have driven attention of the researches for the need of more effective therapeutic alternatives to treat epilepsy and its comorbidities. In this context, we have used in vivo models of post-status epilepticus (SE) for investigating plasticity of neural circuitries which underpins the epileptogenic process and may contribute for the respective comorbidities. Also, we have used the same models to test new candidates for antiepileptic drug development.

Objectives

. To ascertain the state of the cholinergic projections from basal forebrain (BF) to the amygdala by morphometric analysis in experimental MTLE.

. To ascertain the state of the γ-amino-butyric (GABA)ergic projections form basal forebtrain (BF) to the amygdala by morphometric analysis in experimental MTLE.

. To evaluate natural compounds as potential prototypes for development of antiepileptic drugs.

Methods

Status epilepticus (SE) was induced by chemoconvulsants (kainic acid or pilocarpine) by systemic or intracerebral route. Ethologic analysis was carried out. Sometimes, video-electrocorticogram (v-ECoG) tracks were taken and analyzed. Animals were euthanized either in subacute or chronic period. Tissues were

harvested and stored for cresyl-violet and FluoroJade-B staining, as well as for immunohistochemcal (IH), immunofluorescence (IF) or Western blot (WB) assays. Morphometric measurement of brain structures and cells was carried out in a microscope equipped with appropriate soft- and hardware. Ethanol extraction of plant material, preparative chromatography, ^{2}H and ^{13}C MRI, as well as mass spectrometry assays were carried out. Statistics (goodness of fit, ANOVA, Student's *t*-test, and *post hoc* Bonferroni's or Tukey's tests for multiple comparisons) were carried out in Prism-7 software.

Results

In the context of mesial temporal lobe MTLE epilepsy, oppositely to the hippocampus, there are relatively scarce data of data about amygdala involvement in humans, as well in animal models. We have recently published a paper [1], for the best of our knowledge, the first report of hypertrophy of cholinergic neurons from basal forebrain projecting to amygdala (namely the basolateral complex) in the kainic acid model of MTLE in rats. These pathologic alterations can contribute for the epilepsy *per se* as well as for the inherent cognitive and psychiatric comorbidities. Yet, concerning the model of the systemic kainic acid, we have found a strong correlation between the latency for the first generalized seizure, with loss of postural reflex, after systemic administration of kainic acid with the latency for the first spontaneous recurrent seizure (SRS) after the silent period; as well as a strong correlation of the cumulative length of the convulsive seizures (Racine $\geq$3) during kainic acid-induced status epilepticus (SE) with the number of brain structures damaged [2]. At the same time, we have had accepted for the 14th European Congress on Epilepsy an abstract [3] reporting the antiseizure activity of a proline derivative from natural source in a MTLE model by systemic pilocarpine. The full version of this paper is now waiting for an experiment required by the referees [4]. A "twin" work, about the same proline derivative compound in a model of intracerebroventricular chemoconvulsant was submitted for review recently [5]. Now, we are carrying out a stereological measurement of GABAergic projection neurons in the basal forebrain (BF) in the kainate model of MTLE in order to disclose of possible trophic alterations.

Conclusions

As stated above, the burden of epilepsy is most due to uncontrolled seizures and behavioral and cognitive interictal phenotypes. On this background, our work can contribute for devising new therapeutic targets to mitigate the disease and its comorbidities and, perhaps modify their progression.

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5. Aquino, P E A; Lustosa, I R; Sousa, C N S; Chaves Filho, A J M; Lima, F A V; Santos, A D; Gramosa N V; Silveira E R; Viana G S B. The methanol fraction from Sideroxylon obtusifolium, rich in N-methyl (2S,4R)-trans-4-hydroxy-L-proline shows an anticonvulsant activity, associated to its anti-inflammatory/antioxidant actions (UNDER REVIEW)

XXXIV cycle

Dr. Giulia Rioli

CEM Curriculum: Health Sciences
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THE COMORBIDITY BETWEEN METABOLIC SYNDROME, COLORECTAL ADENOMAS, ANXIETY AND DEPRESSION: A CROSS-SECTIONAL STUDY AMONG OUTPATIENTS ACCORDING TO A PSYCHO-NEURO-IMMUNO-ENDOCRINOLOGICAL (PNEI) PERSPECTIVE

Background

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

Both Metabolic Syndrome (MetS), CRC and psychiatric symptoms, especially anxious-depressive symptomatology, could be related to high levels of psychosocial stress and to an impaired quality of life, through still unclear mechanisms.

A chronic low-grade inflammation state could mediate these associations, according to a PNEI perspective.

Objectives

Primary aim of this study was to measure prevalence and association between colorectal adenomas, MetS, anxious-depressive symptoms and personality traits in outpatients.

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

Methods

In this cross-sectional study, outpatients of both gender, aged between 18 and 80 years, referred to Modena University Hospital for colonoscopy were enrolled. Patients were excluded if they have been diagnosed with disorders or prescribed with medications primarily affecting immune or inflammatory system activity, such as anti-inflammatory drugs. Moreover, patients affected by the following conditions were excluded because their potential of confounding with the major focus of the research: prior stroke, heart attack or other cardiovascular disease; type 1 diabetes mellitus; obesity of genetic aetiology; pregnancy; current psychopharmacological therapy or estro-progestinic medications; positive history of major psychiatric disorders (schizophrenia spectrum or bipolar disorders).

For each enrolled patient, sociodemographic and clinical information were collected: height (cm), weight (kg), waist circumference (cm), arterial blood pressure (mmHg), low and high density lipoprotein cholesterol (mg/dL), triglycerides (mg/dL), fasting plasma glucose (mg/dL), c-reactive protein (CRP) and serum cytokines (IL-1, IL-6, TNFa, 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). Personality traits were assessed by means of The Temperament and Character Inventory (TCI), a self-assessment 240-items test. To assess patients' perception of own general health status, the 36-item Short Form Survey (SF-36), Italian version, with its two subscales (MCS, Mental Component Summary and PCS, Physical Component Summary), was used. Descriptive statistics were performed as means, frequencies, standard deviations and ranges. Correlations were measured via the Pearson' coefficient. Statistical analysis was performed with the software STATA 14.

Results

Sixty-two patients were recruited (M/F 31/31), with a mean age of 60.8 ± 9.4 years. Twenty-eight participants were affected by at least one colorectal adenoma (45.2% of the total sample), 19 (67.9%) of those were males. MetS was diagnosed in 26 participants (41.9% of the sample). Clinically significant anxiety and depressive symptoms were detected in 16 (32.7%) and 9 (18.4%) subjects; 4 (8.2%) patients suffered from combined anxiety-depressive symptoms. Detection of adenoma correlated to male sex ($r=0.32$; $p=0.01$), age ($r=0.34$, $p<0.01$), IL-6 ($r=0.31$; $p=0.03$), CRP ($r=0.27$; $p=0.04$), and diagnosis of MetS ($r=0.28$; $p=0.03$). MetS correlated with age ($r=0.32$; $p<0.001$) and IL-6 ($r=0.37$; $p<0.01$).

Conclusions

Findings support the hypothesis of a link between a proinflammatory atherogenic status, psychological traits, increased mucosal inflammation and metabolic parameters in a sample of outpatients screened for adenomas. Our preliminary results could suggest that such an integrated PNEI approach could help clinicians in the prevention and treatment of their patients considering the issue of multimorbidity, rather than focusing separately on each single component of pathogenesis. Such a comprehensive assessment could be further developed in future projects with larger samples.

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CEM Curriculum: Translational Medicine
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GENOMIC CHARACTERIZATION OF CHRONIC MYELOPROLIFERATIVE NEOPLASMS

Background

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

During the past decade, several aberrations have been identified and characterized in MPN patients, such as the dysregulation of JAK2 signalling in 2005 and the CALR mutations in 2013. Still, the molecular pathogenesis of these malignancies remains incompletely understood and, for most MPNs, allogenic stem cell transplantation is the only curative treatment option. Diagnostic features and outcomes in patients show strong heterogeneity, probably as a consequence of a complex genetic landscape in addition to changes in gene expression. Currently, risk stratification of MPN patients is based mainly on clinical features and the presence of driver mutations. Gene expression data have already been used to provide prognostic indications in haematological diseases, nevertheless, none of the existing models for MPNs integrates transcriptomic data. Therefore, there is a need to better characterize the transcriptomic profile of these disorders in order to elaborate a signature able to add more robustness to clinically widely accepted scoring systems.

Objectives

The main scope of this project is to identify a molecular signature able to distinguish "high risk" MPN patients with inferior overall survival from "low risk" ones. In particular, we intend to compare it with the current predictive models in terms of prognostic power and investigate how, if combined to them as an independent prognostic factor, it can improve the ability to direct patients toward the best available therapeutic strategy.

Methods

We analysed the gene expression profile of granulocytes isolated from 114 MF patients (35 prefibrotic/early PMF, 37 overt PMF, 26 PET and 16 PPV). In order to identify transcripts whose expression is related to survival, we performed a Cox regression analysis using the *survival* package (v.3.1.8) in R (v.3.5.1). Supervised

clustering of samples using survival-related probesets, was carried out using the R package *ComplexHeatmap* (v.1.20.0), with the aim of defining risk classes. The *rms* (v.5.1.4) and *survival* R packages were exploited to conduct survival analysis with the Kaplan-Meier method and to perform log-rank test for survival curves comparison. Clinical data was analysed using the *stats* R package (v.3.5.1) by means of Fisher's exact test or Chi-square test for categorical variables, and Mann-Whitney test for continuous non-normal variables.

In order to optimize the number of probesets and to build a robust classification model, we applied a supervised learning technique, the "nearest shrunken centroids" method, and cross-validated the model using the leave-one-out method. Both methods are implemented in the R package *pamr* (v.1.56.1). Specifically, the following steps were performed:

1) Classification model generation:
   - for each risk class defined with supervised clustering a standardized centroid was calculated. The centroid is defined as the median expression of each probeset in each class divided by the standard deviation in each class;
      - centroids were shrunken towards the overall centroid by a fixed quantity called threshold. Different thresholds were tested to find the one that gives the lowest error rate. If a probeset was shrunk to zero for all classes, then it did not survive the threshold and it was eliminated from the prediction rule.
2) Cross-validation with the leave-one-out method:
   - for each threshold a model was generated using n-1 samples (where n is the total number of samples) and the left out sample was used for testing it;
   - the class of the test sample was predicted on the basis of the nearest centroid;
   - the process was repeated until each sample was used for testing;
   - a cross-validated misclassification error was obtained for each threshold.
3) Further probeset number optimization:
   - steps 1 and 2 were repeated m-1 times (where m is the total number of probesets). Starting from 2 probesets, each time 1 probeset was sequentially added from the top of the rank-ordered probeset list based on hazard ratio.

Each time the model's performance was assessed with the use of misclassification error. At the end of the process, the model with the lowest error was selected. The prognostic value of the optimized model was tested on the same dataset analysing available clinical data.

Results

Cox regression analysis led to the identification of a list of 832 probesets, corresponding to 596 genes, which discriminates high-risk (HR) MF patients from low-risk (LR) ones. In fact, supervised clustering showed 2 main

clusters which we marked as high-risk and low-risk since the frequency of deceased patients was increased in the HR group and, according to the survival curves, the HR group showed a lower median overall survival compared to the LR group (3.39 y vs 6.88 y, Log-rank p-value < 0.01).

The 832 probesets list was used to build a classification model exploiting the "nearest shrunken centroids" method. 30 different thresholds were evaluated during the leave-one-out cross-validation to identify the model with the lowest misclassification error. The process was repeated 831 times, starting from a model with 2 probesets, sequentially adding 1 probeset from the top of the rank-ordered list of 832 probesets. The model with the lowest cross-validated misclassification error was selected among the 831 models. It was obtained using the first 425 probesets of the list, of which 339 (corresponding to 249 genes) survived the threshold of cross-validation. Finding the optimal number of probesets allowed to lower the misclassification error from 9.65% (model with 832 probesets) to 7.02% (model with 425 probesets). We used this optimized model to assign a classification (high-risk or low-risk) to the 114 MF samples of our dataset, resulting in 53 high-risk samples and 61 low-risk samples. We observed a high level of concordance (93.86%) with the classification defined with supervised clustering with 832 probesets. In the HR group defined by the model the frequency of deceased patients was confirmed to be increased (33 vs 16, Fisher's p-value < 0.05) and median overall survival was lower (3.39 y vs 6.88 y, Log-rank p-value < 0.01).

Clinical data analysis showed that the LR group was significantly enriched in prePMF samples, while the HR group was enriched in PPV samples, which represent a more advanced stage of pathology (prePMF: 25 in LR vs 10 in HR, PPV: 4 in LR vs 12 in HR, chi-square's p-value < 0.05). Samples with high molecular risk (HMR) mutations along with samples bearing homozygous JAK2 mutation were concentrated in the HR group, whereas samples with low molecular risk (LMR) mutations and heterozygous JAK2 mutation were concentrated in the LR group (HMR: 26 in HR vs 13 in LR, LMR: 13 in HR vs 34 in LR, Fisher's p-value < 0.05; homozygous JAK2: 24 in HR vs 7 in LR, heterozygous JAK2: 5 in HR vs 16 in LR, Fisher's p-value < 0.05). Moreover, median age at diagnosis was higher in the HR group than in the LR group (69.7 y vs 62.0 y, Mann-Whitney p-value < 0.05). Percentages of patients with haemoglobin lower than 10 g/dL (30.2% in HR vs 9.8% in LR, Fisher's p-value < 0.05), leukocytes higher than 25 x 10^9/L (32.7% in HR vs 3.3% in LR, Fisher's p-value < 0.05) and more than 1% circulating blasts (34.8% in HR vs 13.6% in LR, Fisher's p-value < 0.05) were greater in the HR group. The latter thresholds refer to the reference values of existing MF prognostic scores.

Regarding the comparison with the existing MF prognostic scores, the analysis showed that the HR group was enriched with samples classified as high risk with IPSS, DIPSS and MIPSS70, while the LR group was enriched with samples classified as low and intermediate-1 with IPSS and samples classified as low with DIPSS and MIPSS70. 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 leukaemia transformed into a shorter time frame than the median survival reported for the reference prognostic class.

Survival curves and log-rank test showed that overall survival rates were significantly different between high-risk and low-risk samples defined according to our model, in each of the IPSS, DIPSS and MIPSS70 collapsed risk classes (low and intermediate-1 were collapsed together, as well as intermediate-2 and high) (Log-rank p-value < 0.05). It is worth noting that, when considering non-collapsed risk classes, the difference between high-risk and low-risk samples overall survival rates was always statistically significant in DIPSS and MIPSS70 intermediate categories (Log-rank p-value < 0.05). These represent the most challenging patients' categories, for whom determining the optimal therapeutic strategy is more difficult. Furthermore, multivariable regression analysis showed that our model represents an independent prognostic factor when considered together with the existing prognostic scores (p-value < 0.001).

Conclusions

These results suggest that our model, that exploit gene expression profile of myelofibrosis granulocytes, can add prognostic information to the existing scores based on clinical features and driver mutations. In particular, it is able to identify high-risk patients that would be classified as low or intermediate risk by other prognostic scores. In other words, it can improve the identification of patients' subgroups characterized by poor prognosis. This may allow these patients to be directed towards the most appropriate therapeutic option.

These results should be validated in an independent dataset in order to confirm their predictive power.

Further endeavours will be aimed at converting the model using Real Time qRT-PCR data, more easily applicable in the clinical setting.

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CEM Curriculum: Translational Medicine
Tutor: Dr. Alessia Ciarrocchi

THE NOVEL lncRNA BLACKMAMBA CONTROLS THE NEOPLASTIC PHENOTYPE OF ALK−ANAPLASTIC LARGE CELL LYMPHOMA BY REGULATING THE DNA HELICASE HELLS

Background

Anaplastic Large Cell Lymphoma (ALCL) is the most common subtype of Non Hodgkin T-cell lymphoma ALCLs are classified based on the presence of genetic translocation involving the Anaplastic Lymphoma Kinase locus (ALK). Clinically ALK^- ALCLs display a poorer outcome as compared to ALK^+ ALCL patients with the exception of DUSP22 positive and breast implant-associated ALCL (BIA), which have a favorable prognosis. Poor response to therapy and inferior survival has been observed in patients carrying defects of TP53, TP63 and loss of PRDM1 genes. Hyperactivation of STAT3 is documented in about 40–50% of ALK− ALCL, and contributes to the tumorigenesis. Recent findings also indicate that STAT3 contributes to cancer development and progression by regulating long noncoding RNA(lncRNA). Mechanistically, the molecular mechanisms leading to ALK^- ALCL transformation remain quite elusive.

LncRNAs are molecules longer than 200 nucleotides that play important regulatory functions. LncRNAs were founded to be overexpressed or downregulated in many types of cancer. In lymphocytes, the expression of lncRNAs is a dynamic and cell/stage specific phenomenon. In T-cells, detectable lncRNAs include genes often located within the neighborhood of co-expressed lineage-specific mRNA. So far, a significant number of studies have identified lncRNAs associated to lymphocyte signatures, and they are predicted to control cell differentiation and identity. Although the functional properties of several lncRNA have been dissected in normal lymphocytes, their expression and/or role in lymphoid malignancies are not fully understood. With the intent of defining the role of lncRNAs on ALK-ALCLs and to add new information on the biological mechanisms that underline these tumors we performed a deep expression profiling in conjunction with d*e novo* transcriptome assembly. We identified a panel of novel lncRNAs expressed by ALCL. We further, discovered and characterized a new chromatin-associated lncRNA, selectively expressed by ALK−ALCL lymphoma, named *BlackMamba*. 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, an increase of polynucleated cells and actin cytoskeleton rearrangements. RNA-Sequencing analysis on BlackMamba KD ALK^-ALCL cells identified a list of genes involved in migration, integrin-mediated cell adhesion and regulation of actin cytoskeleton reinforcing the hypothesis that BlackMamba controls the neoplastic phenotype through the regulation of cell

cytoskeleton. Among the *BlackMamba* targets genes we found the lymphoid helicase HELLS which has been implicated in cancer progression both for its helices and transcriptional activities. in ALK-ALCL samples, the expression of BlackMamba and HELLS are significantly correlated. Besides we showed that BlackMamba and HELLS interacts suggesting a high functional relationship between these two molecules. Collectively, our preliminary data indicate a previously unknown tumorigenic role of STAT3 via a lncRNA-DNA helicase axis and reveal an undiscovered role for lncRNA in the maintenance of the neoplastic phenotype of ALK−ALCL.

Objectives

The aim of this project is to characterize the molecular mechanisms through which the axis *BlackMamba*-HELLS regulates ALK$^-$ALCL neoplastic phenotype:
We will pursue this aim through the following tasks:

- The characterization of *BlackMamba*-HELLS-dependent gene expression program in ALK-ALCLs. due to the cell phenotype observed in both BM KD cells, particular attention will be posed to genes involved in cytokinesis and cytoskeleton-organization
- 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 a bioinformatic prediction using JASPAR and PROMO 3.0 tools on starting from the promoter sequences of BM/HELLs identified targets. 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).

Results

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). Functionally, the loss of HELLS led to reduction of cell proliferation and clonogenicity, and increased the number of polynucleated cells and

F-actin cytoskeleton rearrangements. To study the role of HELLS in ALK-ALCL setting, 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 are upregulated and 780 genes are downregulated upon HELLS KD). Among the downregulated genes, we found the significant enrichment of cytoskeleton-remodeling and cytokinesis regulating genes (6) confirming the hypothesis 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 and KLHL21 are also BlackMamba targets.

To get into this signaling we focused on the study of HELLS-*BlackMamba* common downstream targets which are Pak2, RhoA, ANLN and RhoU. Each of these targets, was silenced i 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 Pak2^{KD} did not affect signfically cell growth or the formation of polynucleated cells. Morphologically, loss of Pak2 caused the reorganization of actin filaments increasing the production of lamellipodia. I RhoU^{KD} resulted in a slight reduction of cell growth and an increase of percentage of polynucleated cells. The loss of RHOU also impaired the cytoskeleton architecture leading to the formation of lamellipodia. Similarly to RhoU, RhoA^{KD} resulted in an increase of percentage of polynucleated cells, slightly affecting cell growth. The loss of RhoA also impaired cytoskeleton architecture leading to slight actin disruption/rearrangements. Instead, ANLN^{KD} did not mimic any of the effects observed from *BlackMamba*^{KD} or HELLS^{KD} and for this reason ANLN was excluded from following experiments. Of note, Pak2 shRNA does not affect RhoU or RhoA protein levels or vice versa. Combination Pak2^{KD}+RhoU^{KD} or Pak2^{KD}+RhoA^{KD} or RhoA^{KD}+RhoU^{KD} were able to only partial mimic the complex phenotype observed after *BlackMamba*^{KD} or HELLS^{KD} suggesting that these targets may synergistically cooperate to execution of this program. Thus, to address this hypothesis triple-KD siRNA against these three candidates was performed. As expected, the simultaneous KD of Pak2, RhoA e RhoU determined a phenotype which closely resembled the one observed in *BlackMamba*^{KD} or HELLS^{KD} with reduction of cell growth, an increase of polynucleated cells and strong changes in actin organization.

To better investigate how HELLS and BlackMamba exert the transcriptional effects on cytoskeleton and cytokinesis, we performed ChIP experiments and we observed HELLS binding on the promoter regions of many of the genes identified by RNA-seq. We selected the promotorial sequences of these targets and then we performed a bioinformatics prediction of bound transcriptional factors (TFs) by Jaspar2018 and PROMO 3.0 tools to establish potential HELLS TFs partners in the regulation of these genes. We selected 6 top-scoring TFs able to bind almost of 40% of target gene promoters which are: E2F1, YY1, ETS1, TFAP2a, FOXP3 and CEBPB. We tested the basal expression of these TFs in a panel of ALK$^-$ALCL cell lines, albeit with a variable expression, 5/6 TFs were expressed in all cell lines tested, whereas FOXP3 was not detectable. To prove an

effective association between HELLS and TFs, we performed Co-Immunoprecipitation and demonstrated that HELLS binds YY1.

Conclusion

Our findings define a new role of the DNA-helicase HELLS in the transcriptional control of cytoskeleton and cytokinesis master regulators in ALK-ALCLs.

Collectively, our data highlighted that *BlackMamba*-HELLS helicase loop is one of the most important mechanism that govern ALK$^-$ALCL neoplastic transcriptional program by regulating the main cytoskeleton and cytokinesis-related target genes such as RHOA, PAK2, RHOU, ANLN and that these genes are at the base of ALK-ALCL neoplastic phenotype.

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CEM Curriculum: Nanomedicine, Medicinal and Pharmaceutical Sciences
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DATA-DRIVEN TECHNOLOGIES FOR DRUG REPURPOSING: INTEGRATION OF *IN SILICO* APPROACHES TO PREDICT NOVEL THERAPEUTIC INDICATIONS FOR KNOWN MOLECULES

Background

A well-established alternative to *de novo* drug discovery is the identification of novel therapeutic indications for marketed drugs (*drug repurposing*). Drug repurposing provides several advantages, such as reducing the times and costs required to bring the investigated compounds to the market (1). In addition, repurposing strategies can also help to identify new biological targets for already synthesized compounds, allowing to maximize their value for drug discovery.

In recent times, the increase in biological and chemical data has enabled the progress of novel attractive drug repurposing opportunities. Accordingly, the large-scale use of integrated *in silico* approaches has proven to be an efficient and cost-effective strategy (2).

Objectives

The main objective of the project is to devise and apply effective computational strategies for drug repurposing campaigns. The project will make use of the joint application and integration of ligand- and structure-based approaches, as well as the development of machine learning models.

In a first task, we carried out a repurposing campaign on a library of previously synthetized ligands based on a hexahydrocyclopenta[c]quinolinic scaffold. We devised a computational protocol integrating both ligand- and structure-based approaches. Results were validated by *in vitro* assays.

In a second task, machine learning approaches were applied to build models trained on reported data on the well-studied human Carbonic Anhydrase family. The aim of this task was to build models able to efficiently predict the selectivity profile on these therapeutically relevant targets.

Methods

Task 1: The 3D shape-based similarity profile of a library of hexahydrocyclopenta[c]quinoline derivatives was first evaluated with respect to the ligands reported in the DrugBank, PDB and ChEMBL databases, by using the ROCS software. Then, a structure-based screening was performed on the most promising targets by running molecular docking calculations with the Glide software into representative crystallographic structures. Finally, a list of potential compounds was tested *in vitro* on a panel of human Carbonic Anhydrases (hCA) and Estrogen Receptors (ER) α and β.

Task 2: A dataset of hCA isoforms II, IX and XII bioactivities was extracted from ChEMBL release 26. Activities were filtered, and the RDKit software was used to calculate 118 molecular descriptors for each molecule. Machine learning (ML) models were built to assess the hCA activity profiles at given activity thresholds. The python Scikit-learn modules were used to build, fine-tune and validate all ML models. For each model, the dataset was split into training and testing dataset using a 75:25 ratio. The training dataset was further subjected to a 10-fold cross-validation. The testing dataset was used to evaluate the final model. Performances were evaluated using the A*ccuracy* and the *Matthew Correlation Coefficient* metrics.

Results

In the first task, we developed an integrated *in silico* protocol to reposition a previously identified library of molecules (3). We performed a shape-based screening against 3 public databases and identified a list of targets to be further validated by structure-based methods. Results analysis led to the identification of human Carbonic Anhydrase (hCA) and Estrogen Receptor (ER) as the most promising targets. Literature findings suggested that the dual hCA/ER inhibition could be of aid to anticancer therapy. Thus, a new series of derivatives was designed to obtain a dual hCA/ER modulation and is currently under evaluation. Preliminary results showed that one of the investigated compounds and one of the newly synthesized derivatives are potent and selective hCA II and cancer-related isoform hCA IX inhibitors, respectively.

In the second still ongoing task, machine learning models were trained on activity data for hCA isoforms II, IX and XII, extracted from the latest ChEMBL release. Models were built using 10 different classification algorithms and tested against previously unseen data using 2 performance metrics. Preliminary results showed that the best models could correctly predict the selectivity profile of molecules with an 80-90% accuracy. These models will be then used to screen commercial databases, and the most promising molecules will be tested to experimentally validate the predictions.

Conclusions

Overall, the research results herein reported provide evidence of how *in silico* approaches can be efficiently used to identify new therapeutic uses for already known molecules. Indeed, preliminary results have shown the successful repositioning of an existing molecule as a hCA inhibitor. Subsequent derivatization has led to the discovery of a potent and selective inhibitor of a cancer-related isoform. The development of dual hCA/ER inhibitors and the use of innovative machine learning techniques are currently being pursued.

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EPIDEMIOLOGICAL FEATURES AND RISK FACTORS OF INTERSTITIAL LUNG DISEASE IN PATIENTS WITH RHEUMATOID ARTHRITIS: THE LIRA (LUNG FIBROSIS IN RHEUMATOID ARTHRITIS) STUDY

Background

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

ILD is often underrated, particularly in its earliest stages. High resolution computed tomography (HRCT) represents the gold standard for the diagnosis of ILD, but its routine use for screening program is not advisable for both the high cost and X-rays exposure. Recently, we validate the use of VECTOR as a simple and non-invasive tool to aid clinicians to perform an early screening for ILD in RA patients. The use of VECTOR may improve early diagnosis and a better management of the disease.

Moreover, the natural history of ILD in RA patients remains an international gap of knowledge.

The prevalence of ILD in RA ranges from 4 and 60%. This wide variation reflects differences in study design, study populations and definition of RA-related lung disease. Unfortunately, all the available studies on this topic are retrospective, with small series of patients and numerous bias, and therefore not reliable.

Finally, several therapeutic agents have been suggested for the treatment of RA-ILD, but nowadays there are no randomized controlled clinical trials to support therapeutic guidelines.

On these bases, there is an urgent unmet need of prospective studies to clarify some crucial points such as the incidence and prevalence of ILD, its clinical features (onset features and clinical evolution), radiological characteristics, the possible predictive factors and the causal relationship with some drugs commonly used for the treatment of RA.

Objectives

The purpose of the Lung fIbrosis in Rheumatoid Arthritis (LIRA) study is to evaluate incidence and prevalence of ILD in patients with RA. Moreover, it aims to assess radiological features, clinical onset and natural history of ILD-RA, as well as the risk factors for the development of ILD in RA patients.

Methods

The LIRA study is an international prospective multicentre observational study enrolling patients with a diagnosis of RA and evaluating the possible presence of pulmonary interstitial involvement.

All consecutive patients with a diagnosis of Rheumatoid Arthritis (RA) satisfying EULAR/ACR criteria, aged more than 18-year-old, will be enrolled. The enrolment stage will take 1 years, the duration of the study (follow-up for each patient) will be 3 years per subject.

In our project, all RA patients will be screened for signs or symptoms suggesting for pulmonary involvement (cough, dyspnea, velcro crackles, suspected ILD in a chest x-ray performed for other reasons) during the outpatient visit at rheumatologic center. Patients with suspicion of pulmonary disease will undergo HRCT, the gold standard for the diagnosis of ILD. No additional HRCT will be required in patients who already have a diagnosis of ILD.

For each patient, clinical and serological data will be recorded at baseline and every 6 months. Patients with a diagnosis of ILD periodically perform pulmonary function tests to monitor lung function evolution.

Results

LIRA was approved by the local ethical committee in April 04, 2019.

Recruitment's initiation by the coordinator center started in April 2019. At now, a total of 10 participating centers throughout Italy were activated. Moreover, two Spanish and a French rheumatologic centers are going to be activated.

To date, 205 RA patients were enrolled in the study (female/male 161/44, mean age 64.8±12.9 years, mean disease duration 14.2±8.9 years). Anti-citrullinated peptides antibodies (ACPA) and rheumatoid factor (RF) were positive in 77.1% and 78.1%, respectively. The prevalence of ILD was 21% (43 patients). In other 13 patients the HRCT is ongoing; therefore, we could suppose up to a prevalence of 27.3%. Patients with ILD were symptomatic in 53.5% of cases (23 patients), they are more frequently males and were older than patients without ILD (mean age 73.2±7.4 and 62.7±13.2; $p<0.0001$, female/male ratio 139/23 vs 22/21; $p<0.0001$) without significant differences regarding disease duration, positivity for ACPA or RF.

Conclusions

ILD is one of the most common extra-articular manifestation of RA, and its management is challenging, for the deterioration of quality of life, the high mortality and utilization of healthcare resources.

Despite its clinical relevance, the prevalence, incidence and survival of RA-ILD is unknown and supposed on the base of retrospective data or registry-based studies. Moreover, no proteomic or serologic biomarkers are available to improve our armamentarium for both diagnostic and prognostic purpose in RA-ILD. Non-homogeneous and sometimes discordant results regarding risk factor for RA-ILD have been described, and, finally, randomized controlled clinical trials to support therapeutic decisions in RA-ILD patients are still missing.

In summary, with our prospective study we expect to clarify these crucial points in the field of RA-ILD. Overcoming these gaps of knowledge is compulsory for the design of target therapies and to improve patient management and quality of life.

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

Objectives

- To 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 advanced PD patients operated on with bilateral STN-DBS.
- To assess the evolution of axial symptoms in a group of advanced PD patients who have undergone STN-DBS surgery.
- To evaluate the influence of anatomical location and parameters setting of the active STN-DBS contact on axial symptoms.

Methods

At first, we are evaluating retrospectively data from 30 PD patients operated on with bilateral STN-DBS at the Neurology Unit of OCSAE Hospital, Modena, 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 Hoen and Yahr scale (H&Y) and the four parts of the Unified Parkinson's Disease Rating Scale (UPDRS) score and subscores (akinesia, rigidity, tremor and postural instability and gait disorders 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). The above mentioned analyses has been carried out at the Motion Analysis Laboratory (LAM), Neuromotor and Rehabilitation Department, Correggio Hospital, AUSL-IRCCS of Reggio Emilia. Furthermore, each patient will undergo a complete neuropsychological assessment and a [^{18}F]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. The study was approved by the ethics committee in May 2019 and was authorised by the AUSL – IRCCS of Reggio Emilia at the end of June 2019.

Results

Eight patients were recruited from September 2019 to February 2020 (50% males; mean age: 61 years, SD ± 6.26) with a mean follow-up after surgery of 6.25 years (SD ± 0.88 years). Unfortunately, due to the COVID-19 outbreak the patients' recruitment has been necessarily interrupted at the end of February 2020. The study procedure was tolerated in all the eight patients studied. Due to the small sample no statistical analysis was performed. Comparing the three postoperative conditions the combination of both stimulation and medications led to an improvement of motor score and subscores. Concerning the gait preliminary analysis, both the 10-meter walk test and the Timed Up and Go Test (TUG) showed a similar trend with the longer duration (in seconds) in the off-stimulation/off-medication condition, meaning that both levodopa and stimulation improved gait. It is interesting to note that the dual task condition performed in the on-stimulation/on-medication condition affected severely 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.

Conclusions

Even if in a small 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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FEASIBILITY AND SAFETY OF PHYSICAL EXERCISE IN PROSTATE CANCER PATIENTS RECEIVING ANDROGEN DEPRIVATION THERAPY AND RADIOTEHRAPY

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. We conducted a systematic literature review on the effectiveness, feasibility, and safety of PE on bone health in patients with PCa receiving ADT.

Aim 2. In patients with PCa, we are collecting data on lifestyle and motivation or perceived barriers against the adoption of a healthier lifestyles.

Aim. 3. We will evaluate the feasibility and safety of PE in PCa patients 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 feasibility of a PE program was measured through recruitment, retention, dropout, and adherence rates. The safety of PE was measured through the number, type, and severity of adverse events. The components, setting, intensity, frequency, and duration of PE programs were extracted.

Aim 2. An observational monocentric cross-sectional study is ongoing in an Italian hospital setting. Adult patients newly diagnosed with PCa are interviewed through open-ended questions. We exclude patients who have undergone major surgery in the latest three months. We are collecting demographic, clinical, and anthropometric data, habitual level of physical activity, and smoking, eating, and drinking habits. We are also collecting information on each individual's motivation to change towards a healthier lifestyle and the perceived barriers to this change.

Aim 3. A non-randomized feasibility and pilot study has been submitted to the Institutional Ethics Committee for approval. A single group of patients with PCa receiving ADT and RT will be 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 will be the feasibility and safety of the PE program. We will also record 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 and mood disturbances.

Results

Aim 1. Nine RCTs were included, none of which focused on the risk of accidental falls and fractures. Only two trials reported beneficial effects of PE for lumbar spine BMD (0.014 g/cm^2, 95% CI 0.001–0.027), hip BMD (g/cm^2 right 0.015, 95% CI 0.003–0.027; left 0.017, 95% CI 0.002–0.032), or femoral shafts BMD (g/cm^2 right 0.018, 95% CI 0.004–0.032; left 0.024, 95% CI 0.005–0.044). 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. To date, nineteen interviews have been conducted. Data collected will inform a PE intervention whose feasibility will be tested in this population.

Aim 3. We expect to start patient recruitment in September 2020. At the moment we have no available data.

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, but caution should be used in prescribing football training for safety reasons. 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 results of this study (Aim 2 and 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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EFFECTS OF DYSMETABOLISM ON CARDIOVASCULAR AND REPRODUCTIVE SYSTEMS: POSSIBLE INTERVENTIONS IN EXPERIMENTAL AND CLINICAL MODELS

Background

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

Objectives

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

Methods

The pregnant murine models of MS and Obesity were obtained using a transgenic eNOS-/+ female and a WT female, respectively. Those animals were fed with high fat diet for 4 weeks to induce MS and obese phenotype, then bred with WT males. On gestational day (GD) 1, dams were randomly allocated to the

treatment or control groups. Maternal weight and systolic blood pressure (SBP) were obtained at GD 18, using a calibrated, 8-chamber, tail-cuff system, then dams were sacrificed for fetuses and placentas collection. Carotid arteries were dissected out for the in vitro-vascular studies using a wire-myograph system. The vessels were mounted in the chamber between two force transducers and bathed in Krebs solution at 37 degrees Celsius and pH of 7.4. The tension generated by the vascular tissue was recorded and acquired by a power lab computer software. Responses to the contractile agent, phenylephrine (α1 adrenergic agonist) in the presence and absence of a non-specific nitric oxide synthase inhibitor (NG-nitro-L-arginine methyl ester) were obtained. For the vasorelaxation responses, acetylcholine (endothelium-dependent vasorelaxant agent) and sodium nitroprusside (endothelium independent vasorelaxant agent) were evaluated. Masson's trichrome staining was used to evaluate connective deposition in maternal heart.

To evaluate the effects of a lifestyle intervention in pregnant women with MS and obesity, we selected singleton pregnant women with body mass index (BMI) ≥ 30. Women were recruited between the 9-12th weeks of pregnancy at the Policlinic Hospital of Modena. They were randomized to receive either a lifestyle intervention (LI) of hypocaloric, low-glycemic, low-saturated fat diet with an average intake of 1800 Kcal/day and physical activity prescription (30 minutes walking 4 times/week) or standard care (SC), namely standard recommendations for a healthy diet. Women in both groups were scheduled to have specific follow-up evaluations for adherence to the study at the 16th, 20th, 28th, and 36th weeks of pregnancy. Maternal body composition was evaluated at each appointment through the bioimpedance analysis (BIA), measuring the weight, the total fat mass (FM) and visceral fat mass (vFM). The plasmatic lipid profile (Total Cholesterol, HDL, LDL, Triglycerides) and cytokines (IFN-γ, IL-1α/β, 2, 6, 8, 10, 12p70, TNF-α) were measured at enrolment and at 36th week. A 75 g oral glucose tolerance test (OGTT) was performed between the 16-18th weeks and repeated between the 24-28th weeks, to diagnose the onset of gestational diabetes mellitus. Maternal and fetal outcomes were evaluated for women who completed the study and attended all the follow-ups.

Results

1. Main findings of the Animal Study

Gestational weight gain (GWG) was similar between treated and untreated dams. SBP was decreased in eNOS$^{-/+}$ MS (P=0.01) and WT obese dams (P=0.001) by inositols and antioxidant supplementation. In the MS dams, the supplementation decreased the %Max contractile response to PE and L-NAME (P=0.01) and improved ACh vasorelaxation (P=0.05). In WT obese dams, %Max response to PE was similar in either treated or untreated groups. Incubation with L-NAME increased vascular PE response in the untreated pregnant mice, which was lowered by the supplementation (P=0.0001). No differences were seen in SNP relaxation in both animal models.

2. Preliminary findings of the Human study

A total of 390 women were included in the study. Patients were divided into Obese (Ob) and MS groups, if they presented at least 3 of the 5 criteria defined according to the International Diabetes Federation. The Ob group counted 312 women of which 158 were assigned to the LI group and 154 to the SC group. While the MS group included 78 women, of those 40 were in the LI group and 38 in the SC.

There were not significant differences between groups compared as Ob (LI vs SC) and MS (LI vs SC) in the socio-demographic features, while pre-pregnancy BMI resulted higher among MS women enrolled in the LI group compared to SC (LI: 39.1 ± 6.5 vs SC: 36.5 ± 4.6 kg/m2, p=0.05). At the BIA measurement, pregnancies with MS in LI presented a higher FM at baseline compared to those in SC, while among obese women the two groups were comparable. The rate of GDM and gestational hypertension were similar between groups among Ob and MS women. FM and vFM at 36 weeks of pregnancy were significantly higher in MS women assigned to the SC group (FM p=0.009; vFM p=0.04). Maternal weight at delivery was higher in MS women allocated to the SC group (LI: 104.4 ± 12.6 vs SC: 112.7± 16.54 kg, p=0.03). While neonatal birthweight was similar between groups either among obese or MS women. Interestingly, a significantly higher rate of Large for Gestational Age (LGA) infants was found in MS women receiving the SC when compared to those in the LI group (p=0.05).

Conclusions

1. In animal models of obesity and MS, the supplementation with inositols and antioxidants during pregnancy improved maternal SBP and vascular response by targeting other pathways beside the nitric oxide. In conclusion, our findings suggest that inositols, fucoxanthin and hydroxytyrosol could represent a new non-pharmacological approach for the treatment and/or prevention of vascular disorders in pregnancy complicated by obesity and MS. Their beneficial action on maternal vascular profile is mediated by an improvement in endothelial function and reduction in oxidative stress. Future studies are warranted to investigate the effect of MDFH treatment on pregnancy metabolic profile and their mechanism of action in the antioxidant balance.
2. An early lifestyle intervention constituted of a hypocaloric, low- glycemic, low-saturated fat caloric restriction associated to a constant PA, could be a strategy to limit the detrimental role of dysmetabolism related to obesity and MS in the delicate “fetal programming” process. A healthy lifestyle started early in pregnancy improved body composition in MS women at term of pregnancy and reduced the rate of LGA infants.

Dr. Veronica Mantovani

CEM Curriculum: Nanomedicine, Medicinal and Pharmaceutical Sciences
Tutor: Prof. Nicola Volpi

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

Background

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

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

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

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

Objectives

This project is focused on GAGs qualitative and quantitative characterization in a murine MPS II model (iduronate-2-sulfatase knock-out, I2S KO) non-treated, treated with ERT or treated with genistein, analyzed at different weeks after the beginning of therapy.

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

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

Methods

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

GAGs were extracted from urine, plasma and organs sample according to a standardized protocol guideline. The protocol step included protein digestion with specific protease, treatment with sodium borohydride and sodium hydroxide (Sigma-Aldrich) to release GAGs from their core proteins, purification on anion-exchange resin and filtration on YM-3 centrifugal filters having a molecular mass cutoff of 3000 Da (Amicon). The crude retained GAGs fraction was digested with chondroitinase ABC and heparinase I, II, III (Sigma-Aldrich) to isolate HA, CS, DS and HS disaccharide units. Disaccharides were lyophilized and tagged with AMAC fluorophore (Sigma-Aldrich). Finally, derivatized disaccharides were separated and quantified by capillary electrophoresis (Agilent) interfaced to laser induced fluorescence.

During the last year, we focused on plasma results performing quantitative and qualitative analysis of extracted GAGs. In particular, we analyzed total concentration of GAGs (ng/ml plasma) and total concentration of CS-DS, HS, HA. We evaluated the GAGs prevalence analyzing the CS/HS parameter and perform qualitative analysis investigating structural parameters. We considered, Charge density (evaluates the sulfation level of GAGs, indicated as the average of sulfate groups for each disaccharide unit), disaccharides sulfation at different position in CS-DS structure (CS in mammals is variably sulfated at carbon C-6 of N-acetyl-galactosamine, while DS at C- 2 or 4 of iduronic acid, the 4s/6s and 2,4s parameters can give us information about the prevalence of CS or DS) and disaccharides sulfation at different position in HS structure (N acetyl, Ns, 2s, 6s).

Results

Compared to WT, the I2S KO subjects showed from the first weeks significant differences ($p < 0.05$) for all quantitative and qualitative parameters analyzed. 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. Moreover, CS / HS ratio analysis showed an imbalance towards HS accumulation in plasma, while CS was the main GAGs accumulated in WT subjects. From a qualitative point of view, we observed an increase in charge density for both CS-DS and HS, 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. Furthermore, HS presented an increase in disaccharides sulfated in position Ns and 2s, compared to sulfation in the N-acetyl position.

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. We evidenced a significant decrease in the concentration of all analyzed GAGs, a decrease in charge density and a partial restoration of sulfation profile, similar to WT subjects. 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. These results support the I2S KO mouse model as a good experimental model for Hunter Syndrome. We can also evidence how the increase in disaccharides sulfated in position 2,4s indicates an accumulation of DS in plasma, 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 plasma accumulation and increase catabolism at the cellular level of I2S KO mice. These findings further strengthen the coherence of this animal model to MPSII pathology. Despite the positive effects described, it should be emphasized that ERT is not able to induce total normalization of the parameters, consequently the presence of pathological manifestations in treated subjects cannot be excluded. 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. In conclusion, from this data in plasma we can show how this animal model is a promising model for the study of MPSII in plasma. In the next stage of the study we will analyze the performance of this animal model also in urine and different organs. Furthermore, we will have more indications on the molecular effectiveness of different therapies in reducing GAGs accumulation, providing the basis for future clinical trials on humans.

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CEM Curriculum: Translational Medicine
Tutor: Prof. Enrico Clini

TRANSESOPHAGEAL AND DYNAMIC TRANSPULMONARY PRESSURE MONITORING IN PATIENTS WITH DE NOVO RESPIRATORY FAILURE UNDERGOING NON-INVASIVE MECHANICAL VENTILATION

Background

Acute hypoxic respiratory failure (ARF) represents the clinical model of acute lung injury. In patients developing acute ARF a trial of non-invasive mechanical ventilation (NIV) might be offered in the appropriate clinical setting. Among the other potential predictors of NIV failure, the role of spontaneous breathing (SB) is still debated. In this study, we explore the hypothesis that in patients with moderate to severe DRF undergoing a NIV trial, the SB effort might be a major determinant of NIV failure.

Objectives

In this study, we explore the hypothesis that in patients with moderate to severe DRF undergoing a NIV trial, the SB effort might be a major determinant of NIV failure.

Methods

Thirty consecutive patients with AHRF admitted to a single center and candidates for a 24-hour NIV trial were enrolled. Clinical features, tidal changes in esophageal (ΔPes) and dynamic transpulmonary pressure (ΔPL), expiratory tidal volume, and respiratory rate were recorded on admission and 2-4-12-24 hours after NIV start, and were tested for correlation with outcomes.

Results

ΔPes and ΔPes/ΔPL were significantly lower 2 hours after NIV start in patients who successfully completed the NIV trial (n=18) compared to those who needed endotracheal intubation (n=12) [mean=13.4 (SD=5.6) cmH2O vs 30.7 (7.8) cmH2O, p<0.0001] while other variables differed later. ΔPes was not related to other predictors of NIV failure at baseline. NIV-induced reduction in ΔPes of 10 cmH2O or more after 2 hours of treatment was strongly associated to avoidance of intubation and represented the most accurate predictor of treatment success (OR=15, 95%CI 2.8-110, p=0.001, AUC=0.97, 95%CI 0.91–1, p<0.0001).

Conclusions

The magnitude of inspiratory effort relief as assessed by ΔPes variation within the first 2 hours of NIV was an early and accurate predictor of NIV outcome at 24 hours. If confirmed in larger trials, these findings suggest that monitoring of esophageal pressure might assist clinicians in the timing of intubation for patients with ARF undergoing a NIV trial.

Dr. Vittoria Tarantino

CEM Curriculum: Translational Medicine
Tutor: Prof. Stefano Luminari

PROSPECTIVE COLLECTION OF DATA OF POSSIBLE PROGNOSTIC RELEVANCE IN PATIENTS WITH INDOLENT NON-FOLLICULAR B-CELL LYMPHOMA FROM FIL NF10 CLNICAL TRIAL

Background

Marginal zone lymphoma (MZL) is the third most frequent indolent lymphoma subtype accounting for about 7% of newly diagnosed NHL. It comprises three distinct diseases according WHO classification subclassified as splenic, nodal and extra-nodal subtypes (SMZL, NMZL, ENMZL).
As a low grade lymphoma, MZL displays many common characteristics such as long lasting asymptomatic phase, and an excellent response to treatment when needed. During the last decade, mainly due to advances in immunotherapy, an excellent progression free survival (PFS) and overall survival (OS) were achieved. However, patients' response is heterogeneous and the course of the disease may be aggressive reflecting in some of them a high risk profile disease.
In 2010 the Fondazione Italiana Linfomi (FIL) launched a project to prospectively collect in a large observation multicenter study, patients with indolent non follicular lymphoma, the NF10 trial with the aim of better characterize the heterogeneous clinical course of the disease.
The accrual was recently completed and here a brief report of the whole collection of data of possible prognostic relevance is described

Objectives

The aim of the project is to verify whether a prognostic collection of data would allow the development of a more accurate prognostic assessment for non-follicular low grade B-cell lymphomas. Moreover, the NF10 trial represents the basis for an international project with the aim of investigating the role of the FDG PET in MZL as prognostic tool for whom current definition remains largely undefined. During the second year of PhD course the protocol was prepared and recently approved by the International extranodal lymphoma study group (IELSG) involving Italian, French and Swiss Group (IELSG44 trial). The efforts of the study is to put together a large series of cases of MZL coming from archives of prospective clinical trials or population/hospital based registries.
The study will be part of the final research PhD project.

Methods

The NF10 Project is a prospective international registry of INFL including consecutive patients with a histologic confirmed diagnosis of INFL.

The primary endpoint of the study was the 5-year progression-free survival for the treated cohort. Secondary endpoints were 5-year overall survival.

PFS was defined for all patients and calculated as time from the date of initial diagnosis to progression, re-treatment, or death due to any cause. Overall survival was calculated from the date of diagnosis to the date of death for any cause. Registration of patients in the study and data collection was performed on-line with Electronic Case Report Forms (ECRFs). Specific eCRF was created for IELSG44 trial. In these latter study, as a significant proportion of patients considered will be retrieved from previous observational and interventional clinical studies; thus data on clinical presentation, treatment and follow-up will be obtained from the existing dataset of the previous protocols. For the additional cases identified from clinical practice data will be collected from patient chart.

Results

Between July 2010 and March 2020, 1523 patients were enrolled in the NF10. 1328 were eligible for the analysis after central histological review. Among the whole cohort, 322 were classified as ENMZL (25%), 250 SMZL (20%), 79 NMZL (6%), and 104 defined as disseminated MZL (8%). The median follow-up was 43 months (range 1-102). 774 pts (51%) were addressed to a watch and wait strategy whereas 743 (49%) received an active anti-lymphoma treatment. Overall 5-years PFS was 61% (95%CI 57-94). According to INFL subtype 5-yr PFS was 76% (95%CI 68-91), 54% (95%CI 46-62), 58% (95%CI 40-72) and 62% (95%CI 57-94) for ENMZL, SMZL, NMZL and Disseminated cases, respectively. Overall 5-year OS was 87% (95%CI 84-90), 95%, 84%, 80% and 78% for ENMZL, SMZL, NMZL and disseminated. A significant different PFS and OS was measured for patients who experience an early relapse within 24 months from diagnosis (POD24) Three-year OS for patients with POD24 was 53% with a HR of 19.5 (95%CI 8.4-45) compared with patient without POD24 (3 yr OS 95%).

The exploratory analysis for IELSG44 study identified more than 150 cases retrieved from NF10 study for whom a FDG PET evaluation was made. These represent the starting series for the Italian cases analyzed to correlate CT and FDG pet for stage definition and to evaluate the prognostic role of metabolic response.

Conclusion

Marginal zone lymphomas represent a heterogeneous group of indolent non follicular lymphoma with an excellent response to treatment when needed resulting in a very good PSF and OS.

However, differences in clinical behavior exists among different subtypes. Recently assessment of POD24 stratifies subsequent outcome in MZL and should be considered as a surrogate for OS in clinical research and for patient's management.

Further analyses on the prognostic role of metabolic response, already planned, may contribute to better clarify the role of novel tools to define the prognostic profile earlier during the course of the disease.

Dr. Elia Paradiso

CEM Curriculum: Translational Medicine
Tutor: Prof. Manuela Simoni

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

Background

Sphingosine-1-phosphate (S1P) is a lysosphingolipid highly represented in plasma and lymph, as well as in the ovarian follicular fluid together with glycoprotein hormone gonadotropins. 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 maintain 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 essentially the same 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 G protein-coupled receptors (GPCRs), known as S1PR1-5. S1PR1 and S1PR3 are expressed in human primary granulosa lutein cells (hGLC), as well as in the immortalized human primary granulosa cell line hGL5. S1PRs-mediated signals are activated at nanomolar S1P concentrations, resulting in the activation or inhibition of a number of intracellular signaling pathways.

Objectives

This study aims to characterize the role of S1P- and gonadotropins-induced signaling in determining ovarian follicle development *in vitro*. To study how FSH and LH influence/substitute for each other and interact in cells expressing one or both receptors. Obtaining cell lines stably expressing FSHR and LHCGR or both receptors induced by a promoter inducible to control both temporally and quantitatively the expression of the receptors in the human granulosa cell lines. Receptor homo/heterodimerizations and coupling to intracellular interactors will be also evaluated.

Methods

Human primary granulosa lutein cells (hGLC), the immortalized human primary granulosa cell line hGL5, human granulosa cell line KGN and other cell models, such as COS-7 and HEK293 cells, will be used *in vitro*. Intracellular signaling pathways activation, such as production of cAMP, kinase activation, as well as intracellular calcium ion increase, were characterized by treating cells with hormones and/or selective agonists. This analysis was performed by ELISA, Western blotting and bioluminescence resonance energy transfer (BRET). Moreover, the role of S1P/gonadotropins-dependent steroid hormones synthesis and gene expression were investigated in the presence of specific inhibitors and ligand antagonists. These intracellular endpoints were analyzed by immunoassay and real time PCR.

During my 6-months internship at the Prof. Pascale Crepieux's laboratory, Dep. Physiology of Reproduction and Behavior, INRAE Centre Val de Loire, were obtained two plasmids encoding FSHR and LHCGR under the control of distinct inducible promoters to obtain a cell line stably expressing FSHR, LHCGR or both receptors. It was monitored the inducible expression of the receptor transcripts by Reverse Transcriptase PCR and expression at the cell surface by flow cytometry in presence of increasing doses of tetracycline and different times. Functionality of the receptors was evaluated by assaying cAMP production and progesterone synthesis by homogenous time resolved fluorescence (HTRF). The number of copies of receptor cDNA inserted into the genome and the sites of insertion will be determined by reverse PCR. The activation of kinase will be evaluated by Western blotting and Ca^{2+} mobilization by BRET.

Receptor homo/heteromerizations and coupling to G proteins and β-arrestins will be assessed.

Results

It was evaluated the signaling cascade activated by S1P and specific agonists in hGLC and hGL5 cells. It was demonstrated potently induced-CREB phosphorylation by S1P in granulosa cells. No cAMP production was detected and pCREB activation occurred in presence of the PKA inhibitor H-89. 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 EGF-like epiregulin-encoding gene (EREG) expression (two-way ANOVA and Bonferroni post-test; $p<0.05$; $n=3$) in granulosa cells.

During my internship at the Department of Physiology of Reproduction and Behavior of INRAE Centre Val de Loire, I obtained several clones of KGN and HEK293 cells selected with puromycin, which have stably integrated the plasmid both FSHR and LHCGR by TetOn system. Expression of the FSHR and LHCGR was induced by doxycycline inducible promoter. I demonstrated the inducible expression and integration of each receptor by RT-PCR after 24 hours.

In various conditions of doxycycline, I also demonstrated the presence of receptors FSHR-KGN, LHCGR-KGN and FSHR-HEK293 cells at the cell surface by flow cytometry after 24 and 48 hours of induction.

The cell line stably expressing LHCGR is responsive in terms of cAMP production, indeed, an increase of cAMP accumulation is detected in LHCGR-KGN cells after stimulation by 10 nM hCG (Kruskal-Wallis test; $p<0.05$; n=3). I measured progesterone production by competitive assay based on HTRF demonstrating that LHCGR-KGN cells have a steroidogenic response. Moreover, I proved cAMP production in FSHR-KGN and FSH-HEK293 cells in presence of 10 nM FSH (Kruskal Wallis test; $p<0.05$; n=2).

Conclusions

It was 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.

Dr. Tommaso Filippini

CEM Curriculum: Public Health
Tutor: Prof. Marco Vinceti

EXPOSURE TO PARTICULATE MATTER AND RISK OF CONVERSION FROM MILD COGNITIVE IMPAIRMENT TO DEMENTIA: A COHORT STUDY IN MODENA AND REGGIO EMILIA PROVINCES

Background

Neurodegenerative dementias are severe medical conditions that are prevalent worldwide and expected to increase in the upcoming years [1]. The cognitive and functional decline characterizing all clinical forms of dementia and commonly occurring in later life is now considered result of a process that begins earlier in the life course [2]. In addition to genetic susceptibility, the environmental and nutritional risk factors have been suggested to play an important role in dementia etiology [3]. Particularly, exposure to particulate matter has been linked to increased risk of neurological disorders, including Alzheimer's disease.

Objectives

This project aims at investigating the association between outdoor air pollution on risk of onset of dementia in a cohort of subjects with mild cognitive impairment of non-vascular origin.

Methods

We recruited a cohort of 53 subjects newly diagnosed with mild cognitive impairment residing in Modena and Reggio Emilia provinces at the time of diagnosis. Using a Geographical Information System, we assessed outdoor air pollutant exposure, by modeling air levels of particulate matter ≤10 μm (PM_{10}) from motorized traffic at geocoded subjects' residence. We investigated the relation of these concentrations to subsequent conversion from mild cognitive impairment to any dementia using a Cox proportional hazards model. We computed hazard ratio (HR) and 95% confidence interval (CI) according to increasing PM exposure, adjusting for sex, age, and educational attainment.

Results

During a median follow-up of 42 months, 19 participants developed Alzheimer's dementia, three frontotemporal dementia and two Lewy body dementia. PM_{10} exposure levels were 9.6 $\mu g/m^3$ on average. Using PM_{10} levels below 5 $\mu g/m^3$ as reference, we found a dose-response increase in any dementia risk with HR of 1.04 (95% CI 0.41-2.66) at 5-10 $\mu g/m^3$, 1.32 (95% CI 0.36-4.92) at 10-20 $\mu g/m^3$, and 1.38 (95% CI 0.14-13.13) above 20 $\mu g/m^3$, respectively

Conclusions

Using a prospective cohort study design, our results suggest that exposure to outdoor air pollutants increase the risk conversion from mild cognitive impairment to dementia, though the low number of participants suggests caution in the interpretation of study findings.

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CEM Curriculum: Translational Medicine
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MOLECULAR AND CELLULAR ACTION OF REPRODUCTIVE HORMONES

Background

Ovarian physiology is regulated by tandem action of gonadotropins and sex steroids, both necessary for the completion of follicle growth, maturation and ovulation. During the menstrual cycle, the selection of the dominant follicle occurs, as a result of variations in gonadotropins and steroid hormone levels and in receptors expression on the surface of granulosa cells. Nevertheless, mechanisms regulating this event are still poorly understood and it is supposed to be due to low estrogen levels, decline of follicle-stimulating hormone (FSH) levels and receptor (FSHR) expression, 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). However, it was demonstrated that the FSHR and LHCGR genotype impact the ovarian response to hormones. These data suggest the ability 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.

Objectives

The aim of the project is to better understand the role of estrogens/gonadotropins and their membrane receptors in regulating ovarian physiology and the selection of the dominant follicle. I will characterize how interactions between GPER- and FSHR/LHCGR mediate receptors internalization, recycling and signaling cascades, which regulate pro and anti-apoptotic signals underlying human reproduction.

Methods

The cDNA of GPER mutant-FLAG and hLHR-HA were inserted in the plasmid pcDNA3.1 by cloning experiments and their expression on the surface of HEK293 cells was then tested by immunofluorescence and flowcitometry. The presence of heterodimeric and homodimeric receptor structures was evaluated by photo-activated localization microscopy (PALM), while cell signaling events as cAMP and IP-one accumulation was analyzed in HEK293 upon FSH, estradiol, LH and hCG treatment by homogeneous time-resolved fluorescence (HTRF) and by bioluminescence resonance energy transfer (BRET).

All these *in vitro* experiments were performed during my 1-year internship at the Prof. Aylin C. Hanyaloglu's laboratory, Dept. of Surgery and Cancer (now changed to Dept. of Metabolism, Digestion and Reproduction) of the Imperial College London (London, UK). The internalization and the possible recycling of the receptor dimers will be investigated by immunofluorescence in HEK293, hGLC and hGL5. The same cell lines will be used for the gene expression evaluation by real time PCR and advanced digital PCR as well as cell signaling events by BRET and Western blotting. Several intracellular endpoints such as cAMP, Ca^{2+} and pERK1/2 activated by endosomal compartment will be investigated by BRET as well. Life/death signals will be evaluated by analysis of procaspases cleavage, MTT assay and immunostaining.

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. During my 1-year internship at the Dept. of Surgery and Cancer of the Imperial College London I demonstrated by PALM that GPER is capable to heterodimerize with LHCGR on the surface of HEK293 as well (cell=3). While the GPER/LHCGR complex does not affect the LH and hCG- induced cAMP production (LH $264.6x10^{-9}$±$69.6x10^{-9}$M *versus* $306.5x10^{-9}$±$55.03x10^{-9}$M and hCG $1539x10^{-9}$±$319.5x10^{-9}$M *versus* $1824x10^{-9}$±$437.9x10^{-9}$M; Mann-Whitney's U-test; $p<0.05$; n=4; mean±SD), an important decrease of IP1 accumulation is detected in LHR-GPER co-expressing HEK 293 cells upon LH and hCG compared to LHCGR-expressing cells (LH $2.7x10^{-9}$±$0.2x10^{-9}$M *versus* $31.8x10^{-9}$±$2.1x10^{-9}$M and hCG $11x10^{-9}$±$1.1x10^{-9}$M *versus* $52.7x10^{-9}$±$2.5x10^{-9}$M; Mann-Whitney's U-test; $p<0.05$; n=4; mean±SD). As a negative control of the GPER-FSHR/LHCGR interaction, a mutated form of GPER was generated and its cDNA inserted in a pDNA3.1 plasmid. Using BRET and PALM I demonstrated that mutations on transmembrane segments 6 and 7 of the receptor disrupted the ability of GPER to heterodimerize both with FSHR ($r^2=0.7x10^{-3}$; p=0.86; linear regression; cell=9 in PALM) and with LHCGR (cell=3), causing both FSH-induced cAMP production (FSH $152x10^{-3}$±$24x10^{-3}$ *versus* basal $5x10^{-3}$±$9x10^{-3}$ BRET changes; Mann-Whitney's U-test; $p<0.05$; n=4; mean±SD) and apoptosis in FSHR-GPER mutant co-expressing cells, and IP1 accumulation (LH $39.8x10^{-9}$±$11.6x10^{-9}$M *versus* $31.8x10^{-9}$±$2.1x10^{-9}$M and hCG $52.8x10^{-9}$±$7.2x10^{-9}$M *versus* $52.7x10^{-9}$±$2.5x10^{-9}$M; Mann-Whitney's U-test; $p<0.05$; n=4; mean±SD) in LHCGR-GPER mutant co-expressing HEK293 cells.

Conclusions

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

Nevertheless, further investigations are needed. These results provide important steps forward in the knowledge of the ovarian physiology and may have important applications in assisted fertilization protocols.

Dr. Lucia Marchetti

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

Background

Nowadays, it is estimated that over 450 million of people in the world are suffering from type 2 diabetes mellitus (DM). The main cause is the unhealthy lifestyle, involving diet with high contents of sugars and fats. Type 2 DM is characterized by hyperglycemia, and complications such as cardiovascular diseases, retinopathy and nephropathy. The current therapy mainly involves oral hypoglycemic drugs, including biguanides (metformin), sulfonylureas, thiazolidinediones (rosiglitazone and pioglitazone), which are not well tolerated by the organism. In fact, nausea and diarrhoea, are common side effects as well as excessive blood sugar lowering and weight gain. Alpha-glucosidase inhibitors (AGIs) commercial drugs, e.g. acarbose and miglitol, are very effective for reducing the postprandial blood glucose level by inhibiting the breakdown of oligosaccharides, mediated by maltase, sucrase and α-amylase located in the intestinal brush border. However, the excessive inhibition of these enzymes results in gastrointestinal disorders, due to an increase of undigested carbohydrates and intestinal fermentation. On the other hand, natural AGIs from plant sources, which exert a lower inhibitory activity (and minor side effects), can be promising and effective agents for the treatment of DM type 2. Mulberry (*Morus alba* L.) leaves and fruits are traditionally used in the Far Eastern Asia to control blood sugar levels. Recent studies have shown an effective inhibition on α-glucosidases, which results in the reduction of postprandial hyperglycemia [1]. The main compounds of the plant are polysaccharides, phenols and flavonoids (resveratrol, chlorogenic acid, mulberroside, moracin) anthocyanins and alkaloids. Among the alkaloids, iminosugars are analogues of sugars, in which an atom of nitrogen replaces the ring oxygen atom; this substitution results in inhibition of glycosidases and glycosyltransferases [1]. 1-Deoxynojirimycin (1-DNJ) has been widely studied for its selective and competitive α-glucosidase inhibition, preventing normal carbohydrate metabolism and lowering glucose blood levels and fat accumulation [2].

Objectives

Current research on 1-DNJ has been mainly carried out in the Far Eastern Asia, while few data are available on the content of 1-DNJ in mulberry cultivars adapted to European pedoclimatic conditions. The main

objective of this work was therefore to identify the highest-yielding mulberry varieties and the best leaf extraction conditions to maximize the amount of 1-DNJ in order to obtain a standardised extract to be used alone or in combination with other drugs in the control of hyperglycemia in type 2 DM. The aim of the study was also to investigate the inhibitory potential of mulberry extract on carbohydrate digesting enzymes, as models unravelling the mechanism of crude extract and its major active ingredient 1-DNJ and extrapolate the findings on humans.

Methods

Plant material of 10 different cultivars was provided by CREA – Research Centre for Agriculture and Environment, laboratory of sericulture (Padova, Italy). *Morus* leaves have undergone a mild drying process in oven at 50 °C, until reaching constant weight. A representative portion was ground and extracted by 3 consecutive steps with a solution water:ethanol (50:50) under magnetic stirring at room temperature. The 1-DNJ determination was carried out by UHPLC-ESI-MS analysis. The chromatographic separation of components is based on the Hydrophilic Interaction Liquid Chromatography (HILIC), which provides strong retention of very polar molecules that are typically unretained under conventional reversed phase conditions. The concentration of 1-DNJ in samples was obtained through the calibration curve built with the pure standard compound. The method showed a good linearity ($r^2 > 0.9995$) within the selected range of concentrations. Lyophilized extracts (sequential dilutions) were dissolved in phosphate-buffered saline (PBS) solution pH 6.9 and tested *in vitro* on porcine α-amylase (EC 3.2.1.1) and yeast α-maltase (EC 3.2.1.20), key enzymes responsible for the digestion of dietary carbohydrates into glucose. Colorimetric assays were adapted from the method described by Spinola et al. with slight modifications [3]. 1-DNJ was used as positive control and the IC_{50} values were determined from the least-squares regression line of the logarithmic concentrations plotted against percentage inhibition.

Results

The content of 1-DNJ in the different cultivars ranged from 0.42 ± 0.02 to 0.99 ± 0.05 mg/g of dry leaf ± SD, resulting in good agreement with previous findings. *In vitro* studies revealed that, in our experimental conditions, a significant inhibition of α-amylase and α-maltase was exerted at concentrations higher than 3 mg/mL of crude extracts, with slight differences among cultivars. Further assays will be performed also in association with the standard drug acarbose, in order to compare the activity of the extracts with those of the reference compound and to better understand the potential of these extracts to control hyperglicemia in diabetic patients in combination with conventional drugs used in type 2 DM.

Conclusions

In mulberry raw extract, 1-DNJ could act together with flavonoids and alkaloids as effective phytocomplex, exerting a synergistic antidiabetic effect, enabling the use of reduced amounts of drugs. These findings could provide new insights into a rational use of natural AGIs as new therapeutic approach for the prevention and treatment of type 2 DM and its complications, with only limited side effects.

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PI3K/AKT/mTOR PATHWAY INHIBITION SENSITISES FLT3-ITD ACUTE MYELOID LEUKEMIA (AML) CELLS TO TARGETED THERAPY USING RTK INHIBITORS

Background

AML has a very poor 5-year survival of just 16% in the UK. 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, and predictive of an adverse prognosis. 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 (LSCs) drive AML leukaemogenesis, responsible for drug resistance and disease relapse following conventional chemotherapy. Growing evidence recognises that FLT3-ITD mutation leads to the constitutive activation of FLT3 kinase and its downstream pathways, including PI3K/AkT/mTOR signaling, strongly associated with LSC activity. 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, primary patient blasts and our established patient derived xenograft (PDX) murine models.

Objectives

To address our central hypothesis, we aim to

- determine the efficacy of PI3K/Akt/mTOR inhibitors (PF-04691502 and BAY-806946) in FLT3-ITD+ versus FLT3 wildtype (wt) AML cell lines;
- determine the efficacy of FLT3 inhibitor (quizartinib) and standard chemotherapy (cytarabine) in FLT3-ITD+ versus FLT3 wildtype (wt) AML cell lines;
- determine the efficacy of PI3K/Akt/mTOR inhibitors in the combination therapies with quizartinib or chemotherapy agent in FLT3-ITD+ vs. FLT3wt AML cell lines and primary patient blasts.
- establish a 2D co-culture system using human stromal cell line (HS-5) with AML primary patient blasts and determine the impact of microenvironment on protection of AML cells from PI3K/Akt/mTOR inhibition.
- perform global gene expression analysis using RNA-seq to identify genes or molecular pathways regulated by PI3K/Akt/mTOR inhibitors or combination with quizartinib.

- validate identified targets following signaling pathway inhibition which may represent novel targets in FLT3-ITD AML using pharmacological inhibition and/or genetic manipulation.

Methods

Human AML cell lines, such as MV4-11 and 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 (dual PI3K/mTORi PF-04691502, pan-PI3Ki BAY-806946, FLT3i quizartinib, and standard chemotherapy cytarabine) for a time course experiment between 24h to 72h to monitor their proliferation. Cell proliferation and IC50 of inhibitors used 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). Further detailed investigation of cell phenotypes was determined by apoptosis and cell cycle assays in cells treated with an increasing concentrations of drugs for 48h around their respective IC50 using flow cytometry Annexin V FITC/DAPI staining and PI/RNase staining. To determine the effect of combination therapy on cell proliferation, resazurin-based assays were performed; and combination indices (CI) were calculated using CompuSyn.

Results

The IC50 value for quizartinib treatment at 48 hr time point for MOLM-13 and MV4-11 was at the nanomolar range (0.72 ±0.18 nM and 0.58 ±0.12 nM, respectively), whereas THP1 cells were insensitive to quizartinib. The IC50 value for PF-04691502 and BAY-806946 treatment at 48 hr was 0.06 ±0.01 μM and 0.04 ±0.02 μM for MOLM-13, 0.04 ±0.02 μM and 0.06 ±0.01 μM for MV4-11; and 0.47 ±0.16 μM and 0.12 ±0.03 μM for THP1, suggesting that these cell lines are sensitive to PI3K/Akt/mTOR inhibitors irrespective FLT3 status. Based on the IC50 concentrations obtained from resazurin-based assays, detailed cellular phenotype in response to inhibitor treatment was evaluated by apoptosis or cell cycle assays. Cell cycle assay showed that BAY-806946 treatment at 400 nM concentration induced G1 cell cycle arrest in MOLM-13 and MV4-11 detected by a significant increase of G1 phase compared to vehicle control (26.9% ±3.2; p=0.0014 and 25.7% ±2.9; p=0.0016). Furthermore, apoptosis assay showed that PF-04691502 at 1 μM concentration induced 18.8% ±1.2 (p=0.0194) apoptosis in MOLM-13 cells. Combination of quizartinib and BAY-806946 or PF-04691502 at respective IC50 value at 48 hr improved reduction of cell viability in FLT3-ITD cell lines compared to quizartinib treatment alone (37.5% and 39.4% for MOLM-13; 44.6% and 36.74% for MV4-11) and this effect was synergistic based on a CI<1 (0.48 and 0.44 for MOLM-13; 0.48 and 0.52 for MV4-11).

Conclusions

In summary, I have characterized the profile of FLT3-ITD+ vs FLT3wt human AML cell lines in response to PI3K/Akt/mTOR pathway inhibition and FLT3i. I observed that all cell lines tested are sensitive to

PI3K/Akt/mTOR inhibitors irrespective FLT3 status. Flow-cytometry based cell cycle and apoptosis assays showed that BAY-806946 reduces cell viability in FLT3-ITD+ cells via induced cell cycle arrest and PF-04691502 in part via apoptosis in MOLM-13. Dual targeting of FLT3 and PI3K/Akt/mTOR induced synergistic lethality in FLT3-ITD+ cells, suggesting a potential combination strategy for FLT3-ITD+ AML.

Dr. Laura Turco

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

Background

Cirrhosis represents the end-stage of any chronic liver disease, with a course characterized by a transition from an asymptomatic compensated stage to a symptomatic decompensated stage, two different entities that cannot be combined neither in clinical or research setting. Decompensation is mainly driven by portal hypertension, with a portal pressure, as determined by the hepatic venous pressure gradient (HVPG), ≥10 mmHg. A close relationship between portal hypertension and HCC has been described, identified baseline HVPG as independent predictors of HCC development.

Nonselective b-blockers (NSBB) have been the main- stay of therapy of portal hypertension, because, by reducing portal pressure through decreasing the splanchnic vasodilation and lowering the cardiac index, they may prevent complications of portal hypertension. However, all studies carried out so far, combined results from patients with both compensated and decompensated cirrhosis.

It has also been demonstrated that not only portal pressure measured by HVPG, but also the cardiodynamic alterations that occur in patients with cirrhosis play an important role in determining decompensation and death. During the past years, a new entity of "decompensation" has been identified, that is acute on chronic liver failure (ACLF) that has an important interrelationship with portal hypertension and systemic inflammation. However, the impact of cardiodynamic alterations, that are one of the target of NSBB, in ACLF developing has not been yet studied.

Only few studies carried out so far have tried to understand if modifications of portal hypertension induced by NSBB may change the risk of HCC development. These studies demonstrated that patients on NSBB had a lower incidence of HCC, however none of them has provided evidence about the HVPG level modifications during treatment to understand if the lower risk of HCC is related to the hemodynamic response more than to other pleiotropic effects of NSBB.

Objectives

First study

To assess the benefits of NSBBs apply equally in patients with compensated or decompensated cirrhosis separately.

Second study

To evaluate the impact of portal pressure and cardiodynamic state, the targets of NSBB, on fatal ACLF development in liver cirrhosis.

Third study

To evaluate the role of portal hypertension on HCC development and whether NSBB may have an impact on HCC relative risk in cirrhotic patients.

Methods

First study

We performed a meta-analysis to pool data from patients with cirrhosis included in studies (randomized controlled trials or other) that assessed the difference in clinically relevant outcomes between HVPG responders and non-responders relating to the two main prognostic stages of cirrhosis, compensated or decompensated, which in this study we defined as the absence or presence of ascites, respectively.

Second study

Retrospective analysis of a prospectively observed cohort of patients with cirrhosis referred to the Department of Clinical Physiology and Gastro Unit Hvidovre Hospital, Denmark, between 2002 and 2016, for per protocol hemodynamic assessment. Patients were followed until October 2017. Since the ACLF definition according to the CLIF-consortium was established in 2013 and the ACLF diagnosis was therefore established retrospectively. Causes of death that were recorded as liver failure, sepsis and multiorgan failure were classified as fatal ACLF. Non-specified shock, cardiovascular disease, malignancy, and unknown causes were classified as non-ACLF deaths.

Third study

Nested study of an already ongoing prospective cohort study looking at predictors of HCC in patients with cirrhosis when undergoing at baseline complete clinical, radiologic, endoscopic and hemodynamic work up. Patients are followed up on 6-months basis with ultrasound in order to identify HCC lesion. Goal of the study is to identify risk factors for HCC development. In the nested study, we envisage to add the evaluation of the modification of these predictors of HCC development in patients requiring NSBB.

For patients requiring NSBB therapy a second evaluation is performed 30 days after baseline, including a second HVPG with combined right heart catheterization in order to assess the decrease in portal hypertension and the cardiodynamic changes.

Results

First study

We obtained data from investigators of 15 studies of primary or secondary prophylaxis of variceal hemorrhage (VH) that had reported on VH and death in HVPG responders vs. non-responders (HVPG response was defined as a decrease >10-20% from baseline or to <12mmHg) for a total of 1113 unique

patients. Of the 1113 patients included in the studies, 968 patients (87%) had been treated with NSBB. In 993 patients (89%), HVPG response was defined as a decrease of more than 20% from baseline (>10% in 11% of patients) or to less than 12 mm Hg.

In the 661 patients without ascites, responders (n=329; 50%) had significantly lower odds of events (ascites, VH, or encephalopathy) than nonresponders (odds ratio [OR], 0.35; 95% CI, 0.22–0.56). Odds of death or liver transplantation were also significantly lower among responders than nonresponders (OR, 0.50, 95% CI, 0.32–0.78).

In the 452 patients with ascites, responders (n=188; 42%) had significantly lower odds of events (VH, refractory ascites, spontaneous bacterial peritonitis, or hepatorenal syndrome) than nonresponders (OR, 0.27; 95% CI, 0.16–0.43).

Overall, odds of death or liver transplantation were lower among responders (OR, 0.47; 95% CI, 0.29–0.75). No heterogeneity was observed among studies.

Second study

208 patients (73% male, 77% alcoholic cirrhosis, median age 60 years) were included in the final analysis. Eighty-two patients died (39%) and 50 (24%) developed fatal ACLF. Median time to all-cause mortality was 18 months and fatal ACLF was 17 months. None of the patients underwent liver transplantation or transjugular intrahepatic portosystemic shunt placement.

Based on cut-offs of cardiac index as previously applied, 84 (40%) patients showed hypodynamic (<3.2L/min/m^2), 69 (33%) patients normodynamic (3.2-4.2L/min/m^2), and 55 patients (27%) hyperdynamic (>4.2L/min/m^2) cardiodynamic state.

ACLF was the cause of death in 68%, 66%, and 45% of hyperdynamic, hypodynamic, and normodynamic patients, respectively. The cumulative probability of fatal ACLF in the three groups was 35%, 25%, and 14% ($p = 0.006$). After one year, the numbers were 20%, 8%, and 3% ($p < 0.01$), respectively. The all-cause mortality at the end of follow up and after one year was also higher in the hyperdynamic group and lowest in the normodynamic group. Hyperdynamic state was strongly associated with systemic inflammation.

In total, 39 patients (19%) were treated with NSBB. The proportion of patients treated with NSBB was highest in the hypodynamic group (n=18, 21%) and lowest in the hyperdynamic group (n=8, 15%). There was no significant difference between the three cardiodynamic groups with respect to NSBB treatment.

Third study

445 consecutive patients with cirrhosis prospectively enrolled from July 2013 at Azienda Ospedaliero-Universitaria, Policlinico di Modena, Gastroenterology Unit, when undergoing HVPG, and then followed-up every 6 months. Median follow-up was 40 months. 61 patients died during follow-up, 34 developed HCC

(incidence 4-5% per year). At univariate analysis, with preliminary data, HVPG>15 mmHg, large esophageal varices, viral vs. non-viral etiology, and albumin were statistically associated with HCC development (HVPG/large varices collinear). In HVPG>15 multivariate model, none of these factors was significantly associated with HCC development while in large esophageal varices model, the latter were independently linked with it (HR 2.258,95% CI:1.135-4.494). Albumin had borderline significance (HR 0.586, CI% .337-1.018).

Conclusion

First study

Patients with cirrhosis, with or without ascites, who have reductions in portal pressure after treatment with NSBBs are at reduced risk for adverse events or death. NSBBs should not be avoided in patients with ascites. By showing that reductions in portal pressure induced by NSBB-based pharmacological therapy improve outcomes and decrease mortality, our study supports the use of NSBB in all clinical settings (primary or secondary prophylaxis) and in both patients with or without ascites.

Because ascites is the hallmark of cirrhosis decompensation, our study shows that decreases in portal pressure are associated with better outcomes in both patients with compensated and decompensated cirrhosis and is proof that portal hypertension is a major mechanism in the development of both decompensation and further decompensation.

Second study

This study shows that, in patients with cirrhosis, cardiodynamic state plays an important role not only in the development of decompensation and death as previous demonstrated, but also on the development of ACLF. Cirrhotic patients with hyperdynamic or hypodynamic circulatory state have a higher risk of fatal ACLF. Hyperdynamic state is strongly associated with systemic inflammation, which independently predict fatal ACLF development. In our study, there was no significant difference between the three cardiodynamic groups with respect to NSBB treatment, but this new pathophysiological view represents the rational basis for further studied exploring the impact of NSBB on ACLF developing in cirrhotic patients with portal hypertension.

Third study

These are preliminary data and preliminary results of an ongoing study confirming the role of portal hypertension in the development of hepatocellular carcinoma. Patients with severe portal hypertension (that are those with HVPG>15 mmHg and those with large varices) have higher risk of HCC development. The reduction of portal pressure induced by NSBB may play a role in decreasing HCC risk.

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IDENTIFICATION OF KEY MOLECULES AND MECHANISMS DURING CELLULAR AND TISSUE REGENERATION

Background

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

Objectives

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

Methods

Punch biopsies of the skin were obtained from patients and immediately frozen in liquid nitrogen at −196°C and stored until use. Tissues were disrupted using TissueLyser II (Qiagen) and homogenized using QIAshredder (Qiagen) to reduce viscosity. RNAs were purified by using RNeasy Mini Kit (Qiagen). Quantification were performed by using RNA 6000 Nano Kit (Agilent Technologies) on Agilent 2100 Bioanalyzer. All libraries were sequenced by NovaSeq 6000 Sequencing System at deep of 30^6 bp. Data were

analyzed by RStudio software using EdgeR package. The genes identified as differentially expressed showed an FDR<0.05 (False Discovery Rate) and $|\log_2 FC > 1|$ (log2 fold changes).

Results

To elucidate how the injection of AD-MSC induce the tissue regeneration we performed RNA sequencing assays on punch biopsies of the skin from 3 patients before and after lipofilling treatment. A total of 305 genes showed differential expression (DE) after the treatment. Of these genes 175 were up-regulated (log2FC >1) whereas 130 were down-regulated (log2FC <1). Following these results, we performed a pathway enrichment analysis using the Reactome database. As results, we found that WNT pathway (*WNT9B, WNT3, WNT4, WNT16*) was highly enriched (p<0.01) using the list of upregulated genes, while using the list of downregulated genes we found that several pathways involved in the process of muscle contraction (*MYH2, MYBPC1*), keratinization (*KRT74, KRT72, LCE3D, LCE1E*), neuronal transmission (*CHRNA9, CACNA1S*) and extracellular matrix (*COMP, COL4A3*) were highly enriched (p<0.02).

Conclusions

By carrying out transcriptome analyses of skin biopsies from scleroderma's patients, we found that most of DE genes enriched numerous pathways that were involved in the regeneration process. Wnt/β-catenin signaling was involved in the proliferation of skin cells and contribute robustly to wound healing, also serve as signals for the skin stem cells that contribute to this process. Also, the decreases of muscular contraction and ECM production can contribute significantly to the reduction of fibrosis and the hardening of the skin. This data shows that after the treatment with fat enriched of AD-MSC the tissue regeneration process was successfully activated. Moreover, many genes that encode for membrane receptors involved in the transmission of signals were found. These possible targets will have to be analyzed in depth to understand if they can be used for future and more specific treatment of the disease.

Dr. Maria Luisa Introvigne

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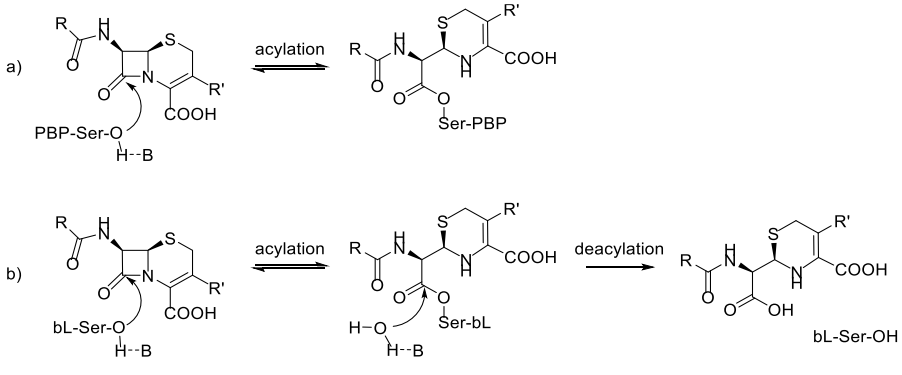
FACE TO FACE WITH ANTIBIOTIC RESISTANCE USING BORONIC ACID TRANSITION STATE INHIBITORS

Background

Antimicrobial resistance

"*Never has the threat of antimicrobial resistance been more immediate and the need for solutions more urgent*" says Dr. Tedros Adhanom Ghebreyesus, Director-General of the World Health Organization (WHO).[1] Antimicrobial resistance (AMR) is one of the most important problems facing human health: it increases health-care costs, the length of stay in hospital, morbidity and mortality. Moreover, new therapeutic agents against resistant bacteria are lacking.[1] The WHO Priority List assigned as critical *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacteriaceae*[2] which are part of the "ESKAPE" pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, and *Enterobacter* species). These bacteria currently cause the majority of world-wide hospital infections and "escape" the effects of antibacterial drugs due to the high prevalence of cephalosporin and carbapenem resistance, and to their ability to survive in adverse environmental conditions.[3]

Despite the high incidence of antibiotic resistance, β-lactam antibiotics remain the most used class of antibacterial drugs due to their safety. Different classes (penicillins, cephalosporins, carbapenem, monobactams) share the same mechanism: their β-lactam ring forms a stable adduct with the bacterial penicillin binding proteins (PBPs), enzymes involved in the synthesis of the bacterial cell wall. The bacterium is, therefore, no longer able to synthesize the cell wall and die. One of the most important mechanisms of resistance is the production of β-Lactamases, bacterial enzymes capable to neutralize β-lactam antibiotics: in close analogy to PBPs, they open the β-lactam ring, forming an adduct that in this case is easily hydrolysed by a water molecule and, therefore, the drug is inactivated (Scheme 1).



β-Lactamase enzymes are

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

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

β-Lactamases inhibitors and BATSIs.

Two new β-lactam/β-lactamase inhibitor combinations recently entered the market (Figure 1), namely the diazabicyclooctane avibactam with ceftazidime (Avycaz-2015)[4] and the boronic acid vaborbactam with meropenem (Vabomere-2017)[5]. Avibactam shows activity against class A, class C and some class D enzymes, but it lacks activity against class B metallo-β-lactamases. Moreover, there have been increasing evidences of resistance also against this inhibitor. Vaborbactam proved to be a good inhibitor of class A and class C enzymes, but it is not active against class B or D β-lactamases with carbapenemases activity. Therefore, these combinations fail in the treatment of infections caused by multidrug resistant strains of *Acinetobacter* and *Klebsiella pneumoniae*.[6]

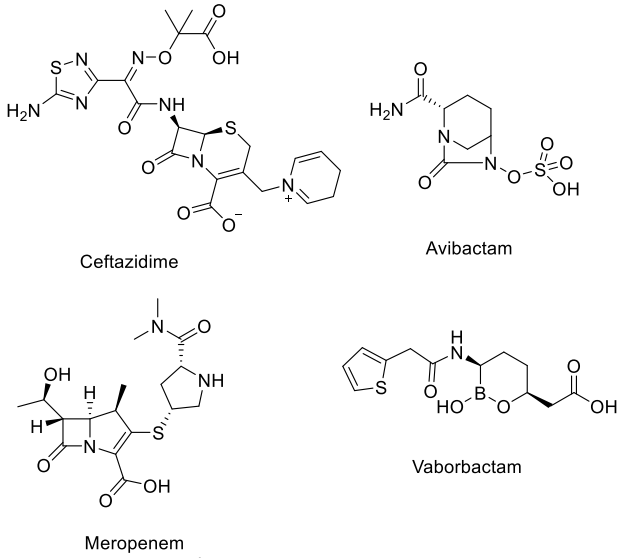
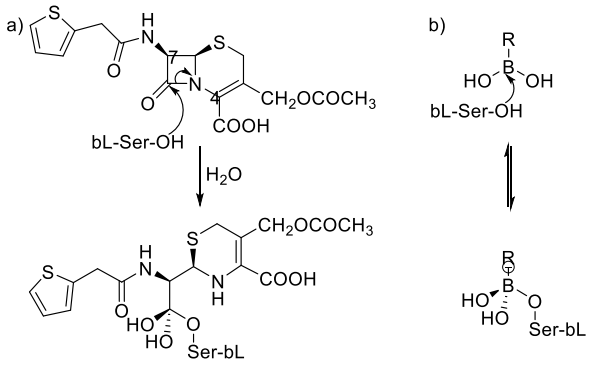
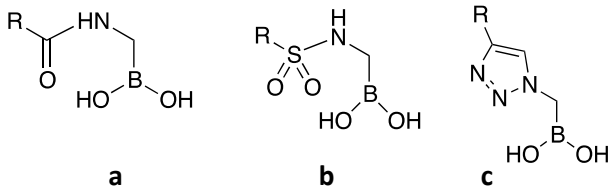


Figure 1. β-lactam/β-lactamases inhibitor combinations.

Boronic Acid Transition State Inhibitors (BATSIs) are known reversible covalent inhibitors of β-lactamases, due to the electrophilic character and *sp²* geometry of the boronic moiety, which upon attack of the nucleophilic serine, forms a tetrahedral adduct with the enzyme (Scheme 2, **b**), mimicking the one formed with the β-lactam/antibiotic complex (Scheme 2, **a**). In this case, de-acylation does not occur and the enzyme is inhibited (Scheme 2).



Scheme 2. **a)** Mechanism of action of a β-lactamase with a β-lactam (the antibiotic Cephalothin). **b)** Mechanism of action of a β-lactamase with a boronic acid, highlighting the similarity with the high-energy intermediate of the β -lactam antibiotic in the (de)-acylation step.



a **b** **c**

Figure 2. General structures of **a)** α-acylamidoboronic acids, **b)** α-sulphonamidoboronic acids and **c)** α-triazolyl-boronic acids.

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:[7] in particular, α-acylamidoboronic acids are characterized by the presence of an amide side chain bearing substituents typical of commercially available β-lactams (Figure 2, **a**).

Starting from α-acylamidoboronic acids (**a**) our research group replaced the amide group with different bioisosters, such as a sulphonamide (**b**) or a triazole (**c**) and generated compounds active against ADC-7, a highly resistant class C β-lactamase in *A. baumannii*. The second step was the synthesis of chiral compounds introducing into the boron-bearing carbon atom a carboxy-substituted ring system, able to interact with different regions of the enzymes. Two compounds demonstrated to bind effectively and inhibit β-lactamases: S02030 (Figure 3) and the related MB076 (structure not shown). The first recognizes and inhibits class A and class C enzymes; notably, its activity has been confirmed against class A KPC-2 (IC_{50}= 0.084 μM) and SHV-1 and class C ADC-7 (Ki = 0.044 μM), structurally different β–lactamases. The latter demonstrated a good activity against ADC-7 (Ki = 0,1 μM) too.

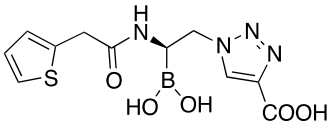


Figure 3. S02030

Objectives

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

Methods

Design and synthesis. Starting from data obtained for S02030 and MB076, the first goal was to obtain new classes of boronic acids with improved activity profile against class A and/or C. These new compounds were specifically designed keeping into account not only the determinants important for class A and C recognition, but also new substituents able to gain interactions with class B and/or class D enzymes. To obtain this result we synthesised and tested on ADC-7 and on KPC-2 the 1,2,3-triazolylmethaneboronic acids. The second step is to find compounds active against class D and/or class B enzymes and to reach this objective we synthesized β-triazolylboronic acids and α-sulphonamideboronic acids and we are going to test their activity.

Tests. Microbiological analysis, kinetics studies, docking analysis and crystallographic experiments are performed in collaboration with prof. Robert A. Bonomo research group at the Cleveland Medical Center (Ohio, USA).

Analysis. In collaboration with the Department of Chemistry of the Grand Valley University (Michigan, USA) the X-ray crystal structures of ADC-7 in complex with α-triazolylboronic acids were determined in order to understand the conformation of the inhibitor in the active site and the role of substituents, interacting with different regions of the enzyme.

Results

1,2,3-Triazolylmethaneboronic acids active against ADC-7 (class C) and KPC-2 (class A).

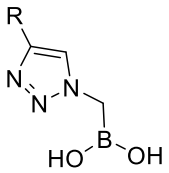


Figure 4. 1,2,3-Triazoles 1,4-disubstituted.

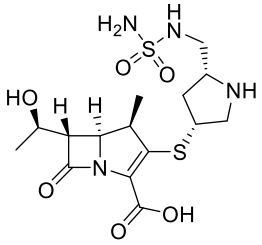
1,2,3-Triazoles 1,4-disubstituted (Figure 4) are amide bioisosters easily accessible through 1-3-dipolar Cu-catalyzed Azide–Alkyne Cycloaddition (CuAAC). The Cu-based process employs click chemistry, which proceeds in mild reaction conditions, using inexpensive reagents, with high efficiency and simple product isolation. A library of 26 α-triazolylboronic acids was synthesized and characterized via kinetic analysis and

microbiological assays performed by prof. Robert A. Bonomo at the Cleveland Medical Center (Ohio, USA). They demonstrated an extraordinary inhibitory activity against ADC-7 (Ki values spanning from 0.090 μM to 33 μM) that could be compared with vaborbactam binding affinity of 0.72 μM (IC_{50} 14.6 μM). Additionally, the X-ray crystal structures of ADC-7 in complex with 5 of these compounds confirmed the conformation of the inhibitor in the active site demonstrating that the triazole is an effective amide bioisoster in ADC-7.[8]

Fourteen of these triazolylmethaneboronic acids were also tested against resistant strains of *K. pneumoniae* producing KPC carbapenemases to determine their inhibitory activity and their ability to restore antibiotic susceptibility in combination with cefepime (FEP). All compounds show very good inhibition of KPC-2 (Kis ranging from 1 nM to 1 μM) and most of them were able to restore cefepime activity. The best KPC-2 inhibitor exhibited a Ki value of 0.030 μM and it restored FEP susceptibility in KPC-Kpn cells (MIC = 0.5 μg/mL) with values similar to vaborbactam (Ki 0.020 μM, MIC in KPC-Kpn 0.5 μg/mL). 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.[9]

β-triazolylboronic acids as a new scaffold for class D β-lactamases inhibitors.

The OXA- β-lactamases were among the earliest β-lactamases identified and initially they were rares and plasmid mediated. In particular, OXA 24/40 was initially found in isolates of *A. baumannii*, but recently the genes encoding for these enzymes have been identified in other *Acinetobacter* species as well as in *P. aeruginosa* and *K. pneumoniae*. These enzymes are able to hydrolyse both penicillins, chephalosporins and the last generation carbapenems, such as Doripenem (Figure 5).[10]



Doripenem

Figure 5. Last generation carbapenem, Doripenem.

Crystallographic studies highlighted that direct hydrophobic interactions between the Tyr-112 and Met-223 side chains create a hydrophobic barrier that restricts access to the cleft, defining a tunnel-like entrance to the active site.[11]

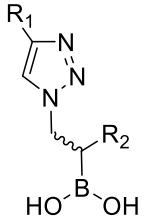


Figure 6. β-triazolylboronic acids

Starting from these evidences, we designed new inhibitors with one more carbon atom between the boronic acid and the side chain bearing an amide or its bioisoster (Figure 6). A new stereoselective synthetic process has been optimized to obtain achiral and chiral β-triazolylboronic acids. These molecules will be tested by prof. Bonomo for the microbiological profile and the complex inhibitor/β-lactamase(s) will be determined and analyzed in *silico* to verify their affinity and ability to bind the OXA 24 enzyme. Once the structure-activity (SAR) relationship will be understood, the next step will be the rational design of inhibitors bearing an amide or a sulphonamide in lieu of the triazole or bearing different substituents in R1 and R2 positions.

α-Sulphonamideboronic acid as multi target inhibitors.

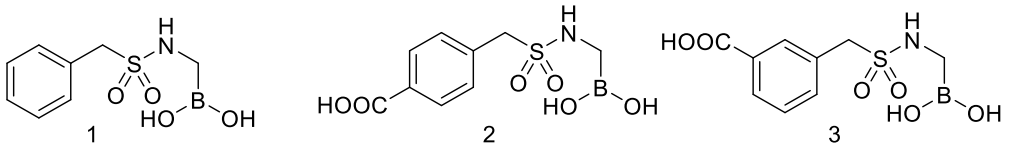


Figure 7. α-Sulphonamideboronic acids

Sulphonamideboronic acids (Figure 7) were previously tested against class C β-lactamases and proved to bind the enzymes with Ki in the nanomolar range and to restore antibiotic susceptibility in MIC analysis.[12]

Compounds **1**, **2** and **3** (Ki 70 nM, 25 nM and 1.3 nM respectively) show the best inhibitory activity and restored the activity of ceftazidime (MIC 4 μg/mL, 8 μg/mL and 1 μg/mL) and cefotaxime (MICs 1 μg/mL, 2 μg/mL and 0.5 μg/mL).[12,13]

Encouraged by these results, compound **3** was tested on ADC-7, KPC-2 and OXA 24/40 and inhibited these enzymes in the low micromolar range. Therefore, we designed a series of chiral compounds bearing a small substituent in R2 position to verify if they could improve the activity on multiple enzymes and if there is activity also on class B metallo-β-lactamases (Figure 8). These compounds were synthesised and are being tested by prof. Bonomo and his research group.

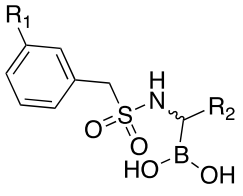


Figure 8. New α-sulphonamideboronic acids

Conclusions

In order to overcome antimicrobial resistance, the objective of my doctoral programme is to obtain inhibitors active not only against class A and class C β-lactamases, but also against class B and/or class D enzymes. To do that, different classes of compounds were synthesised and tested. Kinetic studies were

performed to determine the affinity for the enzymes and microbiological tests to analyse the ability in restoring antibiotic susceptibility. Docking studies and crystallographic experiments show how boronates actually can interact with β-lactamases active sites. Boronic acids are known inhibitors of β-lactamases and our results confirmed the ability of boron in forming a tetrahedral adduct, mimicking the β-lactam/antibiotic complex and restoring antibiotic susceptibility. The results obtained on the first class of compounds synthesised (triazolylboronic acids) demonstrated that the triazole is an excellent amide bioisoster that enhances the activity of BATSIs against ADC-7 (class C) and KPC-2 (class A) enzymes. We optimized the synthesis of β-triazolylboronic acid and synthesised new chiral α-sulphonamideboronic acid to gain affinity and inhibitory activity against class D and hopefully class B enzymes. Sulphonamideboronic acids previously synthesised were able to bind effectively and inhibit class A, C and D β-lactamases in the micromolar range. Therefore, these new molecules are expected to improve the multi-target activity. All data collected on different structures shorten the distance to achieve our objective and enhances our knowledge in fighting resistant bacteria.

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TREATMENT OF ANGIOIMMUNOBLASTIC T-CELL LYMPHOMA IS STILL AN UNMET NEED FOR MOST PATIENTS: LONG TERM RESULTS FROM THE PROSPECTIVE INTERNATIONAL T-CELL LYMPHOMA PROJECT

Background

Angioimmunoblastic T-cell lymphoma (AITL) is a rare subtype of peripheral T-cell lymphoma (PTCL) accounting for approximately 2% of all non-Hodgkin Lymphoma and about 15-20% of PTCLs. Clinical presentation of AITL is various and usually characterized by older age of patients, advanced stages and aggressive disease course. Curative treatment modalities are still under debate. The optimal treatment modalities for patients with AITL has not been found yet and are still debatable.

Objectives

A key objective of the T-cell Project (TCP) was to better define the clinical characteristics and survival outcomes of different peripheral T-cell lymphoma entities, such as AITL. To the best of our knowledge, this sub-study represents the largest international cohort of patients with AITL to date, with a focus on response to treatment and prognostic factors. The present study focuses on characterization and evaluation of outcomes of 282 cases of AITL registered in the TCP, an international prospective cohort study, designed to more accurately define prognosis and treatment outcomes of patients with mature T-cell and NK cell lymphomas.

Methods

The study was conducted in compliance with the Helsinki Declaration, and approval was obtained from the institutional review board at the coordinating center (Modena Cancer Center, University of Modena and Reggio Emilia, Italy). Eligible patients were aged 18 years and older, had adequate tissue biopsies for diagnosis, and had clinical data including baseline information on disease staging and laboratory parameters at diagnosis, types of treatment received, and follow-up. The primary and secondary endpoints were 5-year overall survival (OS) and 5-year progression-free survival (PFS), respectively. Additionally, we analyzed prognostic factors and POD24. The T-Cell Project is registered on ClinicalTrials.gov, NCT01142674.

Results

Out of 1,553 patients eligible for analysis in T-Cell Project, a diagnosis of AITL was reported in 282 cases. The median age was 64 years (range, 22-88), with 63% of cases older than 60 years. Men prevalence was observed in 60% of patients and 90% had advanced stages. The vast majority of patients (81%) received anthracycline containing chemotherapy regimens and 27 (12,5%) underwent HDT with ASCT as consolidation. According to IPI, PIT, and PIAI, the majority of cases were in the high-risk categories. 5-year OS and PFS were 44% and 32%, respectively. CR were achieved 106 patients, and 27 (25%) of them underwent consolidative ASCT. Patients treated with ASCT, exhibited a superior OS (89% vs 52%, p = 0.05) and PFS (79% vs 31, p = 0.02) compared with a control group of 56 patients who did not undergo ASCT.

In multivariate analysis, older age (p=0,003), ECOG PS >2 (p=0.0001), CRP>ULN (p=0.003) and β2 microglobulin >ULN (p=0,002) showed an independent prognostic value on PFS. Five-year PFS was 41%, 29%, and 14% for patients with 0-1, 2, and 3-4 risk factors (p=0,0002). Finally, POD24 resulted a powerful predictor of outcome. 5-year PFS was 2% and 48% for patients with or without POD24 (p=0.0001).

Conclusions

Our data confirmed the aggressive course of AITL, with dismal outcomes especially in patients exhibiting POD24. However, a superior survival was observed in patients consolidated with ASCT in CR1. Finally, a prognostic score based on age, ECOG PS, CRP, and β2 microglobulin allowed the identification at time of diagnosis of patients at different risk groups and prognosis. Analyzed data gives a better understanding about the need to continue prospective collection in real-world population.

Dr. Simonetta Lugari

CEM Curriculum: Translational Medicine
Tutor: Prof. Francesca Carubbi
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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 skeletal/bone disease represent the main features of type 1 GD, the most frequent phenotype of the disease. Moreover, GD patients present a peculiar metabolic profile characterized by an increased energy expenditure, peripheral insulin resistance, lipid metabolism disorders and hyperferritinemia. Liver involvement in GD is frequent and ranges from hepatomegaly to liver fibrosis/cirrhosis with an increased risk of hepatocellular carcinoma. Enzyme replacement therapy (ERT) and substrate reduction therapy (SRT), available for a long time, have led to great results on visceral and bone alterations and to a significant improvement of expectancy and quality of life. Moreover, a significant weight gain in GD type 1 patients on stable ERT has been reported. Considering this background, aging GD patients, as for the general population, may be exposed to unhealthy lifestyle with a potential impact on morbidity and mortality, especially on cardiovascular and liver-related complications.

Objectives

1. To characterize the metabolic profile, the cardiovascular risk and the liver disease burden in a large cohort of adult type 1 GD patients mostly on stable ERT/SRT;
2. To evaluate the role of GD severity, ERT/SRT and/or lifestyle on the metabolic profile (i.e. body composition, glucose and lipid 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

This observational study relies on the enrollment of a cohort of adult type 1 GD patients, mainly on stable ERT/SRT, 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, in particular we collected 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 features: clinical, anthropometric, biochemical and metabolic data; body composition evaluated with DEXA (model Hologic Discovery); standardized questionnaire for nutritional and lifestyle habits ('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 ®) with measurement of liver stiffness and controlled attenuation parameter (CAP) for non-invasive liver fibrosis and steatosis assessment; magnetic resonance imaging
- Cardiovascular risk: cardiovascular risk score; non-invasive diagnostic methods (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

Twenty consecutive adult type 1 GD patients were enrolled. A complete baseline assessment for each patient about GD severity, anthropometric and metabolic features (including DEXA *and* standardized questionnaire) and liver involvement (including liver stiffness and CAP measurement) was performed. Preliminary results showed that the majority of patients were on stable ERT and presented mild disease according to severity score. Metabolic comorbidities were widely represented in our cohort: 40% was overweight/obese, *50% had arterial hypertension, 15% presented insulin-resistance and 20% had metabolic syndrome. Regarding liver involvement, 8 patients (40%) had* significant steatosis and 4 patients (20%) had significant fibrosis. In particular, GD patients with significant steatosis showed a worse metabolic profile (overweight/obese, insulin resistance and metabolic syndrome), while *GD severity, dose and duration of ERT were not associated with significant 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 significantly associated with metabolic syndrome components*. Unfortunately, data collection and statistical analysis (especially about body composition and lifestyle habits) have been temporarily interrupted due to SARS-CoV-2 outbreak.*

Conclusions

Even if the complete evaluation of features of GD patients is still ongoing, we observed that metabolic alterations and liver steatosis are prevalent in our cohort. These alterations seem to depend on several

factors and unhealthy lifestyle could have a significant negative impact on metabolic status. Moreover, the metabolic derangements observed in GD patients may contribute to liver disease progression and cardiovascular complications. If confirmed, these results could improve the follow-up and therapeutic approach with particular regard to lifestyle changes, which should be strongly recommended to all GD patients.

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CEM Curriculum: Translational Medicine
Tutor: Prof. Gianni Cappelli

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

Background

Central-venous-catheter-related bloodstream infections (CRBSIs) are a concerning clinical issue because associated with morbidity, mortality, and cost among patients on maintenance hemodialysis. CRBSIs manifest with bacteremia that often evolves in severe septicemia. The incidence of catheter infections varies from about 0.6 to 6.5 episodes per 1000 catheters per day. The incidence of catheter infections varies from about 0.6 to 6.5 episodes per 1000 catheters per day. The factors favoring an infection include diabetes mellitus, recent hospitalization, and inadequate hemodialysis. The microorganisms that are commonly isolated are Gram-positive (52-84%), followed by Gram-negative (27-36%) and fungal infections (<10%). Staphylococcus Aureus is the most frequently detected pathogen, its prevalence has been evaluated to vary between 21% and 43% among several studies. CRBSIs appear to be preceded by early colonization of the catheter by the biofilm, which is made up of proteins and polysaccharides where bacteria and fungi can survive and proliferate. The interplay between microorganisms and biofilm is recognized as the main virulence factors for CRBSIs. The physical proprieties of the extracellular matrix composing biofilm protect antimicrobials from conventional antimicrobial agents because avoid drug penetrance within it. Furthermore, the pressure given by the use of antibiotics favors antimicrobial resistance of the microorganisms that arise by mutation or transfer of resistance genes.

Objectives

Since microorganisms and biofilm concur together to determine CRBIs, the objective of our research project is widening the knowledge of their interaction in patients on maintenance hemodialysis. In particular, our goal will be to evaluate whether the microorganisms that cause severe complications including sepsis, endocarditis and osteomyelitis, such as S. Aureus, possess peculiar virulence factors that can explain their increased pathogenicity compared to other bacteria of the same species. Molecular typing of their genome would allow verifying if bacteria responsible for CVC infections are the same germs that normally colonize the mucous membranes or the exit side of the CVC of the patients on chronic hemodialysis. In addition, our study would evaluate the ultrastructural composition of biofilm detected on CVC removed from patients with CRBSIs. This analysis would allow evaluating whether some morphological features of the biofilm (structure, thickness) or localization (intra- or extra-luminal) are linked with poor responsiveness to both systemic and

local antibiotics. Finally, the identification of the microorganisms located inside the biofilm would allow assessing the presence of polymicrobial flora, another well-known mechanism of antibiotic resistance.

Methods

The study will be conducted at the Nephrology Unit of the University of Modena. We will enroll patients on chronic maintenance hemodialysis patients older than 18 years with CBRI. According to the Infectious Diseases Society of America guidelines of 2009, the diagnosis of CBRI is based on (i) direct microbiological identification of the microorganism from a segment of the catheter if this latter is removed or (ii) on catheter cultures. Approximately 30 patients are expected to be enrolled in 12 months. For each patient demographics, clinical and laboratory data will be collected from the diagnosis of catheter infection. Blood cultures of aerobic and anaerobic germs, nasal swab and exit-side will be analyzed by microbiology laboratory (Department of Diagnostic, Clinical and Public Health Medicine) of the University of Modena for microbial identification. It will occur through microbial culture, thereafter, molecular typing through polymerase chain reaction should identify their virulence factors. In about 30% of the patients, their CVC will be removed for infections nonresponsive to antibiotics. In these cases, we will perform an ultrastructural investigation of the biofilm by means of transmission electron microscopy to evaluate the spatial organization of biofilm.

Expected results

CRBSi is a concerning issue in clinical practice because associated with an increased rate of morbidity and mortality. Knowing the microorganism and biofilm virulence factors could provide significant novelties in the management of CRBSi. The results of this study can be an impulse for innovation in material science of CVC and pharmacology of agents able to prevent catheter infection. The knowledge of the structure of biofilm could be a impulse for technologies aimed to prevent microbial adherence and subsequent biofilm formation. On the other side, the development of drugs active in both prophylaxis and treatment could reduce the severe morbidities associated with CRBSi

Dr. Camilla Reggiani

CEM Curriculum: Translational Medicine
Tutor: Prof. Cristina Magnoni
CoTutor: Prof. Giovanni Pellacani

INTRAOPERATIVE DIGITALLY STAINED FLUORESCENCE CONFOCAL MICROSCOPY IN MICROSCOPICALLY CONTROLLED SURGERY

Background

Ex vivo fluorescence confocal microscopy (FCM) is a novel imaging technique that enables the capture of real time digital images of freshly excised tissue with resolution comparable to that of histopathology. FCM offers some advantages over hematoxylin and eosin (H&E) frozen section analysis, especially in terms of reduced time-intensity. FCM has been investigated for its application in intraoperative diagnosis of skin tumors, such as BCCs and squamous cell carcinomas. FCM instrument is a laser equipped with both reflectance (wavelength of 830 nm) and fluorescence (wavelength of 488 nm) modalities and produces separate black and white (BW) images (reflectance and fluorescence). The newer generation FCM enables the visualization of both reflectance and fluorescence modalities simultaneously (fusion mode); high resolution images are available either separately (BW mode) or in combination, where a green filter is applied for the fluorescence and a white filter for the reflectance modes (GW mode). The newer generation FCM is also equipped with software that enables the visualization of the fusion mode with a digital stain, similar to H&E (H&E-like mode). Previous generation FCM was largely investigated in terms of accuracy and limits of the methodology, but there are no data on the diagnostic accuracy of the newer generation FCM.

Objectives

To evaluate the diagnostic accuracy of the 3 FCM visualization modalities compared to gold standard H&E frozen section.

To establish the diagnostic accuracy according to the evaluators' experience according to the visualization methodology used.

Methods

Retrospective analysis of adult patients scheduled for microscopically controlled surgery with diagnoses of primary or recurrent basal cell carcinoma (BCC). Acquired FCM images of tumor margins were assessed by 2 pathologists and 2 dermatologists.

FCM image acquisitions were performed with the Vivascope 2500 4th Gen®, (MAVIG GmbH, Munich), which has two lasers of varying wavelengths; reflectance (785 nm wavelength) and fluorescence (488 nm

wavelength) modes. ImageJ is the software that enables the visualization of the fusion mode with a digital stain similar to hematoxylin and eosin (H&E-like)[25].

Evaluator agreement was assessed with Cohen's kappa (k) and the diagnostic performance of all evaluators with the receiver operating characteristic (ROC) curve and the area under the curve (AUC).

Results

The H&E-like image modality achieved the best diagnostic accuracy, confirming 94% overall correct diagnoses among all evaluators.

At ROC curve analysis, the highest AUC was associated with the H&E-like mode, reporting the highest sensitivity of 83.3% and a specificity of 96.1%.

Conclusions

This present evaluation suggests that FCM H&E-like images are associated with the highest diagnostic accuracy, interpreted by both dermatologists and pathologists. The future application of FCM in BCC microscopically controlled surgery may decrease the number of further surgical stages, thereby reducing total surgical time and related costs. Larger studies are necessary to confirm these outcomes, and should include the evaluators' FCM image interpretation experience for estimates of diagnostic accuracy in different clinical settings.

XXXV cycle

Dr. Alessandra Odorici

CEM Curriculum: Public Health
Tutor: Prof. Elisabetta Blasi
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ANTIMICROBIAL EFFECTS OF A TOOTHPASTE, BASED ON MICROREPAIR, AND CHEWING GUM CONTAINING SELECTED PROBIOTICS ON ORAL PATHOGENS

Background

Dental plaque is a multispecies biofilm, consisting of a heterogeneous community of microorganisms, embedded in a self-produced polysaccharide matrix; well-known is the close adhesion of microbial cells to each other, to the tooth and to the mucosa, as well as to abiotic surfaces, often present in the oral cavity. Innovative strategies are continuously being proposed to facilitate everyday oral hygiene and in particular to counteract dental plaque. As an example, a novel toothpaste formulation, based on microrepair (toothpaste Pb) and chewable gums, containing selected probiotics (gum P) should be used in combination to potentiate the antiseptic effects of the oral cavity treatment. The efficacy of domestic oral hygiene procedures becomes particularly important on patients with orthodontic devices that are difficult to be cleaned, thus enhancing the risk of microbial plaque formation and deposition.

Objectives

The purpose of this study is to evaluate *in vitro* the effects of the combination "toothpaste Pb + gum P" on microorganisms commonly present in the oral cavity of healthy subjects; particularly, we will focus on specific microbiological parameters, such as total microbial load and biofilm formation onto orthodontic elastics treated or not with the toothpaste Pb.

Methods

Saliva from healthy volunteers will be collected and used to contaminate *in vitro* orthodontic elastics; then, the latter will be treated or not with the toothpaste Pb and subsequently assessed at different times for microbial load and biofilm formation. Six healthy individuals will be asked to provide their saliva in at least 3 sessions (one every 2 weeks). Briefly, 3 volunteers will masticate for 15 minutes a traditional chewing gum (chewing gum A) and the other 3 volunteers the experimental gum (chewing gum P); after that, the first group will receive the gum P and then the gum A, for additional 15 minutes plus 15 minutes of chewing. During that times, their saliva will be collected in different sterile containers. Then, at the laboratory of Microbiology, the saliva samples will be pooled (to obtain two experimental samples: Saliva A and saliva P) and used to contaminate *in vitro* orthodontic elastics. At different times post-contamination, the elastics will be treated or not with toothpaste and then analyzed to establish: i) microbial adhesion, ii) total microbial

load and iii) biofilm formation/persistence ((by live/dead fluorescent assay, Colony Forming Units (CFU) assay and microscopy analysis) and iv) microbial species (by standard procedure for species identification).

Expected results

In order to perform the above described study, an Ethical Committee approval has to be required. Accordingly, our request is currently being processed by the AVEN-CE, Policlinic of Modena. Based on the proposed protocol, the microbiological analysis will allow to establish whether and to what extent microbial biofilm occurs onto orthodontic elastics *in vitro* exposed to saliva from healthy donors. Also, the effects of the toothpaste Pb and gum P will be investigated, with the aim of establishing their efficacy in limiting microbial growth and plaque formation onto orthodontic devices. By this *in vitro* study, we hope to provide the rational for future clinical analyses on subjects undergoing orthodontic therapy and concomitantly treated with specific domestic hygiene procedures.

Dr. Luca Bedetti

CEM Curriculum: Translational Medicine
Tutor: Prof. Alberto Berardi

THERAPEUTIC HYPOTHERMIA IN NEWBORSN WITH HYPOXIC HISCHEMIC ENCEPHALOPATHY: OUTCOME FROM AN ITALIAN AREA-BASED STUDY

Background

Moderate or severe hypoxic ischemic encephalopathy (HIE), caused by intra-partum asphyxia, is one of the most important cause of death and neurological sequelae in neonates. Even if in the last decade therapeutic hypothermia (TH) has become the standard of care for brain injury control in term infants with HIE, up to 20% of infants are still affected by major neurological disabilities, particularly cerebral palsy. However, area-based data regarding infants undergoing TH in Italy are lacking.

Objectives

This area-based prospective cohort study involves all Neonatal Intensive Care Units in Emilia Romagna, an Italian region with a population of around 4 million inhabitants.

The primary aim is to evaluate the neurodevelopmental outcome at 2 years of life of infants with HIE who underwent TH. The secondary aim is to describe differences in terms of TH and infants' management between the Neonatal Units.

Methods

A common data collection form on a web platform was created, including perinatal data on neurodevelopmental follow-up (up to 24 months of life). We enrolled prospectively all surviving infants with moderate to severe HIE born in Emilia Romagna at ≥35 weeks' gestation (from January 2016 to June 2020), who underwent TH according to Italian Guidelines. They were evaluated through a neurological examination (according to the Amiel-Tison neurological assessment) and either through the Griffiths Mental Developmental Scales or the Bayley Scales of Infant and Toddler Development, depending on the local protocols. The primary outcome measure was a severe functional disability at 2 years of age, defined as the presence of cerebral palsy, cognitive score <2 SD, bilateral blindness (visual acuity < 6/60 in better eye), or bilateral deafness (requiring bilateral hearing aids or unilateral/bilateral cochlear implants). In relation to the secondary aim, data were collected regarding methods of cooling, electroencephalographic monitoring, adverse effects of hypothermia and timing of neuroradiological investigations.

Expected results

This study is currently the first Italian area-based study about HIE, TH and neurodevelopmental outcome. Considering a total of 32.000 live births per year in Emilia-Romagna, and an incidence of HIE of 1.5/1000 live births (predicted on the basis of preliminary data coming from the NICU of Modena), we expect to enroll around 50 patients per year. By estimating a drop out of 15%, we expect to have a full follow-up in 200 patients at the end of the study. These results will provide information about neurologic outcome at 2 years of age in infants with moderate or severe HIE undergoing therapeutic hypothermia in Italian Neonatal Intensive Care Units.

Dr. Carolina Castro Ruiz

CEM Curriculum: Translational Medicine
Tutor: Prof. Giacomo Borgonovo

EFFECTIVENESS OF PLATELET GEL IN THE MANAGEMENT OF DEHISCENT WOUNDS AFTER COLOSTOMY/ILEOSTOMY CLOSURE

Background

The most common complication after ostomy closure is the infection and dehiscence of the surgical wound often aggravated by chronic pain. It affects 4 out of every 100 patients and carries a mortality of up to 40%. The healing time of this type of wounds can be long, becoming an economic burden to the National Health Care System and most important worsening the quality of life of the oncologic patient.

Recently there has been an increasing application of platelet-rich plasma (PRP) in the management of chronic and infected wounds. PRP has not yet been studied in the management of ostomy closure dehiscence. PRP is a biological product defined as a portion of the plasma fraction with a platelet concentration above the baseline. As a result, PRP contains not only a high level of platelets but also the full complement of clotting factors, growth factors (GFs), chemokines, cytokines, and other plasma proteins. These properties have been proven to reduce the healing time of wounds in general and to enhance healing in chronic wounds.

Furthermore, it has been proven that PRP has antimicrobial activity against E. Coli, S. aureus, C. albicans, and C. neoformans.

PRP has an analgesic function that derives from the serotonin release by the dense granules of activated platelets.

Objectives

We will study the efficacy of Platelet Rich Plasma products (PRP) in the reduction of healing time, infection, and pain rate in dehiscent wounds after ostomy (colostomy/ileostomy) closure.

Specific aims

- Aim 1: Test the efficacy of Platelet Gel in the reduction of healing time of dehiscent wounds after ostomy closure. Using the Liao X et al equation we will establish the wound healing rate.
- Aim 2: Determine the impact of PRP in the reduction of the infection rate of the dehiscent wounds after ostomy closure.

- Aim 3: Establish the impact of PRP in reducing pain in the dehiscent wound after ostomy closure and therefore in the quality of life of our patients. This aim will be tested using the Mankoski Pain Scale.

Methods

We will carry a prospective study over a 3-year period where patients >18 years-old who develop dehiscence of the surgical wound after ostomy closure will be included. Patients who develop post-operatory complications that require surgery, who are under immunodepressant therapy, who are affected by collagenopathies or HIV positive patients will be excluded from the study. Patients will be medicated with Platelet Gel (PG) regularly and registration of the wound dimension (length, depth, width, and volume), Mankoski Pain Scale and microbiological exams will be performed in 3 different periods, that we will define as phase I (wet wound), phase II (semi-dry wound) and phase III (near-dry wound). Statistical analysis will be performed on the collected data.

Expected results

Aim 1: We expect to see a reduction in the healing time of dehiscent wounds after ostomy closure of at least 30%.

Aim 2: we anticipate a reduction of infected wounds with less use of antibiotics.

Aim 3: we expect a reduction in pain with a faster return to everyday activities.

Dr. Giovanni Merolla

CEM Curriculum: Translational Medicine
Tutor: Prof. Giuseppe Porcellini
CoTutor: Dr. Paolo Paladini

GRAMMONT-STYLE PROSTHESIS COMBINED WITH L'EPISCOPO TRANSFER VS ONLAY CURVED-STEM REVERSE TOTAL SHOULDER ARTHROPLASTY: A PROSPECTIVE KINEMATIC AND ELECTROMYOGRAPHIC STUDY

Background

Kinematic studies exploring shoulder function after reverse arthroplasty, so far published, have described active and passive mobility, with related effects on the activity of daily living, and the contribution of scapulothoracic rotation to overall shoulder motion.

Research findings concerning electromyography (EMG) of shoulder muscles after reverse total shoulder arthroplasty (RTSA) are poor; furthermore, EMG studies that analyze the effects on shoulder muscles activation after Grammont RTSA with tendon transfer or after reverse prosthesis with lateralized humeral component are lacking.

Objective

The Scope of the project was to compare kinematic and EMG data of Grammont humeral design with L'Episcopo procedure and onlay curved-stem RTSA.

Methods

This was a prospective laboratory study of 25 consecutive patients who underwent kinematic analysis and EMG after RTSA; 13 subjects received a Grammont style RTSA with combined latissimus dorsi and teres major (LDTM) transfer (L'Episcopo procedure) (Aequalis II Transfer group) and 12 an onlay curved-stem RTSA (Ascend Flex group). The inclusion criteria were a preoperative diagnosis of cuff tear arthopathy with shoulder pseudoparalysis (i.e forward elevation < 90°) associated with dropping and hornblower's signs. Constant-Murley score (CS) and active shoulder mobility (flexion, abduction, external rotation [ER] and internal rotation [IR]) were pre and postoperativeley recorded. Upper limb kinematic was recorded using a Vicon stereophotogrammetric system. The tasks selected as the main movements of the humerus with respect to the trunk were flexion (FLEX) for the sagittal plane and abduction (ABD) for the scapular plane. EMG activity of the anterior and posterior deltoid (AD and PD), upper, middle and lower trapezius (UT, MT, LT), and the upper and lower latissimus dorsi (LD) muscles were recorded concomitantly with upper limb

kinematics. Single-differential bipolar surface electrodes were positioned according to SENIAM recommendations.

The distribution of humeral elevation angles and of CS were assessed separately for Aequalis II Transfer and Ascend Flex group. A two way repeated measures ANOVA were performed to assess the differences between the two independent variables and CS. EMG activity of the two groups was compared using the Mann-Whitney test. The level of significance was set at 0.05.

Expected results

Kinematic data failed to show significant difference of humeral angles in FLEX, ABD and ER between the two groups. Results of surface EMG showed a trend of early muscle activation during flexion movement in Ascend Flex patients compared with Aequalis II Transfer group; the difference was significant for the upper LD, MT and LT muscles ($p = 0.0037$, $p= 0.005$ and $p=0.042$ respectively). Significant early activation of lower LD muscle was recorded during abduction movement in Ascend Flex patients ($p= 0.0166$). Early activation of PD during ER was detected in Ascend Flex group compared with Aequalis II Transfer patients ($p = 0.0038$).

Dr. Cristel Ruini

CEM Curriculum: Translational Medicine
Tutor: Prof. Giovanni Pellacani
CoTutor: Prof. Lars E. French

LINE-FIELD CONFOCAL OPTICAL COHERENCE TOMOGRAPHY FOR THE NON-INVASIVE, BEDSIDE AND REAL-TIME DIAGNOSIS OF SKIN DISEASES

Background

Skin cancer and its early stages are the most common cancer in the general population, whose incidence is constantly increasing. An accurate and early detection could significantly improve the overall survival and life quality of patients, with an extreme positive benefit on the global health costs. The diagnosis of skin cancer and many other skin pathologies must still be confirmed by histopathology, so that a time consuming and expensive surgical biopsy is needed, with discomfort for the patient. New non-invasive diagnostic techniques such as OCT and RCM have proven to be reliable in improving the detection of skin cancer and main skin diseases, but they still have limits such as the impossibility of combining high resolution and penetration depth. LC-OCT could solve those problems and drastically change the non-invasive diagnostics in dermatology.

Methods

The project will involve Modena as Italian study center, and two study centers in Germany at the Department of Dermatology of the University of Augsburg and Munich. At least 200 skin lesions including healthy skin, melanoma, non-melanoma skin cancer, benign melanocytic and non-melanocytic tumors and bullous diseases will be included in the study. Healthy skin and skin lesions will be evaluated through clinical pictures and dermoscopy; RCM and OCT images will be collected through the commercially available devices Vivosight and Vivascope 1500. LC-OCT will be performed using the device prototype of DAMAE Medical. After their excision, the histological examination will be performed. Diagnostic criteria for main skin pathologies in LC-OCT will be assessed.

The LC-OCT images will be compared with histology, RCM and OCT data.

Objectives

Aim of this project is the non-invasive in vivo investigation of healthy skin, skin tumors and main skin pathologies using the new diagnostic system provided by LC-OCT, in order to assess the diagnostic sensitivity and specificity of the new device compared to the standard methods and histology.

Expected results

The following hypotheses should be confirmed:

LC-OCT is reliable in the non-invasive imaging of healthy skin, skin tumors and main skin inflammatory diseases compared to the gold standard of histopathology. LC-OCT is equal or superior to its predecessors OCT and RCM concerning sensitivity and specificity. In particular, LC-OCT is able to identify epithelial tumors with equal or higher sensitivity and specificity than OCT and is superior to OCT regarding melanocytic lesions. Compared to RCM, LC-OCT has an equal or higher sensitivity and specificity for melanocytic lesions and is superior for basal cell carcinomas. Inferiority of LC-OCT in the above mentioned tasks should be interpreted as a failure.

The clinical applications of this novel diagnostic technique can potentially widely enrich the actual knowledge on in-vivo diagnosis of skin diseases, providing new details, which cannot be collected by means of other non-invasive tools such as OCT and RCM, with immense benefits for patients and health system.

Dr. Cecilia Catellani

CEM Curriculum: Translational Medicine
Tutor: Dr. Maria Elisabeth Street

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) and its main peripheral effector, insulin-like growth factor (IGF)-I, 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 and often inadequate receiving an excessively low or high GH dose.

Measured growth rate does not always coincide with the expected rate and the degree of correlation between clinical-auxological parameters and GH dose is extremely variable depending both on the 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 (Safety and Appropriateness of GH treatment in Europe) European 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. An increased risk of colorectal cancer has been reported in acromegalic patients which present GH hypersecretion due to pituitary adenomas.

MicroRNAs (miRNAs) are epigenetic 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 global 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 explored.

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 these miRNAs in oncogenic processes in *in vitro* cell models.

Methods

AIM 1: The enrolment of 18 normal-weight, prepubertal patients with idiopathic isolated GHD (9 males and 9 females) is ongoing at the Pediatric Endocrine Clinics in Reggio Emilia (AUSL-IRCCS di Reggio Emilia) and

Modena (Azienda Ospedaliero-Universitaria di Modena) and it includes the collection of plasma samples at two time points before the beginning of GH treatment and at 3 months on treatment. The patients have been treated with GH, according to the indications of the Italian Regulatory Drug Agency (AIFA Note 39). Total RNA enriched in small RNAs will be extracted from plasma samples by using the miRVana™ PARIS miRNA isolation Kit. The miRNAs will be reverse transcribed to cDNA by using the TaqMan™ Advanced miRNA cDNA Synthesis Kit. MiRNA expression profiling will be 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) and possible gender-specific differences. MiRNAs showing a p-value≤0.05 in the 2 time points before treatment will be excluded. Then the selected miRNAs of interest will be validated and quantified by Taqman MicroRNA assays from RNA samples purified from plasma samples of other 30 GHD patients. Correlation analyses between miRNA levels and clinical and auxological parameters will be performed. Statistical analysis will be performed by using SPSS v24.0 software as appropriate.

AIM 2: The *in silico* analysis will be performed by using MiRWalk v3.0 for the gene target analysis. Furthermore, DAVID v6.8, and DIANA miRPath v.3 for the Gene ontology (GO) analysis and KEGG for the pathway analysis will be used.

The *in vitro* study will be conducted on appropriate cells (human intestinal adenocarcinoma Caco-2 cell line and primary culture of human osteoblasts). The effect of dysregulated miRNAs on oncogenic processes will be analyzed in both cell types. The tumor cell line will be transfected with blank (negative control) or the appropriate miRNA mimics/inhibitors: the up-regulated miRNAs following GH treatment in the patients will be inhibited, and the down-regulated miRNAs will be overexpressed. Primary cells will be transfected with blank or the appropriate miRNA mimics/inhibitors, according to their change of expression following GH treatment in the patients. Cell proliferation, differentiation, apoptosis, invasion and migration will be analyzed. In detail, proliferation will be evaluated by using the MTT assay. Differentiation will be assessed by evaluating carcinoembryonic antigen (CEA) content, and alkaline phosphatase (ALP) activity for the Caco-2 cells, and ALP, collagen type I (COLL-I) levels, and Von Kossa staining for the osteoblasts. Apoptosis will be assayed by measuring Bcl-2 and annexin 5 contents for Caco-2 cells, and caspase 3 activity and annexin 5 content for osteoblasts. Cell migration and invasion rate will be determined using the Boyden chamber assay, considering matrix metalloproteinase-2 (MMP-2), and by using the scratch wound healing assay.

Expected results

This study will offer new insights into the effects of GH treatment on miRNAs. We expect miRNA changes to be useful to predict the response in terms of growth to GH treatment, and to identify any specific gender-effects, yet unknown.

This study will also give the chance to translate the results obtained in pediatric GHD patients undergoing GH treatment into *in vitro* cell models allowing to explore and clarify any potential oncogenic effect of GH.

Dr. Veronica Manicardi

CEM Curriculum: Translational Medicine
Tutor: Dr. Alessia Ciarrocchi

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 metastatization are still poorly defined.

The systematic functional analysis of the genome has revealed in the recent years an intricate network of non-coding regulatory elements as crucial for gene expression regulation. Recently, the ENCODE project showed that more than 80% of the non-coding genome has a biochemical function and cooperates to control gene expression to the point that many disease-associated single-nucleotide polymorphisms (SNPs) were found to reside within non-coding functional elements. Therefore, it has been postulated that sequence alterations in key regulatory elements may affect their regulatory function and cause aberrant gene expression programs, influencing cell behavior.

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.

Preliminary Results. Relying on this assumption, we hypothesize to map the metastatic evolution of melanoma by performing a genome wide analysis of the changes in the non-coding genome associated with this process. To this end we have selected a retrospective cohort of 20 primary and 20 metastatic melanomas from the Research Biobank of the AUSL-IRCCS of Reggio Emilia. We performed a deep sequencing profiling of H3K27Ac (marker of active transcription) in these samples. Differential analysis between the obtained profile of primary and metastatic lesions identified 1753 differentially activated regulatory elements, assigned to 1051 target genes. Gene Onthology (GO) analysis on predicted target genes pointed out a significant enrichment in metastasis associated pathways. 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. Moreover, two subgroups emerged within the class of primary melanomas, possibly underlining a different activation of genomic loci during metastatic progression.

Objectives

The aims of the project are:

- To identify ENHs aberrantly activated during melanoma metastatic progression.
- To point out upstream signals converging on the aberrant regulation of these ENHs during melanoma metastatic spreading.
- To outline gene expression patterns, affected by the identified regulatory regions, that orchestrate melanoma metastatization.
- To define a gene-expression based model as prognostic tool to early predict metastatic progression and improve melanoma patients' risk-based stratification.
- To assess the presence and clinical relevance of genetic mutations in genomic regulatory regions functionally associated with melanoma metastatic progression.

Methods

First, we propose to carry on a RNA-sequencing analysis on the same 40 primary and metastatic melanomas used in the ChIP-seq analysis. This profile will provide confirmation of the prediction performed based on ChIP-seq data and help nail down primary pathways involved in melanoma metastatization.

Using bioinformatic approach we will build an interactome network that will also establish a functional hierarchy in the gene expression program that underlies melanoma progression and will help us in defining the most promising targets to be validated *in vitro* models.

To this regard, we are setting to use paired primary and metastatic melanoma cell lines (WM115-WM266 and A101D-HS 294T) as in vitro model for these analyses. On these cell lines, we are currently setting ChIP-seq analysis against histone markers (H3K27ac, H3K4me1, H3K4me3) to investigate differences in chromatin functional status and to validate and integrate our *in vivo* results.

Next, we will focus on Super-ENHs that have been shown to be relevant in driving expression of crucial genes in cancer. To this end the ROSE algorithm will be applied on our ChIP-seq data from patients 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.

To consolidate the proposed model, the expression of the top scoring metastasis-associated genes will be assessed *in* vivo on a retrospective cohort of 80 FFPE tissues using Nanostring technology to verify their involvement in melanoma progression. In parallel, deep sequencing analysis will be performed on the same retrospective cohort of 80 FFPE tissues to obtained the mutational profile of metastasis-associated ENHs and promoters, thus differential analysis will define whether mutations in the identified non-coding regulatory

elements occur in human melanoma patients and whether they associate with melanoma progression and aggressiveness.

Expected results

Overall this study is expected to provide new insights into the intricate molecular mechanisms governing melanoma progression and clarify the role of non-coding genome in regulating gene expression to sustain metastatization. Moreover, we expect to define a metastasis-associated gene signature and develop a genetic mutations panel as prognostic and diagnostic tools to improve management and risk-based stratification of melanoma patients.

Dr. Ilaria Ottonelli

CEM Curriculum: Nanomedicine, Medicinal and Pharmaceutical Sciences
Tutor: Prof. Giovanni Tosi
CoTutor: Prof. Barbara Ruozi

NANOMEDICINE TO CROSS BARRIERS

Background

In the past few decades, the interest and research of nanomedicine has progressively increased. This is due to the advantages of nano-sized delivery systems, such as high loading and protection of encapsulated drugs, reduced side effects and possible increased drug accumulation into specific tissues through tailored delivery by means of conjugation with targeting moieties. Several applications of nanomedicine focus on releasing therapeutic molecules across the Blood Brain Barrier (BBB), especially by binding targeting peptides on the surface of nanomedicines, such as polymeric nanoparticles (NPs). Nevertheless, the fate of NPs once endocytosed into brain parenchyma remains uncontrolled, as they could be subject to a number of events which strongly affect NPs destiny including: endocytosis trafficking or tunneling nanotubes (TNTs) forming connections between both normal and unhealthy cells.

Objectives

Current limitations of nanomedicine-based approaches lie in the destiny of nanomedicines once they are up-taken by cells. If nanomedicine trafficking is limited to endosomal or lysosomal uptake, diminished effect could be observed as the drug delivery would be confined only to those cells. Therefore, the scope of this project is to investigate taking advantage of TNTs-mediated trafficking to create selective drug accumulation in sick cells without it being transferred to healthy ones. This aim will be pursued by designing, producing and testing a new kind of polymeric nanoparticle that selectively exploits TNTs dynamics, maintaining the ability to target a specific tissue, e.g. the BBB, but also hopefully to increase the specificity of therapeutic effects.

Methods

Starting from a well-established formulation procedure to obtain brain targeted engineered nanoparticles using the FDA-approved polymer poly-lactide-co-glycolide (PLGA), the protocol will be adapted to encapsulate molecules able to hamper or enhance TNT formation. In a second phase of the project, the potential and limits of TNTs in a relevant biological environment such as neurons/astrocytes or cell cultures will be investigated by assessing the TNT formation rate caused by the formulated NPs. At the end of the

project, the most promising NPs will be tested in biodistribution assays for their in vivo effects inside the targeted tissue.

Expected results

The first expected result is the successful formulation of NPs displaying targeting moieties on the surface able to both cross the BBB and to release loaded drugs with controlled kinetics. In vitro results will help understand the potential of both approaches (inhibition or enhancement of TNTs) and to select NPs samples, while in vivo experiments will finalize the proof if the NPs reach the Central Nervous System, are up-taken by cells, and most importantly if they can be exploited to change TNT formation dynamics to yield a novel insight on a possible therapeutic scenario, in which spreading drugs among diseased cells can affect therapeutic potential and NP safety profiles.

Dr. Marta Perin

CEM Curriculum: Public Health
Tutor: Dr. Ludovica De Panfilis

IMPLEMENTATION AND FIRST EVALUATION OF A MULTIDISCIPLINARY CLINICAL ETHICS COMMITTEE

Background

Clinical Ethics Support Service (CESSs) are defined as 'services provided by an individual ethicist or an ethics team or committee to address the ethical conflicts involved in a specific clinical case' (Fletcher, 1996). A CESS is an ethical intervention which aims to promote a personalized care approach by reducing conflicts within the healthcare relationship, promoting the ability of health care professionals to recognize and manage ethical issues and supporting ethical decision-making.

CESSs have been widely implemented among Europe and USA, with different forms and methodologies (Rasoal et al. 2017). A recent Cochrane review shows that there are little and no consistent evidences regarding their effectiveness in clinical practice in terms of: a) reduction of conflicts affecting the decision-making process and health care professionals' moral distress, b) patient's involvement in the decision-making process, c) improvement of patient's quality of life d) development of health care professionals' ethical skills e) patient's satisfaction with the treatments and care provided. (Schildmann et al., 2019)
According with the review, further studies need to provide more information: a) on theories that support interventions of this type, with particular reference to the description of the intervention's goals; b) on the procedures and models of ethics consultation; c) and on the justification of the outcomes chosen to evaluate its effectiveness (Schildmann et al., 2019).

The development, implementation and evaluation of ethics intervention in clinical practice is one of the aim of the Bioethics Unit (BU) implemented by the USL-IRCCS Company of Reggio Emilia since 2016. The BU's general scope is to promote quality of care for patients, familiars and health care professionals, proposing useful interventions which integrate empirical, qualitative and scientific analysis with ethical analysis (Huxtable R. and Ives J, 2019).
The main BU's activities consist in:
1.the development and evaluation of research projects related to ethical issues in clinical practice;
2. the provision of retrospective and prospective ethics consultation for both health care professionals and care team;
3. the provision of training programs and ethics education for health care professionals.

This research project evaluates the impact of the first implementation of a multidisciplinary Clinical Ethics Committee (CEC). The BU will be responsible for its composition, management, activities and evaluation. The CEC will support the activities already promoted by the BU and will represent an additional resource to help healthcare professionals to deal with ethical complexities in their clinical practice.

Objectives

The research project is aiming at collecting data to evaluate a multidisciplinary Clinical Ethics Committee (CEC) in terms of:

- identification of the useful components of the intervention in an Italian health care environment.
- number and types of ethics consultation requests;
- reduction of ethical conflicts and moral dilemmas affecting the health care relationship
- development of health care professionals' ethical skills
- patients and their familiars' satisfaction with care

Methods

The research project is a complex intervention due to the presence of multiple, related components that potentially contribute to the success of the intervention itself. (Schildmann et al., 2016).

We followed the *MRC frame work,* which describes the process of development, implementation and evaluation of a complex intervention through 4 phases: Development, Feasibility, Evaluation and Implementation.

The MRC framework ensures that the intervention is both theoretically and empirically founded. In particular, it highlights the importance of the development phase of intervention design, ensuring that there is an evidence base theory to support the intervention, modeling both the intervention process and outcomes, before it is piloted for feasibility (Dowding, 2017). For that reason, it is particularly useful for the purpose of our research program.

According to the 0-I steps of the MRC frame work, in the 0 step we performed a literature review, aiming to identify the available theories and evidence related to the components of a CESS. We collected data on the different components identified in the CESS implementation process. Preliminary results are described in the Expected results section.

The step 1 will be divided in two studies:

1) an observational, retrospective study with prospective qualitative evaluation

This is a preliminary study for the implementation of the multidisciplinary CEC. The study proposes the quantitative and qualitative evaluation of the BU's activities since its implementation in terms of: research projects, ethics consultations activity, educational programs.

2) *A preliminary evaluation study after 1 year of CEC's implementation*

This study aims to identify the intervention's active components and its effects in the clinical practice related to: the management of ethical conflicts and moral dilemmas affecting the health care relationship; the development of health care professionals' ethical skills; the increase of patients and their familiars' satisfaction with care. We will conduct the evaluation using both quantitative and qualitative methodology. The quantitative and qualitative data collected after the implementation process will then be compared with the data collected in point 1 to identify any processes to be integrated and / or modified.

Preliminary results and Expected results

Preliminary results:

On the basis of the recent Cochrane review (Schildmann et al., 2019) and the additional review of the literature performed during the first six months of the research program, the following elements characterize the CESSs:

a) the presence of a competent and trained ethicist;

b) the elaboration of a conceptual framework for ethics consultation (proactive/request-based timing of the intervention, who can request the intervention, the identification of triggers);

c) a structured process for the consultation (standardized or non-standardized);

d) the identification of the intervention's aim (reducing unwanted and inappropriate treatments through analysis and resolution of ethical conflicts; collecting ethically important information and prevent the development of the ethical conflict; support patients, family members, and health care professionals in dealing with uncertainties or value conflicts) and the related outcomes to measure it;

e) the adherence to international guidelines or legislative indications and national clinical ethics and the need to adapt existing ethical consultancy models to different contexts (Gaucher, Lantos and Payot, 2013; Hurst et al., 2007);

f) the relationship between the level of training of health professionals on topics of clinical ethics and access to the consultancy service (Hurst et al, 2007; Azuraga, 2014, Richter 2001);

g) the identification and evaluation health care professionals' needs related to ethical issues in their clinical practice (Diaz Rivera, 2015, Azuraga, 2014, Meyer-Zehnder et al, 2017, Bruun 2019).

We also elaborated the research protocol of the *observational, retrospective study with prospective qualitative evaluation.* It will be submitted for the ethical approval by CE AVEN on the 3th of August 2020.

The CEC will be implemented at the beginning of June.

Expected results:

a) To identify different components and relative outcomes from literature review

b) To collect quantitative and qualitative data from the local context. We will use this data to adapt the findings from literature review to the local implementation process.

c) To evaluate the CEC's impact in clinical practice in terms of:
-reduction of ethical conflicts and moral dilemmas affecting the health care relationship;
-the development of health care professionals' ethical skills
-the increase of patients and their familiars' satisfaction with care.

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CEM Curriculum: Translational Medicine
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CORRELATION OF BASELINE TMTV/TLG WITH EARLY METABOLIC RESPONSE IN HODGKIN LYMPHOMA AND INTEGRATION WITH iPET GENE-BASED PREDICTIVE MODEL

Background

Classic Hodgkin's Lymphoma (cHL) is highly curable but 30% of patients fails first line treatment and requires intensified salvage therapies. The use of FDG-PET in cHL has shown that early metabolic response during chemotherapy (iPET) is highly predictive of the subsequent risk of progression and death; this has prompted to the definition of response adapted therapy. Therefore, the identification of baseline clinical, biological and PET features to predict iPET response represents a meaningful research question. Total metabolic tumor volume (TMTV) and tumor lesion glycolysis (TLG) measured on baseline PET images have emerged as promising biomarkers of outcome in cHL. Moreover, we recently developed and validated a 5-gene signature (iPET predictive model) from pre-treatment biopsies that anticipates at diagnosis the iPET response in cHL patients.

Objectives

The aim of this study was to investigate the role of baseline TMTV and TLG to predict iPET response and their value in addition to the iPET gene-based predictive model in cHL patients.

Methods

We selected a retrospective cohort of 150 stage I-IV cHL patients who underwent baseline 18F-FDG PET/CT between 2007-2019 with available iPET after two doxorubicin, bleomycin, vinblastine, and dacarbazine (ABVD) courses. TMTV and TLG were computed from baseline PET. iPET was reported according to the five point Deauville scale (DS1-5). iPET were considered positive for DS4-5 (iPET+). TMTV and TLG were dichotomized according to the median value within our cohort. iPET predictive model from our previous study was included (Score positivity ≥-0.93). Univariate and multivariate analyses were performed by generalized linear model to test the relationship between these variables and iPET response. ROC curve was built to determine variables' performance in predicting iPET+. All the analyses were considered significant for $p<0.05$.

Results

Median TMTV was 106 cm3 (range 6-1196) and median TLG was 601 (range 52-6453). iPET predictive model was available for 116 patients. Median age was 38 years (15-79); 64(55%), 18(16%), and 34 patients (29%) had stage I-II, III and IV respectively. Twenty-three patients (19,8%) had iPET+. In univariate analysis, TMTV (OR 2.60, CI 1.00-6.72, p=0.05), TLG (OR 4,29, CI 1.55-11.88, p=0.005) and iPET model (OR 10.34, CI 3.60-29.67, p<0.001) were associated with iPET+. In multivariate analysis, TLG (OR 8.71, CI 1.24-61.40, p 0.03) and iPET model (OR 10.41, CI 3.44-31.50, p<0.001) were confirmed independent predictor of iPET+. No significant correlation of histotype, age, stage and risk group with iPET was observed. Integrating iPET model with TMTV and TLG were able to increase AUC of the model obtained by ROC analysis from 0.76 (CI 0.66-0.86) to 0.83 (CI 0.75-0.92); main difference was observed for specificity that changed from 78% to 81%.

Conclusions

TLG is an independent predictor factor of iPET+ and improves risk stratification of patients with cHL. Integration of TMTV and TLG to iPET gene-based predictive model improved specificity of the model.

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CEM Curriculum: Translational Medicine
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ROLE OF LOW-DOSE RADIOTHERAPY IN INDOLENT LYMPHOMAS

Background

My project will focus on two types of indolent lymphomas (low-grade lymphoma): follicular lymphomas and extranodal lymphomas (follicular and no-follicular). Follicular lymphomas (FL) are the most common subtype of indolent lymphoma and account for 10-20% of all non-Hodgkin lymphomas (1). Histologically, FL is represented by a cell population consisting of centrocytes and centroblasts with diffuse or nodular growth. Treatment strategies for FL may include several therapeutic choices, ranging from a "watchful waiting" approach to stem cell transplantation, mostly depending on staging, age, risk factors, and disease burden at diagnosis.

FL is a highly chemosensitive and radiosensitive lymphoma; however, disease relapse is common, above all, in advanced-stage disease, while early-stage (ES) disease can be cured in a significant proportion of cases. (2-3). In early stage FL standard of care (SoC) consists in exclusive radiation therapy (RT) and, according to historical data, long-term progression-free survival (PFS) rate and overall survival (OS) rate are 40–50 and 50–80%, respectively. With PET/CT staging the curative potential of definitive RT is likely to be higher than previously thought, with 5-year freedom from progression of approximately 74% for stage I and 49% for stage II. However, 30-50% of patients will develop relapse with a median time of 24 months after primary RT. Some data showed that the addition of immune-chemotherapy could increase clinical outcome (PFS but not OS) compared to RT alone, but early and late toxic effects could be unacceptable for patients with indolent disease, low tumor burden and few symptoms, and the SoC today still remains exclusive RT. This because to date we do not have validated criteria that allow us to discriminate the 70% of patients who heal with RT alone and the 30% which instead falls back, and which would benefit from a combination therapy.

Conversely, in advanced stage FL, SoC consists of the monoclonal antibody CD20 Rituximab (R) associated with chemotherapy according to the CHOP scheme or alternatively by the R-Bendamustine therapeutic regimen. In this disease setting, RT can be used as consolidation at the end of chemotherapy on the residual disease site. RT as exclusive therapy is used only in patients unfit for systemic therapies and has a palliative purpose.

Doses of 24 to 40 Gy were considered appropriate in first line curative treatment for localized disease (stages I-II), but several publications investigating low-dose radiotherapy (LDRT) of 4 Gy (2 × 2 Gy) reported an overall response rate surprisingly high.

Studies have shown that exclusive low-dose (4 Gy in 2 fractions) RT has improved the progression-free survival rate with an excellent toxicity profile in advanced or multi-relapsed indolent non-Hodgkin lymphomas (4-5). Currently there is a lack of knowledge on the association between chemo-immunotherapy and low-dose radiation therapy in advanced follicular lymphomas.

Concerning the extranodal lymphomas (ENL), they represent about a third of all non-Hodgkin lymphomas (NHL) and arise in a non-lymphatic organ or tissue (6). ENL can be of various histological types: follicular and marginal lymphomas are the most frequent, while lymphoplasmacytic lymphoma is less frequent. All of these histological types are radiosensitive.

In localized forms of disease, RT is often used as a single definitive therapeutic approach (7-8). The doses of RT have undergone changes after the data obtained from a multicenter English study: today 24 Gy/ 12 fractions represent the therapeutic standard against 45 Gy used previously (9). Recently, a randomized trial compared low-dose radiation therapy (4 Gy in 2 fractions) with the standard of 24 Gy in 12 fractions and found good partial and complete response rates, especially in marginal lymphoma (RC + PR rates 87% for low doses compared to 91% for the standard dose) (10).

To date, there is only one case series of patients with early stage extranodal marginal lymphoma (487 patients), which has benefited from radiation treatment, but at present there is no analysis that includes all the indolent histologies of early stage extranodal lymphoma.

Objectives

The project on indolent lymphomas has two objectives in order to fill the gap of knowledge:

- Prospectively analyze the role of low-dose radiotherapy delivered before systemic treatment in advanced follicular lymphomas to verify whether adding RT increases complete responses to the first line of therapy with a consequent increase in progression-free survival.
- Investigate the effectiveness of exclusive radiation therapy in patients with diagnosis of indolent lymphoma in the extranodal site in the early stage, through the analysis of a large national retrospective and prospective study. Primary endpoint will be progression-free survival at 3 and 5 years and secondary endpoints will be Local control at 3 and 5 years and overall survival at 3 and 5 years.

Methods

For the study on FL, patients with stage II-IV follicular lymphoma will be recruited and underwent low-dose radiation treatment (4 Gy in 2 fractions per day) on all sites of disease at onset. RT will be delivered within

15 days from the signature of the informed consent. Within 7 days from the end of the RT, standard systemic chemo-immunotherapy will be administered for a total of 6 cycles.
Primary endpoint will be the feasibility of this therapeutic approach, evaluating the frequency of patients who have completed the entire treatment sequence (low-dose RT + immunochemotherapy) and respecting the timing and doses of the planned treatments.
As it will be a feasibility study, the sample size was calculated for 20 consecutive patients.
A biological sub-project will be associated, which will evaluate the trend of the cytokines circulating in the plasma before and after RT in order to evaluate the changes in the immune response induced by RT.

The retrospective study on ENL covers all patients with extranodal lymphoma in the early stage and undergoing RT from 2003 to 2018 in our hospital. It will a multi-center study. The following clinical outcome data will be collected: progression-free survival (PFS), Overall survival (OS), local control (LC).
Subsequently a multicenter prospective collection will be planned, in order to confirm the efficacy of low-dose RT in this patient setting (ENL). The search for funds to finance the study is ongoing.

Expected results

New insights into the mechanism underlying the peculiar activity of LDRT (4 Gy in two fractions) recently emerged, together with data suggesting a potential synergistic combination with immunotherapy. These findings may open a new window of therapeutic opportunity for patients with both limited and advanced stage indolent lymphoma, harnessing the immunogenic effects of localized LDRT with negligible toxicity. However, very few clinical and experimental data currently support this hypothesis, and translational studies are strongly needed.

With this PhD project we expect to confirm, in two different settings of patients with indolent lymphoma, the efficacy of low-dose RT both in association with chemo-immunotherapy (advanced FL) and as exclusive therapy in ENL. We also hope that the biological sub-project will provide more data in favor of the systemic immune-mediated effect of RT.

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CEM Curriculum: Translational Medicine
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ENDOGENOUS NEUROSTEROIDS MODIFY THE STATUS EPILEPTICUS DYNAMICS

Background

Neurosteroids are colesterol-derived molecules able to modulate neuronal excitability by directly interacting with various membrane receptors, among which the γ-aminobutyric acid type A ($GABA_A$) receptor is the recognized main target for inhibitory currents. For this reason, the role of neurosteroids in epilepsy is under investigation in the search of new possible antiepileptic drugs. Recently, we discovered that some of them, namely progesterone, 5α-dihydroprogesterone, allopregnanolone and pregnanolone are markedly reduced in the cerebrospinal fluid of patients under treatment for *status epilepticus* (SE). SE is described as a prolonged seizure or a series of seizures without the return to baseline. This refractory neurological incidence affects approximately 41 every 100,000 adults and causes death in 20% of cases. However, it is undetermined if the reported changes in neurosteroid levels could influence the SE dynamics.

Objectives

The aim of this project is to investigate the modification of SE dynamics influenced by changes in neurosteroid levels. We found that progesterone, 5α-dihydroprogesterone, allopregnanolone and pregnanolone were all significantly reduced in comparison to the presumably healthy controls. All the mentioned neurosteroids are known to modulate the γ-aminobutyric acid (GABA) type A receptor ($GABA_A$), and especially 3α-reduced neurosteroids such as allopregnanolone and pregnanolone are potent positive modulators of $GABA_A$-mediated inhibitory currents. For this reason, we hypothesize that the changes observed in central nervous system (CNS) neurosteroid levels could be relevant for the SE onset or evolution. To evaluate the overall function of neurosteroids on the course of SE, we design experiments to modulate CNS steroid production by administering trilostane in the kainic acid model. We use this drug to investigate the effects on neurosteroid metabolisms and the possible changes in SE dynamics determined by the modified CNS neurosteroidal *milieu*.

Methods

Fourty-two adult male Sprague–Dawley rats (Charles River, Calco, Italy) of 175-200 g body weight were used in this study. First, we evaluated if the inhibitor of adrenocortical steroid production trilostane could be able to modify levels of neurosteroids in the hippocampus and neocortex. Notably, trilostane (50 mg/kg in sesame oil) injected subcutaneously 16 h and 2 h before euthanasia. The neocortical and hippocampal levels of

pregnenolone, progesterone, 5α-dihydroprogesterone, allopregnanolone and pregnanolone were evaluated by liquid chromatography tandem-mass spectrometry. Secondly, after having established that trilostane deeply alters brain neurosteroid levels, we characterized the dynamics of SE in presence of the varied neurosteroidal *milieu*. The SE was induced by a single intraperitoneal kainic acid (KA, 15 mg/kg) injection 2 h after the last subcutaneous administration of trilostane or its vehicle. 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.

Expected results

As previously demonstrated, a possible consequence of trilostane administration is the increase in allopregnanolone CNS levels shown in rats. Furthermore, neurosteroid levels in cerebrospinal fluid of patients with SE showed a significantly reduction in progesterone, 5α-dihydroprogesterone, allopregnanolone and pregnanolone levels.

For these reasons, our results will clarify the effect of trilostane on the levels of all the mentioned neurosteroids, as well as reveal the role and importance of changed neurosteroid levels in the SE onset.

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CoTutor: Dr. Alessandro Marchioni

INTEGRATION OF INTERVENTIONAL BRONCHOSCOPY IN THE MULTIMODALITY TREATMENT OF LOCALLY ADVANCED NON-SMALL LUNG CANCER WITH CENTRAL MALIGNANT AIRWAY OBSTRUCTIONS

Background

Despite new therapeutic perspectives, the presence of central airways occlusion (CAO) in patients affected by locally advanced non-small cell lung cancer (NSCLC) is associated with poor survival. Although the palliative role of interventional bronchoscopy has been established, there is no evidence on the impact on survival of endoscopic treatment integrated in the multimodality management of these patients.

Objectives

The aim of this study is to evaluate the differences between patients with central malignant airway obstruction treated only with medical therapy and patients submitted to endoscopic plus medical therapy in terms of prognosis, quality of life and access to health services.

Methods

This retrospective, multicenter, cohort study was carried out in two teaching hospitals over a 10 years' period (January 2010-January 2020). Inclusion criteria included: age >18 years, histologic diagnosis of NSCLC at stage IIIB and CAO at onset of disease, performance status $\leq$ 2. Exclusion criteria were: age > 80, end-stage chronic obstructive pulmonary disease, interstitial lung disease, life-threatening stenosis that needs urgent endoscopic treatments. Primary outcome was 1-year survival. The onset of significant respiratory events, hospitalization, need for palliative treatments, symptoms-free interval and overall survival served as secondary outcomes. Treatment arms were compared using Kaplan-Meier curves and Cox regression analysis after adjusting for confoundable factors.

Expected results

In this retrospective study a significant improvement in one-year survival was found in patients undergoing integrated treatment when compared to standard treatment. Our results are particularly relevant when

considering specific genetical and anatomical phenotypes. Further prospectic investigations are needed in order to confirm the clinical benefit of integrated treatment at one-year survival.

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CEM Curriculum: Translational Medicine
Tutor: Prof. Vincenzo Rochira

MALE OSTEOPOROSIS, A STILL OVERLOOKED ISSUE: THE REAL-LIFE EXPERIENCE OF A TERTIARY ACADEMIC MEDICAL CENTER

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% (1). Moreover, the consequences of osteoporotic fractures in men are more severe than in women, both in terms of morbidity and mortality (2). 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 (3, 4). Furthermore, it has been suggested that secondary causes of osteoporosis are generally underestimated in both sexes, especially in men (5, 6). 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 (7, 8).

Objectives

The objective of this study is to characterize from real-life data male patients seeking endocrinological consultation for bone health evaluation at a tertiary academic medical center. In particular, primary endpoints were the following:

- To define the prevalence of osteopenia and osteoporosis among male patients referring for the first time to the Endocrinologist for bone health evaluation
- To define the rate of osteoporotic fracture occurred before the endocrinological evaluation
- To classify the etiology of osteoporosis (primary or secondary)
- To investigate the presence of osteoporosis-related risk factors
- To stratify patients according to the estimated risk of fracture
- To investigate the relationship between sex steroids and skeletal-muscle systems, comparing bone health status of eugonadal patients to those undergoing androgen deprivation therapy
- To explore the clinical management of primary care physicians (e.g. rate if medical prescription of calcium and vitamin D analogues supplementation)

Methods

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. 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. Biochemical examinations regarding calcium-phosphorus metabolism and gonadal function were recorded. In particular, measurements of serum total testosterone, serum estradiol and gonadotropins were used to classify patients' gonadal status in primary hypogonadism (testosterone <300 ng/dL, high gonadotropins), secondary hypogonadism (testosterone <300 ng/dL, low gonadotropins) and eugonadism. In addition, Sex Hormone-Binding Globulin (SHBG) was recorded, when available, in order to calculate free testosterone through validated formula. Radiological bone parameters assessed by Dual-energy X-ray Absorptiometry (DXA) were recorded to define osteoporosis (T-score ≤-2.5), osteopenia (-2.5< T-score ≤-1.0) or normality (T-score >-1.0). Risk of fracture was calculated through specific algorithms (i.e. FRAX score and DeFRA).

Expected results

This cohort study might provide useful data to better describe epidemiology of male osteoporosis and its related factors, in terms of prevalence and etiology. From a perspective point of view, our findings may contribute to reduce the care gender-based gap in current clinical practice for osteoporosis and change the perception of osteoporosis as an older woman's disease. Given the number of men at midlife and older who have low bone mass and or sustain a low-trauma fracture, it is important to develop a more gendered approach for osteoporosis management. Finally, since many current management strategies to support bone health have not been developed or tested with men, this study can be the basis for future longitudinal clinical trials. In particular, deepening the association between sex steroids milieu and bone heath status be of help in potentiating diagnostic work-up and treatment of specific bone diseases.

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CEM Curriculum: Translational Medicine
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ADVANCES IN WOUND HEALING: ADIPOSE-DERIVED STEM CELLS AND ACELLULAR DERMAL MATRICES

Background

Wound healing is an evolutionary conserved process with the main goal of maintaining skin integrity. However, the skin can only reach 70% of its original strength in a normal wound healing process and often heals with scarring. Chronic wounds can stall in the inflammatory phase because of poor perfusion, poor nutrition, or a myriad of other factors causing excessive buildup of exudates The interest in chronic wound care has recently risen due to an increasing incidence of chronic ulcers, a larger elderly population, and intensive medical care for chronic disorders. Different techniques, such as composite synthetic or biological dressings and skin grafts, have been introduced in the last decades to minimize scar formation and to accelerate the healing process. However, the use of all the above-mentioned strategies is still unsatisfactory for the long healing time, the frequent recurrence of ulcers (67%) and the poor quality of the scar tissue. Extracellular-matrix (ECM) integrity is needed for tissue structure restoration: scaffolding materials, resembling native ECM in terms of mechanical properties and porosity, are needed for the transport of cells, metabolites, nutrients and signal molecules both within the scaffold and the local environment. In this setting both cell-based strategies and the use of acellular dermal substitute have been gaining importance in the last decades. Mesenchymal stem cells (MSCs) are important candidates in tissue engineering because of their plasticity and their capability to differentiate towards a fibroblast-like phenotype and secrete all the major components of the ECM. Adipose-derived stem cells (ADSCs), in particular, are a subpopulation of MSCs contained in the stromal-vascular fraction (SVF) of the adipose tissue, particularly promising because of their relative abundance in the human body and because of the safety of the isolation procedure.

Objectives

- To assess the requirements for *in vitro* integration of MSC in acellular dermal matrices (ADMs) and their pro-epithelizing and pro-angiogenic properties.
- To explore the regenerative potential of the most commonly used ADMs and obtain preliminary data for a clinical study aimed at evaluating their clinical efficacy, ability to attract MSCs and restoration of native extracellular matrix (ECM) architecture.

Methods

ADSCs were obtained from discarded material from patients undergoing elective surgery. The study was approved by the institutional review board and the investigation was conducted in accordance with the Declaration of Helsinki. A pool of ADSCs obtained from 3 different donors was seeded in a 12-well plate with culture medium enriched in ascorbic acid (250µM) for 28 days to obtain ADSC-induced dermis. As a control, the same procedure was repeated with fibroblasts. At the same time, ADSCs (and fibroblasts) were also seeded on ADM (Integra®). The ADSC-induced matrix at day 28 was transferred in 12-mm diameter trans-well plates and seeded with keratinocytes in an air-liquid interface system to promote epithelization. ADSC-induced sheets seeded with keratinocytes were fixed in formalin and paraffin embedded. Several 4-µm-thick sections were obtained using a microtome (Leitz, Germany) and stained with conventional hematoxylin and eosin (HE), Masson's trichrome (MT) and periodic acid-Schiff (PAS). Staining for Ki67 was also performed. Lastly, ADSCs were grown until confluence in complete EGM-2 medium, seeded onto Matrigel-coated (BD-Biosciences) well plates and incubated at 37°C for 6 days. Cells were stained at day 2, 4 and 6 after seeding with carboxyfluorescein diacetate (CFDA). Neo-dermis samples both with and without ADM and CFDA stained cultures were observed by a Nikon A1 confocal laser scanning microscope. The autofluorescence of collagen fibers was detected by exciting the samples with a 488 nm Argon gas laser. The confocal serial sections were processed with ImageJ software to obtain three-dimensional projections, and image rendering was performed using Adobe Photoshop Software.

Results

Keratinocytes can be efficiently seeded on the ADSC-induced matrix, thus confirming the ADSC regenerative potential. In addition to this, Ki67 expression in the basal layer demonstrates active proliferation of basal stem cells, even after only 2 days of co-culture. ADSCs are perfectly incorporated in the scaffold and acquire a homogenous distribution when seeded on ADM. Moreover, ADSCs are capable of active proliferation in ADM. ADSC-induced collagen production is significantly higher when seeded on ADM rather than in control conditions. Those data are also confirmed by semiquantitative analysis through the use of the software Image J. ADSC seeding on Matrigel determined the formation of vessel-like tubular structures even in the absence of specific pericyte and endotheliocyte precursors. In conclusion, ADSCs are proven to be more efficient with regards to their regenerative properties in the presence of adequate scaffolding materials. Collagen-based dermal matrices are a valid support for tissue regeneration, since they represent a guide not only for cell growth but also for differentiation under specific culturing conditions. Those data strongly support the use of ADM in the context of regenerative medicine. However, future studies focusing on the *in vivo* regenerative potential of ADMs in terms of clinical efficacy, ability to attract MSCs and restoration of native extracellular matrix (ECM) architecture are needed.

Dr. Roberto Tonelli

CEM Curriculum: Translational Medicine
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MECHANICAL PROPERTIES OF THE FIBROTIC LUNG DURING ACUTE EXACERBATION TREATED WITH INVASIVE MECHANICAL VENTILATION: THE "SQUISHY BALL" ELASTIC MODEL

Background

Idiopathic pulmonary fibrosis (IPF) is a fibrotic lung disease characterized by progressive loss of lung function and poor prognosis, whose typical histological and radiological pattern is defined usual interstitial pneumonia (UIP). The so-called acute exacerbation of IPF (AE-IPF) may lead to severe hypoxemia requiring mechanical ventilation in intensive care. AE-IPF shares several pathophysiological features with the Acute Respiratory Distress Syndrome (ARDS), a very severe condition characterized by diffuse alveolar damage, and might serve as a mechanical model of elastic behavior for other interstitial lung diseases during acute exacerbation (AE-ILD). Protective ventilation is the cornerstone of treatment of patients with ARDS; however, no studies have yet established the best ventilatory strategy to adopt when patients with AE-ILD are admitted to the intensive care unit. Due to the severe impairment of the respiratory mechanics, the fibrotic lung is at high risk of developing ventilator-induced lung injury, regardless of the lung fibrosis etiology.

Objectives

The purpose of this project is to analyze the effects of mechanical ventilation in AE-ILD and to increase the knowledge on the characteristics of fibrotic lung during artificial ventilation, introducing the concept of "squishy ball lung".

Methods

Consecutive patients with AE-ILD requiring invasive mechanical ventilation (IMV) admitted to the Respiratory Intensive Care Unit (RICU) of the University Hospital of Modena from November 2019 were enrolled. Exclusion criteria were age < 18 years old, established diagnosis of chronic obstructive pulmonary disease, pulmonary embolism, neuromuscular disease, chest wall deformities, body mass index (BMI) > 30 kg/m^2. A multifunctional nasogastric tube equipped with an esophageal balloon and connected with a pressure transducer (NutriVent™ nasogastric polyfunctional catheter; SIDAM, Mirandola, Italy) was placed to perform respiratory mechanics measurements.

The study protocol consisted of two consecutive phases.

The first step was performed assessing the mechanical response of the lung in volume control mode with constant inspiratory flow and low values of positive end-expiratory pressure (PEEP) (lung resting strategy).

The second step was performed after incremental increase of PEEP to target a positive static end expiratory transpulmonary pressure (P_L) (open lung strategy). Mechanical (end inspiratory P_L, driving pressure, chest wall elastance, lung elastance, respiratory system elastance) and clinical parameters (PaO_2/FiO_2) were assessed at each step and correlated with PEEP value.

Preliminary results

Five patients were enrolled. All patients presented a UIP pattern with superimposed ground-glass opacities at CT scan. Patients were all males, aged 62.6 ± 9.1 (age at diagnosis 60 ± 8.5 years). In all patients, with low levels of PEEP aimed at achieving minimal acceptable oxygenation, end-expiratory P_L was negative. When PEEP was set according to open lung strategy, positive value of end-expiratory P_L was obtained. Elevated values of PEEP were correlated with increased driving pressure (+3.2 cmH_20), end inspiratory P_L (+4.9 cmH_20), respiratory system elastance (+21 cmH_20) and lung elastance (+24 cmH_20). Incremental PEEP values were not associated with PaO_2/FiO_2 improvement.

Preliminary results suggest that PEEP is able to counteract alveolar recruitment-derecruitment, but at the price of a remarkable lung parenchymal stress. The mechanical disadvantages determined by high PEEP, suggests that in the fibrotic lung with diffuse alveolar damage, the static strain might play a relevant role. This mechanical behavior might be represented by an elastic solid model of deformation resembling the “squishy ball” toy and claims for further investigation in larger clinical trials.

Dr. Robel Papotti

CEM Curriculum: Translational Medicine
Tutor: Prof. Samantha Pozzi

CLASSIC AND NEW PROGNOSTIC FACTORS IN THE STUDY OF HODGKIN LYMPHOMA

Background

Hodgkin Lymphoma (HL) represents about 1% of all neoplasms and displays some distinct facets, including unique histological and molecular characteristics and a strong connection between tumor cells and microenvironment.

Neoplastic cells, called Reed-Sternberg cells, represent only a very small portion of the tumor, <5% (1).

For a long time, International Prognostic Score (IPS) was widely accepted as a valid method for risk stratification in patients with advanced HL, calculated using: male gender, age >45 years, clinical stage IV, hemoglobin <10.5 g/dl, white blood cells count >15 x 10^9/L, lymphocyte count <0.6 x 10^9/ L and albumin <4 g/dL (2).

After the introduction of the chemotherapy regimen ABVD (adriamycin, bleomycin, vinblastine, dacarbazine) and BEACOPP (bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, prednisolone), classic prognostic and predictive factors, such as IPS, began to lose meaning. Subsequently, imaging-based techniques such FDG-PET-CT introduced a new early evaluation criterion, the interim-PET (I-PET), which allows to distinguish patients who will likely achieve complete remission after further 4 ABVD, without intensifying therapy (3).

Hence, I-PET permits to modulate the subsequent therapy leading to an intensification of the treatment if the I-PET is still positive, nevertheless inflammatory processes and tumor necrosis can affect the results interpretation (3).

Although it appears to be the prevailing prognostic tool, about 20% of patients with negative I-PET will eventually experience progression/relapse, while a part of patients with positive I-PET will still achieve complete remission, without intensifying treatment (3, 4). There is therefore a need to discover new prognostic/predictive factors capable of improving the ones that are currently available.

Objectives

Through an integrated evaluation of the clinical, immunohistochemical, molecular and imaging data, the study aims to achieve the following objectives:

1. To predict I-PET results;
2. To improve the I-PET prognostic significance through the association with other molecular biomarkers;
3. To better understand the connection between tumor Reed-Sternberg cells and microenvironment.

Methods

Approximately 120 patients with new HL diagnosis between March 2007 and March 2017 were enrolled. Formalin-fixed Paraffin-embedded (FFPE) tissue samples were collected from the Pathology Department archive of Modena. All the clinical data were available at onset and with a 2 years' minimum follow-up after the end of 1st line therapy. Patients inclusion criteria were age >18, signed informed consent, availability of complete blood count tests at onset (baseline), availability of initial, intermediate and final PET. Cases that allowed to obtain 10 FFPE samples were identified: two 10 μm slides were used for acid nucleic extraction and eight 3 μm slides for performing immunohistochemical staining.

Results of complete blood counts and the main routine tests were collected, including LDH, Beta2 microglogulin and albumin.

Classic HL prognostic factors (IPS, Absolute Monocyte Count, Absolute Neutrophil Count) were taken into consideration.

The FDG-PET-CT images will be re-evaluated at Nuclear Medicine Departments of University Hospital of Pisa and Modena and considered negative with the Deauville criteria (1-3 negative, and 4-5 positive). Additionally, TMV (tumor metabolic volume) and/or TLG (total lesion glycolysis) will be evaluated. RNA extractions will be performed using the MagCore Genomic DNA FFPE automatic extractor at the Molecular Pathology laboratories of the Pathology Department of Modena. Gene expression profile analysis will be assessed through Affymetrix Microarrays platform at National Cancer Institute CRO (Aviano, PN). Furthermore, expression of specific markers will be evaluated by immunohistochemistry, including FOXP3, P53, BCL2, PD1, which according to literature could play a key role in the interactions between Reed Sternberg cells and the tumor microenvironment.

Moreover, the project has been associated to a prospective side study which aims to verify if the quantitative levels of circulating cytokines (IL2, IL10, IL6, TARC, MDC, sCD163) and Arg-1 enzyme (Arginase-1) correlate with the results of the initial, intermediate and final PET.

According to this goal, serum samples from peripheral blood of HL diagnosed patients will be collected at each PET session.

All data will undergo integrated statistical analysis; for this purpose, Overall Survival (OS) and Progression Free Survival (PFS) will be evaluated.

Expected results

The study expects to improve the prognostic significance of PET/CT (initial, intermediate, final), by evaluating TMV (tumor metabolic volume) and/or TLG (total lesion glycolysis) and by combining it with the results of the immunohistochemical analysis of biomarkers. Furthermore, we presume to improve knowledge about the complex crosstalk between Reed-Sternberg cells and tumor microenvironment and the underlying pathogenetic mechanism. The integration of these data might lead to a better definition of the individual

patient's prognosis at diagnosis and therefore will allow a step forward towards tailored medicine, facilitating the choice of the best therapeutic option.

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Dr. Cecilia Botti

CEM Curriculum: Translational Medicine
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THE EXTENT OF VESTIBULAR INJURY IN SUDDEN SENSORINEURAL HEARING LOSS

Background

Sudden sensorineural hearing loss (SSHL) is defined as the loss of at least 30 dB in three consecutive frequencies. SSHL not only involves cochlear function but may also be accompanied by vestibular disturbances. 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 idiopathic SSHL have been proposed: patient's age, the interval between the onset of symptoms and beginning of treatment, presence/absence of vertigo and tinnitus, audiometric patterns, severity of hearing loss, hearing level in the opposite ear, amplitude of distortion product otoacoustic emissions, vestibular evoked myogenic potentials (VEMPs). 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 canals function can be reliably evaluated with the head impulse test (HIT) in all canal planes. Abnormal caloric result (lateral canal paresis) is a significant negative prognostic factor in SSHL. A recent study showed that HIT results were different in vestibular neuritis and SSHL with vertigo, suggesting different aetiologies of vestibular neuritis and SSHL. Lateral canal function in SSHL without vertigo has been retrospectively studied by caloric test. However, to our knowledge, function of all three semicircular canals in SSHL without vertigo has never been studied before by the means of HIT.

Objectives

The aim of our study is to evaluate c-VEMPs, o-VEMPS and HIT in patients affected by SSHL with or without vertigo. The extent of the inner ear damage in SSHL and the correlation between canal/macular function and the outcome of SSHL will be analyzed. Primary and secondary aims are described in detail.

Primary aim:

- to describe the extension of macular and canal injury in patients affected by SSHL.

Secondary aims:

- to evaluate the correlation between macular injury and the prognosis of hearing function;
- to evaluate the correlation between canal injury and the prognosis of hearing function;
- to evaluate the extension and patterns of inner ear injuries to vascular alterations at head MRI.

Methods

All consecutive patients with SSHL with or without vertigo who refer to the Otolaryngology Unit or to the Audiology Unit of Azienda USL – IRCCS di Reggio Emilia will be recruited for a period of one year.

Inclusion criteria: every age and sex, diagnosis of SSHL. Exclusion criteria: incomplete follow-up.

Patients will be treated by the usual therapy and follow-up protocols, which consist in a complete audio-vestibular evaluation (audiometric test, impedance, HIT, c-VEMPs, o-VEMPs) at admission, followed by head-MRI and follow-up period (audiometric examination is repeated at 7 days, 14 days, 1 month and 3 months). Demographic and clinical data (initial and final audiogram, c-VEMPs, o-VEMPs, HIT, age, gender, comorbidity, associated symptoms, delay of treatment and vertigo, vascular alterations at head-MRI) will be collected. O-VEMPS, cVEMP and HIT test results will be compared with patients' initial and final audiogram, risk factors and alterations at head MRI.

Expected results

We will recruit about one-hundred patients. We expect to find canal and macular injury patterns in patients affected by SSHL. Moreover, we expect to find a correlation between the presence of canal/macular injury and worse prognosis of hearing function. We think that 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. Caterina Vacchi

CEM Curriculum: Translational Medicine
Tutor: Dr. Andreina Manfredi

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) is the most frequent lung involvement in pSS and its prevalence ranges from 6 to 70% of patients. It is associated to an impaired quality of life and physical capacity and to premature 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. Moreover, this complication is often underrated and, nowadays, there are no randomized controlled clinical trials to support therapeutic guidelines.

Non-specific interstitial pneumonia (NSIP) is recognized as the most common ILD disorder, followed by organizing pneumonia (OP), fibrotic NSPI, fibrotic OP, usual interstitial pneumonia (UIP) and lymphocytic interstitial pneumonia (LIP), specifically associated with pSS but less frequent.

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 multicenter observational prospective study involving 9 rheumatologic centers.

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.

Expected results

One hundred and eighty-eight pSS patients have been enrolled up to now. Most of them were female (female/male 177/11). The medium follow-up was 11.53 +- 7.78 years. The median age at diagnosis were 62 (IQR 21).
Among them 35 showed ILD (18.61%), with the following features: three subjects were males and 32 females. Median age was 57 (range 24-80).
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 and a possible different prevalence as regards radiological patterns. Findings about incidence, prognosis, predictive factors and evolutive characteristics could suggest novel screening opportunities and help physicians to better define management and therapeutic algorithms in an increasingly relevant multidisciplinary setting.

Dr. Francesca Combi

CEM Curriculum: Translational Medicine
Tutor: Prof. Giovanni Tazzioli

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

Background

The research project will take place in the *Breast Surgical Oncology* and *Plastic and Reconstructive Surgery* Units of the University Hospital of Modena.

Women who are diagnosed with non-metastatic breast cancer, are treated with surgical excision. A conservative choice (quadrantectomy) or a demolitive one (mastectomy with potential reconstruction) is done depending on the local extension of the disease. Simultaneously a surgical staging of the axillary lymph nodes must be performed, to assess if a loco-regional lymphatic spread is already present. When metastatic involvement of the lymph nodes is found, the surgical choice is complete axillary dissection. To complete the loco-regional treatment some patients undergo adjuvant radiotherapy on the surgical site. 15-20% of women develop upper limb lymphedema after surgical and radiotherapic treatment. In most cases it can be mitigated with rehabilitation but when conservative treatment fails, women experience functional discomfort and a dramatic worsening in quality of life. At the state of the art, no instrument is available to predict risk factors to develop arm lymphedema, nor to tailor the best treatment option for the patient. Microsurgical procedures (lymphatic-venous anastomosis and lymphatic tissue transplantation) have already been described in the treatment of arm lymphedema, nonetheless they are not widespread and the rate of success is frequently disappointing.

At present, no dedicated programs are defined in the University Hospital of Modena, to follow and treat patients who develop arm lymphedema. Some are referred to the rehabilitation service 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 dedicated program inside the Breast Surgical Oncology Unit of the University Hospital of Modena, to evaluate and treat women with upper-limb lymphedema, defining a dedicated space and a “lymphedema team” (breast surgeon, plastic surgeon, radiotherapist, physiotherapist)
2. To elaborate a tool to help surgeons to predict which patients have a higher risk to develop upper-limb lymphedema
3. To elaborate a tool to help surgeons and radiotherapists to early recognize the onset of upper-limb lymphedema

4. To elaborate a tool to define the selected group of patients that may benefit from microsurgical treatment and which technique

Methods

Patients who underwent axillary lymph node dissection in the past five years have been identified from the surgical database of the Breast Surgical Oncology Unit of the University Hospital of Modena. A questionnaire will be created to assess the grade of lymphedema that they are experiencing and what kind of treatments they already underwent. A "lymphedema team" has been identified, composed by a breast surgeon, a plastic surgeon, a radiotherapist and a physiotherapist with experience in conservative treatment of arm lymphedema.

The "lymphedema team" will recruit the patients and evaluate them in a multidisciplinary setting.

Clinical evaluation and arm measurements will be done, together with the filling of the questionnaire by the patient. Apart from the questionnaire, objective data will be collected on the type of surgery and radiotherapy that were performed (date of surgery, number of excised lymph nodes, type of surgery on the breast, dosage, duration and involved structures in the radiant treatment). Physical parameters and potential risk factors (BMI, comorbidities, smoking habits) will also be recorded. After collecting all the data, a statistical analysis will be performed to define a "high risk" category of women. Once the assessment is complete, the team will define the group of patients that can still benefit from conservative treatment options (physiotherapy, manual lymphatic drainage, pressotherapy, compressive sleeves). A dedicated physiotherapist will perform the treatments and a new multidisciplinary evaluation will be repeated every 4-6 months.

For severe cases, that already experienced all the conservative treatments, the surgical indication will be discussed. Patients who will be addressed to surgery will undergo lymphoscintigraphy and Indocyanine green study of the lymphatic patterns before surgery. Surgical treatment will then be performed on this highly selected group of patients from a trained team.

Expected results

We expect that the retrospective analysis of patients that developed lymphedema after breast surgery and radiotherapy will help to define a "high risk" group of patients. This will help to early detect future patients that are more prone to develop the same complication.

We also expect that the thorough evaluation of all the patients that were surgically treated in the last five years, will lead to select only a small group that could benefit from a microsurgical correction of lymphedema, thus permitting to offer the best tailored treatment for each patient, avoiding overtreatment when conservative approach is still feasible.

We believe that the creation of a dedicated “lymphedema team”, that will early take in charge patients right after axillary dissection and radiotherapy, will help to improve and complete the local treatment offer for women affected by breast cancer.

We plan to train a team that will be able to perform lymphatic microsurgical interventions.

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). It is a disorder of the development of movement and posture, often associated with perceptive and cognitive disorders, epilepsy and secondary musculoskeletal problems. Among these, hip luxation represents the most frequent and clinically relevant one, affecting 72% of non-ambulatory CP children. It may cause pain, scoliosis and Quality of Life (QoL) worsening. Hip surveillance programs are recommended and surgery is suggested in case of advanced luxation, but considering risks and burden, it is debated in severe CP children. Thus it is crucial to identify an effective preventive 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 femoral head centering in 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 the trunk aligned and hips abducted), in preventing hip luxation in quadriplegic non-ambulatory CP children.

Methods

Meetings have been organized to share the protocol within the research team, composed by 13 Rehabilitation Sites from Italy. The request for approval to the Ethical Committee is being processed. A total of 102 quadriplegic CP children, aged 1-6 years-old, classified as Gross Motor Function Measure 4 or 5, will be recruited and randomized to usual or experimental sitting, at least 5 hours a day, for 2 years. The primary outcome will be the degree of luxation, measured by means of the Migration Percentage (MP), on pelvic radiography, at 12 and 24 months. Secondary outcomes will include compliance and QoL, using validated tools, hip pain, device cost, MRI lesions and concurrent spasticity treatments (botulinum toxin and baclofen).

Expected results

Experimental sitting is expected to reduce the MP change compared to usual care. It will be of interest to compare compliance, QoL and costs in either groups: aspects affecting the effectiveness. Furthermore, to evaluate correlations between MP and spasticity treatments, MRI lesion type, and other clinical features.

Dr. Tommaso Lo Barco

CEM Curriculum: Translational Medicine
Tutor: Prof. Francesca Darra
CoTutor: Prof. Giuseppe Biagini

GHRELIN AS A BIOMARKER OF RESPONSE TO ANTIEPILEPTIC DRUGS

Background

Despite the continuous development of antiepileptic drugs (AEDs) in the past few decades, about 30% patients with epilepsy still present seizures resistant to drugs. In the clinical setting, testing the response to a specific AED is time-consuming. Uncontrolled seizures and exposure to high doses of multiple medications result in possible developmental delay in children, cognitive decline in adults, and significant increase in the morbidity and mortality. 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 (*Marchiò et al, 2018*). 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; however, mechanism underlying higher levels in drug responders is unknown.

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.

Mostly, we aim to elucidate mechanism underlying this difference, by demonstrating that plasma levels of ghrelin and des-acyl ghrelin increase in pharmaco-responsive children after initiating treatment, and not in those with a drug resistant epilepsy. Secondary, we aim to refute the hypothesis that responders have higher ghrelin and deacyl-ghrelin levels, compared to healthy control subjects and nonresponders, even before the start of drug therapy.

Methods

This is a 24-months-lasting prospective study, conducted in four neuropediatric Italian centers (Modena, Verona, Roma, Firenze).

Inclusion criteria 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: acute or chronic metabolic disorders with rare/sporadic seizures without epilepsy.

In recruited patients, we measured ghrelin and des-acyl ghrelin by immunoassays in blood samples obtained after overnight fast at two different times: before and beyond a month after initiation of drug treatment.

Demographic data, clinical features, epilepsy diagnosis, glycaemia and blood level anti-epileptic drugs were obtained and included in a multivariate statistical analysis, comparing values of ghrelin and des-acyl ghrelin in the following groups:

- patients with positive response to drug treatment;
- patients with drug-resistant epilepsy;
- 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. Control population was constituted of patients enrolled for a suspect of epilepsy which was not confirmed after a minimum of 18 months.

Expected results

In this study we expect to confirm and expand the previous finding of higher plasma level of ghrelin and des-acyl ghrelin in children with epilepsy showing positive response to AEDs, comparing with non-responders and healthy control. In addition, we will define whether the plasma levels of these hormones actually increase following the drug treatment or are already higher before the start of treatment in a part of the subjects under study.

If our hypothesis will be confirmed, measuring plasma level of ghrelin and des-acyl ghrelin may constitute an ecological chance to early detect drug-resistance in children with epilepsy initiating an AED treatment. It will also represent a first demonstration of a peripheral mechanism involved in the response to antiepileptic drugs.

Dr. Rebecca Borella

CEM Curriculum: Translational Medicine
Tutor: Prof. Andrea Cossarizza

ROLE OF T CELLS IN THE THERAPY WITH IMMUNE CHECKPOINT INHIBITORS

Background

Novel treatments based upon the use of immune checkpoint inhibitors have demonstrated impressive efficacy in different types of cancer, including metastatic melanoma. Unfortunately, most patients do not derive benefit or lasting responses. In the last few years, cutting-edge single-cell technologies have been used to interrogate a number of tumor settings with the goal to understand immune responses after treatment with checkpoint inhibitors and identify accessible biomarkers. However, only in few cases this immune cellular response was correlated with a measurable clinical response.

Objectives

Using high-parameter flow cytometry, to identity T cells that could act as possible biomarker(s) of response to anti-PD-1 therapy in patients with metastatic melanoma.

Methods

Polychromatic flow cytometry

A multicolor flow cytometry panel will be optimized to broadly characterizes T cell differentiation and activation along with markers that are target or are involved in immunotherapy response (such as CD3, CD4, CD8, CD45RA, CD197, CD28, CD27, CD127, CD95, CD98, CD71, CD25, HLA-DR, CD38, CD39, CXCR6, CCR4, KI67, T-bet, granulysin, PD1, BTLA, CD244 and ICOS). Moreover, the panel will be optimized to identify the expression of PD1 in T cells obtained from patients treated with human anti-PD1 (Nivolumab or Pembrolizumab) as anti-IgG4 will be used to recognize the human anti-PD1 bound to PD1. Briefly, cryopreserved samples will be thawed in RPMI supplemented with 10% foetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/ml streptavidin, 2 mM L-glutamine, 20 mM HEPES (all from ThermoFisher) and 20 µg/ml DNase I from bovine pancreas (Sigma-Aldrich). After washing with phosphate buffer saline (PBS), cells will be stained immediately with the Zombie Aqua Fixable Viability kit (BioLegend) for 15 min at room temperature. Then, cells will be washed and stained with the combination of mAbs purchased from BD Biosciences, BioLegend, or eBioscience. Chemokine receptors will be analyzed by staining with the adequate mAb for 20 min at 37°C. Intracellular detection of Ki-67, granulysin and the transcription factor T-bet will be performed following fixation of cells with the FoxP3 transcription factor staining buffer set (eBioscience, ThermoFisher), according to manufacturer's instructions, and by incubating with specific mAbs for 30 min at 4°C. A second

panel will be optimized to broadly investigate mucosal invariant associated T (MAIT) cell phenotype, including CD3, CD8, TCR Vα7.2, CD161, CD45RO, CD197, CD28, CD27, CD127, CD95, CD25, HLA-DR, CD38, CXCR4, KI67, granulysin, CD69. All these samples will be acquired on a Cytoflex LX flow cytometer (Beckman Coulter) equipped with six lasers (UV, 355 nm; violet, 405 nm; blue, 488; yellow/green, 561 nm; red, 638 nm; IR, 808nm) and capable to simultaneously detect 21 parameters. Flow cytometry data will be post-compensated by FlowJo, with the approach of single stained controls (BD Compbeads incubated with fluorescently conjugated antibodies).

In parallel, thawed PBMC will be kept at rest for 4 hours at 37°C and then in vitro stimulated with anti-CD3/CD28 (1 µg/ml) (Miltenyi) and suboptimal concentration of IL-12 (2 ng/mL) (Miltenyi) and IL-18 (50 ng/mL) (R&D) and a combination of those. A 11 parameters/10-color flow cytometer panel will be optimized to identify MAIT cells producing granzyme (GRZM) A, GRZM B, TNF-α and IFN-γ after 16 hours of incubation. For detection of cytokines, cells will be fixed with BD Cytofix/Cytoperm Fixation/Permeabilization Solution kit (BD Biosciences) according to the manufacturer's instructions. These samples will be acquired on an Attune NxT acoustic flow cytometer (Thermofisher) equipped with four lasers (violet, 405 nm; blue, 488; yellow/green, 561 nm; red, 640 nm) and capable to detect 14 parameters. Flow cytometry data will be compensated in FlowJo as described above.

High-dimensional flow cytometry data analysis

Flow Cytometry Standard (FCS) 3.0 files will be analyzed by using FlowJo software V 9.6. Aggregates and dead cells will be removed from the analyses and identify CD3+, CD8+ T cells will be gated. Data related to 10,000 CD8+ T cells per sample will be exported and biexponentially transformed in FlowJo V10. Further analysis will be performed by a custom-made script that makes use of Bioconductor libraries and R statistical packages. Data will be analyzed using the Phenograph algorithm coded in the cytofkit package (version 1.6.5) under R (version 3.3.3). Phenograph clusters will be visualized using tSNE. Clusters representing <0.5% will be not analysed in subsequent analysis. New FCS files (one for each cluster), originated from Phenograph analyses, will be imported and analyzed in FlowJo to determine the frequency of positive cells for each marker and the corresponding median fluorescence intensity (MFI). gplots R package will be used to generate the heat map, showing the iMFI of each marker per cluster.

Expected results

Optimization of the methodology for the multiparametric analysis of different types of T cells; identification and characterization of the main T cell populations that are involved in anticancer response.

Dr. Fulvio Massaro

CEM Curriculum: Translational Medicine
Tutor: Dr. Francesco Merli
CoTutor: Prof. Stefano Luminari

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, holding an indispensable role for normal hematopoiesis. An important feature of MSC is their immunomodulating capacity, which contributes to the regulation of the activity of T and B cells, macrophages, natural killer and dendritic cells. This immunomodulatory potential is partially mediated by secreted cytokines and extracellular vesicles. With host aging, MSC also undergo age-related changes, which have been shown to play important roles in the pathogenesis of several diseases of the elderly, often related to a persistent low-grade systemic pro-inflammatory status defined “Inflammaging”. The effect of aging on MSC has been demonstrated for several features, including telomeres length, cell density, proliferation potential, trilineage differentiation potential, epigenetics, mitochondrial function and secretome. Furthermore, aged MSC gradually decline their immunomodulatory ability, an element contributing to the wide process of “Immunosenescence”, the aging-related changes of the immune system. Several trials are using MSC for therapeutic interventions in severe degenerative and/or inflammatory diseases, including Crohn's disease and graft-versus-host disease, alone or in combination with other drugs. MSCs are promising for therapeutic applications given the ease in obtaining them, their genetic stability, their poor immunogenicity and their curative properties for tissue repair and immunomodulation.

Objectives

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

-MSC morphology, phenotype and expansion (size, granularity, population doubling and doubling time, cell cycle);

-Cellular senescence: β-galactosidase activation, CD264 expression and expression of senescence associated genes (p16, p21 and p53);

-Expression of genes implicated in cell proliferation (pRB, CDK2, FosB, Stat1, CDK1, CCNA, CCNB, CCNE), hematopoietic support (MMP2, SDF-1, SCF, IL-6, IL-8, GM-CSF), osteogenesis (Runx-2, OSX, OPN, OPG, BSP),

chondrogenesis (Col2α1, SOX9, ACAN, COMP), adipogenesis (KLF2, KLF5, PPARɣ, CEBPα), immunomodulation (Cox1, Cox2, LIG, GAL1, HGF, TSG6 and IDO);

-MSC response to inflammatory priming: immune-related antigens, surface molecules, TLR and NLR, regulatory factors;

-Immunomodulatory potential of MSC and extracellular vesicles (EVs): lymphocyte T proliferation, NK cytotoxicity and 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 Dulbecco's modified Eagle's medium (DMEM) supplemented with 12.5% fetal bovine serum (FBS), 2 mM L-glutamine and 0.5% antibiotic anti-mycotic solution. Non-adherent cells were removed by replacing the medium 48 h after the initial cell seeding. The medium was changed twice a week until cultures reached 80% confluence. Thereafter, cells were detached with TrypleSelect solution and subcultured at 10^3 cells/cm2.

The population of BM MSC was determined by analysis of cell-surface markers, using fluorochrome-labeled antibodies directed against CD73, CD90, CD105, CD146, CD14, CD34, CD45, HLA-DR. The cells were characterized according to the ISCT criteria.

Prior to reverse transcriptase polymerase chain reaction (RT-PCR), MSC were incubated for 24h in serum-free medium in the presence of IL-1β, IFN-A, IFN-ɣ and TNF-α (15 ng/ml). Total RNA from each cell culture was extracted in a single step using TriPure Isolation Reagent. Complementary DNA (cDNA) was obtained by means of reverse transcription reaction. To avoid potential DNA contamination of RNA samples, DNase treatment was performed. To confirm the absence of DNA contamination, we included a no-reverse transcriptase control during the reverse transcription step. The GAPDH gene was used as a housekeeping gene to quantify and normalize the results. Real-time PCR was performed on an ABI Prism 7900HT Sequence Detection System. We used 25 ng of cDNA in a real-time PCR with SYBR Green PCR Master Mix and 0.32 µmol/L of gene-specific forward and reverse primers.

Expected results

The MSC derived from elderly donors are large, flat and granular and display increased β-galactosidase expression. Cell size and granularity increase with aging and seem to represent senescence features. We also observed a reduced proliferation of MSC from elderly donors as evidenced by population doubling and population doubling time. MSC gene expression profile response to inflammatory status seem to vary according to subjects' age.

Dr. Jacopo Demurtas

CEM Curriculum: Public Health
Tutor: Prof. Elena Righi
CoTutor: Prof. Roberto D'Amico

COVID-GUIDE: A CHATBOT FOR SELF-ASSESSMENT AND TRIAGE OF SYMPTOMS DURING COVID-19 PANDEMIC

Background

The unprecedented COVID-19 pandemic has shown the weaknesses of healthcare systems and the huge potential impact of e-health. Recent literature claims that chatbots, if effectively designed and deployed, could be useful tools to rapidly share information, foster healthy behaviors and help lessening the psychological burden of isolation. Despite the relevant potential benefits, their extensive use may amplify misinformation. Computer decision support software (CDSS) and symptom checkers may help in assessing precociously patients' symptoms and suggesting the appropriate health care service to refer to, although they do not often attain to monitor and take care of people.

Objectives

The aim of this project is to develop and test a safe and reliable CDSS/webapp to help people assessing and monitoring COVID-19 related symptoms on a home based setting in order to suggest a safe, timely and effective referral to the most appropriate healthcare service (namely family physician, emergency service or self-monitoring).

Methods

A multidisciplinary team of physicians, epidemiologists, computer engineers, ITs, methodologists, psychologists and expert patient, based on Switzerland, Germany and Italy was recruited to plan and design the web-app COVID-Guide, a Class I medical device developed in accordance with the European Medical Device Directive 93/42/EEC (including 2007/47/EC). On February 2020 clinical prediction rules for COVID-19 disease diagnosis were outlined by matching data from available literature from China and Northern Italy regarding clinical presentation of COVID-19. On that base we defined the different scenarios of the web-app. The COVID-Guide was based on the software SMASS, which had been already used for a structured medical initial assessment in Germany and Switzerland.

Through the interaction with its artificial intelligence, a first assessment of any symptoms' combination and the subsequent referral to the most appropriate health care service can be obtained.

On April 9th, 2020 the web app COVID-Guide was released free of charge and resulted available at www.covidguide.health in German, French, Italian and English.

Expected results

First interim results will be available in the fourth quarter of 2020 after the development of an evaluation scheme and will be updated regularly thereafter. The evaluation of the use of the COVID-Guide will above all allow epidemiological findings on COVID-19 (suspected) cases in the countries where the tool is used. In addition, conclusions can be drawn about user behavior, which allow statements to be made about the individual intention of use or user-friendliness.

The tool will reduce the workload of primary care physicians and healthcare professionals with pre-filtering requests. Fast (self) identification of emergencies and safety netting for less urgent symptoms provided by the app will represent an innovative, safe and effective system for patient's assessment. The output of the app will help efficient communication, as the documentation of the initial assessment can be presented to the treatment center.

Furthermore, the use of anonymous and geo-locatable clinical data along with the generation of alerts and indicators from the COVID Guide could help as a tool for epidemiological monitoring of the current and future development of the pandemic (Telemedical Syndromic Surveillance).

The digital self-assessment of patients will become more important in the future beyond COVID-19-specific symptoms. Italian Primary Care Physicians, for example, could integrate corresponding tools into their information and care offerings, which will serve as an interface for professional initial assessment and subsequent care.

Dr. Giada Giovannini

CEM Curriculum: Translational Medicine
Tutor: Dr. Jessica Mandrioli
CoTutor: Prof. Giuseppe Biagini

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 often represents 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. 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.

To the best of our knowledge, by now, no studies have extensively explored the usefulness of such a multimodal approach to the evaluation of NCSE in humans.

Objectives

This study aims 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 an acute qualitative or quantitative disturb of consciousness suspected for a non- convulsive SE. 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. These patients undergo to CTP/CTA (Cerebral Tomography Angiography) study immediately after the neurological evaluation in emergency room.

Whenever possible, a cEEG (continuous EEG) monitoring either in Intensive Care Unit (ICU) or in Epilepsy Monitoring Unit (EMU) of our ward is applied for the first 24h or even after if needed per clinical practice. The cEEG allows us to better understand the dynamic and evolving patterns in the so-called ictal-interictal continuum (IIC).

Neurosteroids, neuroinflammation biomarkers (IL-8, IL-6, IL-1ß, TNF- α) and neuronal injury biomarkers (NSE and NF) on sera and, whenever collected per clinical practice, on Cerebrospinal Fluid (CSF), 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. The time point of the measurements is within 24 hours from SE beginning and 7 days after its end.

Moreover, clinical information about the etiology, clinical semeiology, and therapeutic management of these patients are collected too.

Expected results

Based on the available literature data and on our preliminary data on 25 already studied patients, we will expect to find hyper-perfusion patterns involving both cortical and subcortical structures encompassing a single vascular territory with a good spatial correlation with the EEG maximal expression of epileptiform activity related to SE.

Following the results of a previous study by our group (Monti et al Epilepsy Behav. 2015) and literature studies on evidences in SE animal models as well as in patients with SE, we will expect to find an increment of the tested neuroinflammation and neuronal injury biomarkers, an elevated CSF levels of t-TAU as well as an increment of markers of BBB breakdown with a positive correlation with SE severity and overall prognosis.

Finally, we already demonstrated that during SE allopregnanolone and progesterone show decreased CSF levels. We plan to increase our knowledge on neurosteroids changes during SE expanding the analysis to different steroids that have been not characterized so far during SE.

These results could create a standardized multimodal evaluation of NCSE capable of improving SE management in clinical practice.

Dr. Domenico Penna

CEM Curriculum: Translational Medicine
Tutor: Prof. Stefano Luminari

HIGH-CYTOKINE-RISK IN MYELOFIBROSIS: A NEW APPROACH TO DETECT HIGH-MOLECULAR RISK MUTATIONS

Background

Myelofibrosis (MF) is the most aggressive bone marrow cancer in the myeloproliferative neoplasm family. One of the three known driver-mutations (JAK2, CALR, and MPL) usually underlies the molecular pathogenesis of the disease. Other cytogenetic alterations and high-molecular-risk mutations (HMR), such as ASXL1, EZH2, SRSF2, IDH-1, IDH-2, and U2AF1 Q157, can be associated and interact with the first genetic lesion, worsening the prognosis and increasing the risk of leukemic transformation. The result of the altered mutational-landscape is the constitutional activation of the JAK-STAT pathway, which produces neoplastic myeloid cells autonomous of growth factors. The consequence is the clonal expansion, the over-expression of pro-inflammatory cytokines (cytokine-storm), and, ultimately, the onset of bone marrow fibrosis, osteosclerosis, and extramedullary hematopoiesis. The cytokine-storm is also involved in the increase of atherosclerotic risk and leukemic progression.

Cytopenias, hepatosplenomegaly, and constitutional symptoms represent the clinical manifestations of this pathological process, which also influences the overall survival (OS). The median OS for patients diagnosed with fibrotic-phase is six years, while patients diagnosed in the pre-fibrotic stage, or those younger than 60, have a median OS of 15 years. Cause of death is leukemic transformation in just 20% of cases, and patients more often die due to disease progression without acute transformation, thrombosis, infection, bleeding, or complications of portal hypertension. Except for allogeneic hematopoietic stem cell transplant (alloSCT), current treatment modalities do not change the natural history of the disease. Unfortunately, alloSCT in patients with PMF is a procedure with a significant risk of treatment-related death and comorbidities related to chronic GVHD and post-transplant immunosuppressive therapy.

Since 2009, groups of experts have produced prognostic models aimed at identifying patients most suitable for the procedure, carefully evaluating costs and benefits. In particular, the three most modern and effective scoring systems integrate clinical data with the recent molecular pathogenesis acquisitions. The first, MIPSS70, counts nine variables, including three genetic factors; the second, MIPSS70+ version 2.0, an improved version of the previous model, adds a three-level cytogenetic risk stratification and the use of anemia levels adapted for gender and severity; and the last, GIPSS, is based only on genetic markers.

Despite the crucial importance of the mutational-landscape, currently, it is difficult to determine the best time to perform the molecular analysis. HMR mutations often represent a late event in MF, and a too early molecular analysis (NGS) could not detect these additional molecular lesions, underestimating the risk of

leukemic transformation. A recent study conducted in collaboration with Mayo Clinic and focused on the relationship between cytokine deregulation and the genetic setting revealed an intriguing association between high-cytokine-risk and the presence of HMR mutations.

Objectives

- First objective of the study is to confirm the association between High-Cytokine-Risk and HMR.
- Second objective of the study is to investigate if this association retain significance in time points different from diagnosis.

Methods

This cohort study includes patients with MF diagnosis (confirmed through bone marrow biopsy) and known driver mutational status followed at the Hematology department of Reggio Emilia Hospital and disposed to provide written consent for the collection and use of the samples in scientific research. Objective of recruitment is 100 patients.

MF diagnosis has to be according to WHO criteria and confirmed by medical records. Medical records will be used to collect laboratory data and for the extraction of clinical variables. The evaluation will be carried out by a clinician specialized in the field of pathologies associated with myeloproliferative JAK2. Variables taken into consideration include disease phase, current laboratory data, ongoing and previous treatments, comorbidities,

For each patient identified, peripheral blood and a plasma sample will be collected and a researcher with experience in molecular and cellular biology will perform cytokine, cytogenetic, and molecular analysis. In particular, the concentration of 6 plasma cytokines will be determined through an analysis performed with Luminex technology. The cytokines object of the study will be IL-1b, IL-6, IL-8, IFN-a, HGF and MIP-1a. Cytogenetic data will be acquired using standard techniques and described according to the nomenclature criteria of the international system for human cytogenetics and classified as favorable, unfavorable, or very high risk (VHR), following the recently published cytogenetic model revision. Prognostic relevant mutations will be determined using Next Generation Sequencing, and High Molecular Risk (HMR) status will include the presence of at least one among ASXL1, SRSF2, U2AF1 Q157, IDH-1, IDH-2, and EZH2 mutations.

Differences between categories in the distribution of continuous variables will be analyzed with the Mann-Whitney test (for the comparison of two groups) or the Kruskal-Wallis test (for the comparison of three or more groups). In contrast, the examination of differences between groups with nominal variables will exploit the Fisher's exact test. Standard statistical methods will allow the determination of the significance of differences between groups in the distribution of continuous or nominal variables. The statistical study will be reviewed and tested by a second experienced clinical researcher.

Expected results

The first objective of the study is to validate the association between cytokine levels and HMR mutations observed in previous researches on an external population. The second expected result is to prove that this association is independent by the time of diagnosis. These two results would confirm the high-cytokine-risk score value as a dynamic and useful tool for the clinician to monitor the OS disease and a cost-effective way to decide when performing molecular analysis.

These acquisitions would have profound implications in terms of improving the accuracy of current models used to select patients eligible for the transplant procedure. Future studies could clarify the relationship between the altered cytokine status, thrombotic complications, and response to treatments. The same approach could also be used in other MPN diseases, such as polycythemia vera, and essential thrombocythemia.

Dr. Kateryna Solodka

CEM Curriculum: Translational Medicine
Tutor: Prof. Marcello Pinti

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

Background

Multiple Sclerosis (MS) is a chronic inflammatory disorder of the central nervous system (CNS) characterized by progressive axonal demyelination and neurodegeneration. So far, no specific biomarkers exist, and the currently available techniques for diagnosis and monitoring of the disease show relatively low sensitivity. Furthermore, many demyelinating diseases present similar symptoms, which is an additional difficulty in MS diagnosis.

Organic Electronics is a fast-rising interdisciplinary field encompassing organic electronic devices that exhibit mixed electronic and ionic conductivity. These devices represent a truly unique communication bridge across the technology gap existing between the living systems and digital electronics. Biosensing is one of the most scientifically and industrially promising application of organic bioelectronics.

Objectives

The aim of the project is the fabrication and demonstration of an ultrasensitive, label-free electronic biosensor for the detection and quantification of biomarkers associated with the onset and prognosis of Multiple Sclerosis in biological fluids. Because neuroaxonal damage is a key factor contributing to the progression of MS, a particular attention is given to neurodegenerative markers, such as neurofilaments and DAMPs (damage-associated molecular patterns). Based on the results obtained, I will try to identify correlations between the levels of these molecules and the progression of the disease, and get a better understanding of the mechanism underlying MS progression.

Methods

The biosensors will be based on an EGOFET (electrolyte-gated organic field-effect transistor) architecture. The transistors will be characterized by electrical measurements, electrochemical approaches and complementary techniques to investigate modified surfaces. The interfaces of the devices will be functionalized with specific recognition elements and the electronic transduction of the biorecognition events will be optimized. The biosensors will be tested in plasma samples from patients with progressive form of MS, and the obtained results will be validated with standard, state-of-the-art diagnostic techniques, such as ELISA and flow cytometry.

Expected results

To design, develop and implement highly reliable organic bioelectronic sensors for detection and quantification of biomarkers of Multiple Sclerosis in biological fluids, that could be a promising strategy to be implemented for point-of-care applications.

Dr. Massimiliano Salati

CEM Curriculum: Translational Medicine
Tutor: Dr. Andrea Casadei Gardini

THE IMPACT OF CT-BASED BODY COMPOSITION PARAMETERS ON SURVIVAL OUTCOMES IN WESTERN PATIENTS (PTS) WITH RESECTED GASTRIC AND GASTROESOPHAGEAL JUNCTION ADENOCARCINOMA (GEA)

Background

Sarcopenia is a multifactorial syndrome defined by progressive and generalized loss of skeletal muscle mass, reduction of strength and physical performance which has been increasingly correlating with impaired cancer patient outcomes. High accuracy and reproducible results make CT one of the gold standard for body composition measurement. We aimed at assessing the impact of CT-based body composition parameters on the survival outcomes of resected GEA.

Objectives

cT2-T4 and/or N-positive GEA pts undergoing curative-intent resection at our Institution between 2008-2018 were eligible. Presurgical clinicopathological, biochemical, and anthropometric data were retrospectively retrieved, while body composition parameters and their changes over time were derived by CT scan using GE Healthcare AW VolumeShare 7 software. Univariate and multivariate analyses for DFS and OS were performed.

Methods

A total of 107 pts were included in the analysis. Median age was 66.1 years (range 20-85), 61 (57%) were males. 45 pts (42.1%) had stage II, while 62 pts (57.9%) had stage III. 98 (91%) pts had a non-cardia GEA. 85 (79%) pts received adjuvant treatment, consisting of fluoropyrimidine-based doublet in 85% of cases. Mean preoperative BMI was 23.9 kg/m2. CT scans were performed presurgically and from 4 to 15 months after resection. In the whole population, the 3-year DFS and OS were 48% and 49%, respectively. Out of 27 tested covariates, baseline IntraMuscular Adipose tissue Content (IMAC), together with ECOG PS and disease stage, was significantly associated with DFS (HR 1.97; p=0.03) and OS (HR 1.77; p=0.04) at the multivariate analysis. Specifically, pts with high baseline IMAC (≥ -0.42) had a shorter 3-year DFS (35% vs 62%, p<0.018) and 5-year OS (32% vs 55%, p=0.037).

Expected results

We showed for the first time that presurgical IMAC, which reflects the quality of skeletal muscle, was an independent predictor of survival in resected GEA. This easy-to-calculate and largely available CT-derived parameter, may identify high-risk patients in need for a prompt and tailored nutritional intervention aimed at improving their outcome.

Dr. Lisa Anceschi

CEM Curriculum: Nanomedicine, Medicinal and Pharmaceutical Sciences
Tutor: Prof. Federica Pellati

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 Cannabinaceae family [1]. The main classes of compounds present in this plant are cannabinoids, terpenes, and flavonoids. Cannabinoids are mainly synthesized in glandular trichomes, which are more abundant in female inflorescences. Among them, the most representative compounds are Δ^9-tetrahydrocannabinolic acid (Δ^9-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 Δ^9-tetrahydrocannabinolol (Δ^9-THC), cannabidiol (CBD), and cannabigerol (CBG) [2]. Fiber-type *C. sativa* (also known as hemp) is characterized by a high content of CBD and CBG, and a level of psychoactive Δ^9-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 such as terpenes [3,4].

Objectives

The aim of the project is to study the possible role and application of non-psychoactive *C. sativa* extracts as antiproliferative agents and to identify the compounds responsible for this activity. To this aim, it was necessary to fully characterize the bioactive compounds present in different *C. sativa* extracts through high-performance liquid chromatography coupled with high-resolution mass spectrometry (HPLC-HRMS) and to evaluate their antiproliferative activity on different leukemia cancer cell lines such as the chronic myelogenous leukemia K562, the acute myelogenous leukemia U937 and the leukemic T-cell lymphoblast Jurkat. We then evaluated the possible synergic activity of conventional chemotherapeutic agents and the extract or pure CBD in order to evaluate the most sensitive cell line to the compounds.

Methods

HPLC-HRMS was used for the qualitative and semi-quantitative analysis of the decarboxylated 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 antiproliferative activity of the extracts was assessed on K562 leukemia cell line and the CBD-type extract was the one that provided the lowest IC_{50} value. Then, 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.

Expected results

The dose-response curves did not show a significant decrease of the IC_{50} value for imatinib and doxorubicin in association with 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, since the other two did not show the same behavior.

Since, the pharmacological activity of vincristine is related to its microtubule-destabilizing properties, we have hypothesized that some bioactive compounds present in the CBD-type extract might act on the same target, thus enhancing the antiproliferative activity of the vincristine. This hypothesis will be further investigated by means of *in silico* techniques as well as immunofluorescence and drug combination studies.

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