University of Modena and Reggio Emilia

PhD COURSE OF CLINICAL AND EXPERIMENTAL MEDICINE

PhD DAY 2015

Abstracts

March 17

9:00 a.m., Lecture Room H1.1

Dept. Biomedical, Metabolic and Neural Sciences

(287 Campi street, Sect. Physiology and Neural Sciences - Modena)

in memory of Professor Paola Loria

PhD Degree of cycle XXVI – 18 February 2014

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 2014 (cycle XXIX) the PhD Course of "Clinical and Experimental Medicine" is 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

cycle XXVII

Dr. Erica ARTONI

CEM Curriculum: Clinical, Genetic and Molecular Medicine
Tutor: Prof. Clodoveo Ferri

CHARACTERIZATION OF MICRO- AND NANO- PARTICLES AND QUANTIFICATION OF TRACE ELEMENTS IN SERUM AND HAIR SAMPLES OF SYSTEMIC SCLEROSIS PATIENTS

Background

Systemic Sclerosis (SSc) is a chronic systemic disease characterized by the presence of autoimmunity, vascular lesions and progressive fibrosis. The etiology of scleroderma has not yet been clarified. It has been shown that the immune system is involved in the pathogenesis of the disease since specific autoantibodies for each variant of SSc were found as well as a massive presence of immune cells in the organs most affected, such as the heart, kidneys, intestines, lungs and skin. Epidemiological evidence suggests a possible role of environmental factors such as crystalline silica and inorganic metal powders as possible etiologic or prognostic factors of SSc.

Objective

Our preclinical study focused on the evaluation of the presence of micro and nano inorganic particles in serum and on the quantification of trace elements in serum and hair samples of SSc patients. The aim was to investigate their possible role in the etiopathogenesis of the disease.

Methods

We enrolled fifty SSc patients with an average age of 56 years and 30 healthy subjects matched for age and sex to patients with SSc. The patients were all residents in the geographic area of the province of Modena for more than 5 years. A questionnaire was developed on the basis of a literature review and piloting to evaluate the subjects exposure to occupational and environmental dust. The questionnaire was submitted to all subjects included in the study. Information has been collected on clinical and demographic, occupational and residential history of the subjects, as well as on smoking habits and the presence of prosthetic implants. Serum and hair samples were chosen as biological matrices for analysis on the presence of micro and nano particles. A total number of fifty serum samples and thirty hair samples from SSc patients and thirty serum and hair samples of healthy subjects were analyzed. The morphological characterization of the particulates was performed by Environmental Scanning Electron Microscopy (ESEM), while the chemical identification of the particles has been implemented by means of X-ray spectroscopy for Energy Dispersive (EDS). The total quantitative evaluation of trace elements present in serum and hair samples was done by Mass Spectrometry Plasma Atomic Emission (ICP-AES) based on the protocols approved by the ISS (Istituto Superiore di Sanità).

Results

We found a significantly increased risk of SSc associated with occupational exposure (odds ratio, OR = 3.167; Confidence Interval, CI 95%= 1.205 to 8.323). Data on environmental exposure (OR = 1.055; CI 95%= 0.4259 to 2.613), on smoking habits (OR = 0.4828; CI 95%= 0.1921 to 1.213) and the presence of implants or prosthesis (OR = 0.5000; CI 95%= 0.1953 to 1.280) were not significant. ESEM analyzes showed the significant presence of inorganic micro and nano particles in serum samples of SSc patients. The particles were found in singlet and in the form of aggregates with a diameter comprised between 30 nanometers and 3.9 micrometers. The elements most frequently found by means of EDS were Si, Fe, Al and Mg while a lower frequency of occurrence was found for Ti, Zn, Cu, Ba, and Cr. The concentration of trace elements in serum and hair samples of subjects analyzed with ICP-AES showed a significant concentration of Si, Cr, Ti ($p < 0.0001$) in SSc patients compared to the controls. Si was the element with the highest significant concentration (324±168 µg/L) in the samples of SSc patients.

Conclusions

The significant presence of Si and other inorganic metals detected as trace elements in all biological samples of SSc patients would seem to suggest a possible role of these elements, particularly silica in crystalline form, as co-factors in the etiopathogenesis of SSc.

cycle XXVIII

Dr. Regina BARTOLOMEO

CEM Curriculum: Cellular and Molecular Pathophysiology
Tutor: Prof. Andrea Cossarizza

INHIBITION OF LON PROTEASE BY TRITERPENOIDS CDDO AND CDDO-Me ALTERS MITOCHONDRIA AND CAUSES CELL DEATH IN RKO HUMAN COLON CANCER CELLS

Background

Lon is a nuclear-encoded, mitochondrial ATP-dependent protease that assists protein folding, degrades oxidized or damaged proteins and participates in maintaining mitochondrial DNA (mtDNA) levels. Lon is up-regulated in several cancer cells, and its down-regulation causes profound alterations of mitochondrial proteome and function, and cell death. Lon enzymatic activity is activated by ATP, and inhibited by the triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) or by its C-28 methyl ester derivative (CDDO-Me).

Objectives

The aim of the work was to characterize the effects of triterpenoids on mitochondria of cancer cells, paying a particular attention to parameters such as membrane potential, mass, morphology, dynamics and ROS content, and Lon proteolytic activity.

Methods

We used RKO colorectal carcinoma cell line as cellular model. Lon was pharmacologically inhibited by the treatment with CDDO, or its methylated derivative CDDO-Me. The expression level of Lon protease and of its two representative substrates, namely human transcription factor A (TFAM) and aconitase (Aco2), were analysed by western blot. Lon was up-regulated by using a retroviral vector, harbouring the cDNA encoding for Lon protease (pMSCV-Lon), to stably transduce RKO cells. Apoptosis, content of mitochondrial hydrogen peroxide and mitochondrial superoxide, mitochondrial membrane potential and mitochondrial mass were measured by flow cytometry. Mitochondrial morphology was analysed by confocal microscopy.

Results

CDDO and CDDO-Me resulted to be potent stressors for mitochondria. In particular, they inhibited cell growth in a dose-dependent manner and induced apoptosis, increased mitochondrial ROS, depolarized mitochondrial membrane, altered mitochondrial morphology and mitochondrial mass, affected the levels of Lon and those of Aco2 and TFAM, two targets of Lon proteolytic activity. Lon overexpression reduced apoptotic cell death induced by CDDO and CDDO-Me.

Conclusions

Lon is a non-oncogenic molecule, which does not initiate tumorigenesis, but is essential for maintaining mitochondrial functionality and morphology; its pharmacological inhibition could represent a possible new anticancer strategy.

Dr. Stefano CECCHINI

CEM Curriculum: Surgery
Tutor: Prof. Luigi Roncoroni

FAST-TRACK SURGERY IN ELDERLY PATIENTS: ENHANCED POSTOPERATIVE RECOVERY

Background

Since age is a demonstrated risk factor for delayed recovery and consequently prolonged hospital stay after surgery, the elderly patients represent a potential target of enhanced recovery program (ERP), even though effectiveness and feasibility of ERP in elderly patients still remain unclear.

Objectives

Evaluation of safety, feasibility and effectiveness of a novel ERP enhanced by a rehabilitation device in patients 70 years of age or older after colorectal surgery, focusing on recovery of functional exercise capacity and length of hospital stay.

Methods

The present study is designed as a single-center, randomized, parallel-group trial comparing a multimodal ERP enhanced by a rehabilitation device vs. traditional care in elderly patients undergoing colorectal surgery. All patients underwent preoperative physiatric evaluation, six minute walking distance, three minute test on the rehabilitation device, Berg balance scale and SF36 Health Survey questionnaire, in preoperative setting and at time of hospital discharge.

Results

Preliminary data on 18 patients showed that ERP enhanced by a rehabilitation device in elderly patients is safe and feasible. Postoperative pain was lower in the ERP group in po day 3 (VAS 3.00 vs. 0.75; p=0.043) and po day 4 (VAS 1.00 vs. 0.00; p=0.033). Recovery of the bowel function was earlier for ERP patients (first flatus 1.8 vs. 2.6 days, p=0.019; defaecation 2.3 vs. 3.9 days, p=0.039). Early mobilization in postoperative day 2 was achieved in the 44% of ERP patients, with a significant reduction of the rehabilitation time (sitting 1.4 vs.2.3 days, p=0.051; standing 1.5 vs.3.3 days, p=0.03; deambulation 2.5 vs.3.8 days, p=0.032). Recovery of physical performance was lower for ERP patients albeit not significant (6MWD -49 vs. -79.6 meters p=0.484). Length of hospital stay was similar although lower for ERP patients (LOS 8.38 vs. 9.88, p=0.452).

Conclusions

Preliminary analysis suggests that ERP is feasible and safe, with a significant reduction in the time to recovery of the bowel function and physical performance even in the elderly patient.

Dr. Chiara DIAZZI

CEM Curriculum: Clinical, Genetic and Molecular Medicine
Tutor: Dr. Vincenzo Rochira

STUDY OF PITUITARY FUNCTION AND REGULATORY FEEDBACK MECHANISMS IN CHRONIC DISEASES (HIV INFECTION AND THALASSEMIA)

Background

Pituitary function, particularly GH/IGF1 axis, seems to be impaired in patients with chronic diseases (HIV-infection and Beta-thalassemia). GH deficiency (GHD) is frequent in patients with HIV, undergoing Highly Active Antiretroviral Therapy (HAART).

Objectives

To investigate the relationship among gender, body composition and GH/IGF-1 axis, by comparing GH deficient HIV-infected patients to hypopituitary subjects.

Methods

This is the second phase of a prospective cross-sectional clinical study. In phase 1, we compared GH/IGF1 status, and body composition in HIV-infected subjects. In this phase we compared 47 HIV-infected patients prospectively enrolled, with 36 hypopituitary subjects retrospectively selected. We evaluated basal serum GH, IGF-1, GH peak and AUC after standard GHRH+Arginine test; BMI, waist and hip ratio (WHR) and body composition by DEXA. Data were analyzed by nonparametric Mann-Whitney test. Finally, we will evaluate all endocrine axis in Beta-thalassemic patients.

Results

HIV-infected patients had higher GH peak, AUC, and IGF-1 ($p<0.0001$). BMI ($p=0.003$), total ($p<0.0001$) and trunk fat mass ($p=0.0003$) were higher in hypopituitary patients; WHR was higher in HIV-infected patients ($p<0.0001$). GH peak was lower in hypopituitary men than women ($p=0.001$). Men showed higher WHR ($p=0.0082$), total ($p=0.0002$) and trunk lean mass ($p=0.0008$), while women had higher total ($p=0.0017$) and trunk fat mass ($p=0.0176$). GH peak, AUC, and IGF-1 were higher ($p<0.0001$) in HIV-infected than hypopituitary men. No difference was found in women.

Conclusions

GHD seems to be worse in hypopituitary patients, suggesting that a primary pituitary disease affects GH/IGF-1 axis more than HIV infection. Moreover, fat distribution seems to play a role in GH/IGF-1 axis in

HIV-infected patients. Furthermore, men seem to have a worse GHD than women, suggesting a possible role of gender. These differences could help distinguishing functional from clinical GHD in HIV-infected subjects, and better targeting treatment strategies.

Dr. Daniela FARIOLI

CEM Curriculum: Cellular and Molecular Pathophysiology
Tutor: Prof. Aldo Tomasi

SEARCH OF NEW POTENTIAL BIOMARKERS FOR PROSTATE CANCER

Background

Prostate cancer (PCa) is the second most frequently diagnosed cancer in men and the fifth most common cancer overall, additional diagnosis and therapeutic strategies are needed. Although the use of Prostate Specific Antigen (PSA) is widely spread, and during the last 2 decades improved the diagnosis of PCA, serum PSA has several drawbacks: (i) its specificity is limited and 60–70% of PSA elevations are caused by nonmalignant disease; (ii) a number of high-grade tumors are overlooked as patients' PSA levels are in the normal range; (iii) many clinically insignificant PCA are overdiagnosed. New molecular biomarkers are needed to improve prostate cancer (PCa) detection and classification for the development of individual prognosis and progression risk. Circulating tumor DNA (ctDNA) is a promising biomarker for noninvasive assessment of cancer burden. It was demonstrated that concentrations of ctDNA exhibited good correlation with the disease status of gastric, ovarian, and colorectal cancer. CpG island hypermethylation causes gene silencing and could be decisive in prostate carcinogenesis and progression. We wiil investigate its role at multiple gene sites during prostate carcinogenesis and progression.

Objectives

The aim of the present study is to identify tumor-specific biomarkers in ctDNA in plasma of 35 patients with histologically confirmed prostate cancer and 15 healthy donors, and to investigate their clinical relevance as a diagnostic and prognostic tool. We will therefore quantify ctDNA , and evaluate CpG island hypermethylation in 5 tumor associated genes: GSTP1, Reprimo, RASSF1, RARB2, PTGS2.

Methods

We will collect 15 mL of blood from each patient in a serum separator tube and process it immediately. Serum will be separated in aliquots and cryopreserved at -80 °C. DNA will be extracted from serum and ctDNA will be quantified by qPCR. ctDNA will be subjected later to sodium bisulfite modification and we will assess the methylation status of GSTP1, Reprimo, RASSF1, RARB2, PTGS2 using methylation-specific PCR.

Results

We expect that the level of ctDNA will be correlated with the presence or absence of disease and the stage of the disease, and genes hypermethylation will correlated significantly with the pathologic stage and/or Gleason score.

Conclusions

We expect that our results will confirm that the ctDNA quantification and the hypermethylation patterns are helpful in the diagnosis and prognosis of PCa. We expect that increase in CpG island hypermethylation at multiple gene sites occur during progression and indicate early biochemical recurrence after radical prostatectomy.

Dr. Carmela GIORDANO

CEM Curriculum: Clinical, Genetic and Molecular Medicine
Tutor: Prof. Giuseppe Biagini

A MODIFIED 6-Hz CORNEAL STIMULATION PROTOCOL PROVIDES EVIDENCE FOR THE SPECIFIC INVOLVEMENT OF THE HIPPOCAMPAL FORMATION IN NON-CONVULSIVE SEIZURES

Background

Seizures can be distinguished into two major categories: convulsive and non-convulsive. In temporal lobe epilepsy, both types of seizures are observed, but the contribution of the limbic regions to these different seizure types is still undetermined.

Objectives

We aimed at evaluating a modified version of the 6-Hz test in order to characterize behavioral changes in seizure phenotype and alterations in neuronal networks activation in response to repeated corneal stimulation.

Methods

Mice received a corneal stimulation for 3s with a 6-Hz pulse frequency and current intensity of 32mA, (interstimulation interval: 48h; 4 sessions). Seizures were video recorded and scored. FosB/ΔFosB was used as a marker of neuronal activation. Cells expressing FosB/ΔFosB immunopositivity were identified using dual-labeled immunofluorescence with calcium/calmodulin-dependent protein kinase II antibody.

Results

Duration of seizures progressively decreased whereas the seizure severity was significantly increased after the second stimulation. FosB/ΔFosB-immunopositivity was analyzed in the hippocampus, parahippocampal cortices, lateral amygdala and striatum. We observed an abrupt decrease in FosB/ΔFosB immunostaining in the hippocampus and parahippocampal cortices that significantly correlated with the decreases in non-convulsive seizures. In addition, a significant increase in FosB/ΔFosB immunoreactivity was observed in the lateral amygdala. Double immunofluorescence experiments identified FosB/ΔFosB immunopositivity mainly in principal neurons.

Conclusions

These findings suggest a major involvement of hippocampal structures in the genesis of non-convulsive seizures, and that these seizures can be modulated by a protocol of repetitive corneal stimulation.

Dr. Enrico GIULIANI

CEM Curriculum: Surgery
Tutor: Prof. Alberto Barbieri

MARKERS OF NEURAL DAMAGE DURING CAROTID SURGERY – OPEN VS ENDOVASCULAR APPROACH

Background

Stroke is one of the leading causes of death and permanent disability in high-income countries. Significant carotid artery stenosis may be a predisposing factor in stroke, so the surgical treatment of this condition can reduce the risk of recurrent stroke in patients with severe carotid stenosis.

Carotid endarterectomy (CEA) is the gold standard for treating severe carotid artery stenosis, whereas carotid artery stenting (CAS) represent its endovascular alternative. Open surgery has demonstrated, on one hand, a lower periprocedural risk of death and stroke, with, on the other hand, a higher risk of acute myocardial infarction (AMI) and cranial nerve injury, making PTA and CAS second line treatment options for carotid artery stenosis.

The diagnosis of periprocedural stroke relies on clinical parameters and neuroimaging techniques, as with all other forms of acute stroke; similarly to what has been done for early triage and evaluation of cardiac symptoms compatible with AMI, various panels of biomarkers of neural damage have been developed and validated.

Objective

Assessment of the potential neural damage following open or endovascular carotid surgery measured by peripheral blood concentration of three biomarkers: S100β, MMP-9, D-dimer.

Methods

Data for this prospective investigation came from the Carotid Markers study (CAR-MA), which sought to measure the levels of specific biomarkers of neuronal damage and thrombosis on candidates to CEA or CAS presenting at the Department of Vascular Surgery of the Nuovo Ospedale Civile Sant'Agostino-Estense of Modena (Italy) at baseline and at 24 hours after surgery. Relevant medical comorbidities were noted.

Results

A total of 113 consecutive patients were enrolled in the study, 41 in the endarterectomy group and 72 in the endovascular group.

The baseline levels of the studied biomarkers did not show any statistically significant difference between groups with the exception of MMP9, that showed higher concentrations in the endovascular group (median 731 vs. 401, p-value 0.0007), while 24 hours after surgery the endarterectomy group featured significantly higher peripheral blood concentrations of MMP-9, S100β and D-dimer. Conversely, no significant difference was detected in the endovascular group except the D-dimer level.

Conclusions

Neural damage biomarkers demonstrated a substantial difference between open and endovascular carotid surgery, that, if performed in selected patients, may become a less invasive alternative to CEA.

Dr. Shaniko KALECI

CEM Curriculum: Public Health
Tutor: Dr. Roberto D'Amico

METHODOLOGY FOR THE MANAGEMENT AND ANALYSIS OF STUDIES ASSESSING EFFICACY OF HEALTH CARE INTERVENTIONS: THE IRMA TRIAL, AN INTERNATIONAL RANDOMIZED CONTROLLED TRIAL EVALUATING THE EFFICACY OF RADIOTHERAPY IN WOMEN WITH BREAST CANCER

PLEASE ASK TO THE PhD STUDENT OR TO THE SUPERVISOR FOR THE FULL ABSTRACT

Dr. Eliana Valentina LIARDO

CEM Curriculum: Oncology
Tutor: Prof. Massimo Federico

PREDICTIVE VALUE OF BASAL PET/CT SCAN IN DETECTING BONE MARROW INVOLVEMENT IN NON HODGKIN DIFFUSE LARGE B CELL LYMPHOMA. RESULTS FROM AN UNICENTRIC RETROSPECTIVE ANALYSIS

Background

F-18fluoro-deoxy-glucose (FDG) positron emission tomography/computed tomography (PET/CT) has become very useful in management of lymphomas. Hematologists use to perform PET/CTscan in staging, response assessment and follow-up.

In the last years many data emerged on the predictive value of PET in detecting bone marrow (BM) involvement in Diffuse large B-cell lymphoma (DLBCL) that is the most common subtype of aggressive non Hodgkin Lymphomas (nHL) and 30–35% of all nHL[1]. It's avid for FDG and BM involvement occurs in around 17% of cases (3.6% in limited stages)[2]. Up to 2014, guidelines recommended BM biopsy (BMB) in all DLBCL[3]. According to my retrospective analysis presented at past PhD day, new indications emerged from last Lugano meeting on Lymphomas. They suggest that BMB could be safely omitted in PET CT early stage DLBDL[4].

Objectives

To evaluate PET/CT value in detecting bone marrow involvement in DLBCL.

Methods

I reviewed 306 cases of nHL seen in IRCCS/IRST of Meldola/Cesena between 2004 and 2013. I found 198 cases of histologically confirmed DLBCL. 108 had available data about both BMB and PET/CTscan at the onset.

Results

Only 7 patients had positive BM-PET/CTscan. BM involvement was confirmed by BMB in 5 cases; in 2, BM was evaluated as negative but the sample was unsuitable in both cases.

The other 101 patients had a negative BM at basal PET/CTscan. Ninetysix of them had negative BMB.

The 5 patients with negative PET/CTscan and positive BMB had stage III disease. One had anemia and enlarged spleen; one bulky disease and extranodal (pharynx) involvement, one had B-symptoms. None had massive BM involvement (3 minimal, 2 < 20%). All patients (47/47) with stage I/II at PET/CTscan had a negative BM-histology.

In order to homogenize the sample, I selected patients diagnosed between November 2012 and February 2015 who underwent a PET/CT scan with the same PET machinery. We reviewed images of 45 PET/CT scan. Thirty three patients had limited PET/CT stage and negative BMB. Twelve patients had diagnosed a bone marrow involvement at BMB. All of them had at least stage III or extranodal involvement at PET/CT staging; 4/12 had a negative PET/CTscan on bone marrow. In 6 cases they had also a diffuse PET positivity on BM. In two cases there was focal positivity on iliac crest and BM involvement was later demonstrated by "guided" biopsy on PET findings. Similar data emerged from a Korean retrospective review[6].

Conclusions

These data suggest that FDG PET-CT could not replace bone marrow biopsy due to the low sensitivity of FDG PET-CT for detection of bone marrow infiltration in advanced stage DLBCL patients. During next year we're planning to design a perspective study to investigate the positive predictive value of PET/CT in detecting bone marrow involvement in all newly diagnosed subtypes of T and B nonHodgkin aggressive Lymphomas.

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Dr. Federica Francesca MORELLI

CEM Curriculum: Clinical, Genetic and Molecular Medicine
Tutor: Dr. Serena Carra

CHARACTERIZATION OF THE R7S MUTATION OF HEAT SHOCK PROTEIN HSPB3 FOUND IN PATIENTS SUFFERING OF MYOPATHY: UNDERSTANDING THE MECHANISMS LEADING TO DISEASE

Background

HSPB3 is a poorly characterized member of the Heat Shock Protein family that forms a stable complex with HSPB2. The HSPB2/HSPB3 complex is induced during muscle differentiation and might play a role in muscle maintenance. Recently the R7S mutation in HSPB3 has been associated with distal hereditary motor neuropathy type 2C. However so far no data are available concerning the functions of HSPB3 in the neuromuscular system and how mechanistically the R7S mutant causes disease.

Objectives

The present study aims to: a) characterize the subcellular localization of HSPB2 and HSPB3; b) determine the effects of the R7S HSPB3 mutant on protein stability, subcellular localization and complex formation.

Methods

Hela cells were transfected for 48 h with vectors encoding for HSPB2, myc-HSPB3 and myc-R7S alone or in combination. Cells were then fixed and processed for immunofluorescence analysis. Subcellular (cytoplasmic and nuclear) distribution of HSPB2 and HSPB3 proteins and expression levels were analyzed by SDS-PAGE and western blot. RNA transcription has been measured using 5-Ethynyl-Uridine.

Results

Concerning subcellular distribution, HSPB2 forms nuclear aggregates and/or nuclear masses, which alter the distribution of the DNA (visualized using DAPI). HSPB3 is also enriched in the nuclei, where it forms intranuclear (IN) and perinuclear (PN) aggregates. Aggregation propensity is increased by the R7S mutant, while is decreased when HSPB3 and HSPB2 are expressed in complex. Interestingly, we found that the IN and PN aggregates influence the nuclear envelope and the distribution of lamin A/C. Intriguingly, mutations on lamin A/C are associated with neuromuscular disease. Lamin A/C regulates not only the nuclear architecture but they also modulate the chromatin organization and gene expression. Moreover, lamin A/C is recruited in interphase cells within nuclear speckles, dynamic foci that contain splicing factors such as SC35. We thus investigated whether HSPB2 and HSPB3 may affect lamin A/C recruitment at speckles. Unlike HSPB1 and HSPB5, HSPB2-HSPB3 do not colocalize with speckles. Furthermore The recruitment of lamin

A/C into speckles is not impaired by HSPB2 and/or HSPB3; however, we observed that in cells expressing HSPB2 and/or HSPB3 speckles changed shape/number, becoming round (mimicking transcription inhibition). Consistently, cells expressing HSPB2-HSPB3 has reduced transcription activity.

Conclusions

Our results show that HSPB2-HSPB3 affect nuclear structure, which may in turn deregulate RNA transcription. Since remodeling of nuclear lamina is required for muscle differentiation, HSPB2-B3 might modulate muscle differentiation/maintenance by regulating lamin functions and NE stability. Alteration of such functions due to HSPB3 mutation may contribute to disease progression.

Dr. Alessia PICCININI

CEM Curriculum: Surgery
Tutor: Prof. Teore Ferri

NUTRITIONAL ASSESSMENT IN PATIENTS WITH HEAD AND NECK CANCER, AND CORRELATION BETWEEN MALNUTRITION AND POSTOPERATIVE COMPLICATIONS

Background

It is well recognized that malnutrition is a comorbidity condition in patients with cancer. Patients with head and neck cancer have multiple risk factors for nutritional depletion. These include disease effects such as cachexia associated with malignancy, dysphagia and odynophagia, the iatrogenic effect of treatment and detrimental social behaviors, such as heavy smoking, poor diet and alcohol abuse , which are often associated with this patient group.

Objectives

The aim of the study is to evaluate the correlation between an adequate enteral feeding during postoperative period and the incidence of postoperative complication

Methods

Prospective multicentric cohort study . Inclusion criteria for this study comprised all patients with a diagnosis of squamous cell carcinoma of the head and neck, who received surgical treatment and postoperative enteral feeding. The Departments involved are: Otolaryngology and Maxillo Facial Surgery of the University Hospital of Modena, Otolaryngology and Maxillofacial Surgery of the University Hospital of Parma, Otolaryngology IRCCS "Casa Sollievo della Sofferenza",St. Giovanni Rotondo; Traumatology and Maxillofacial Surgery University Hospital Careggi, Florence. Ethics Committee approval was obtained.

Results

We present a preliminary analysis on 69 patients operated in the ENT Department of Parma. We present and summarize the results of univariate analysis of the impact of potential predisposing factors on the primary endopoint (fistula formation). From the analysis, p-values ≤0.05 was present in diabetics patients (p=0.004), and in patients with preoperative albumin and prealbumin serum levels respectively < 3.5 g/dL and < 20 mg/dl (p= 0.005).

Conclusions

Patients with head and neck cancer have an increased risk of malnutrition and may require enteral feeding via nasogastric or gastrostomy tube. In addition to the classical risk factors for surgical

complications we must also consider the poor nutritional status as a risk factor, as assessed by preoperative albumin. Preoperative and periodic postoperative nutritional evaluations are mandatory in patients with head and neck cancer.

Dr. Laura RICCETTI

CEM Curriculum: Clinical, Genetic and Molecular Medicine
Tutor: Prof. Manuela Simoni

THE LUTEINIZING HORMONE AND CHORIONIC GONADOTROPIN ACTION ON THE SAME RECEPTOR RESULTS IN DIFFERENT INTRACELLULAR SIGNALLING IN MOUSE LEYDIG CELLS IN VITRO

Background

Human chorionic gonadotropin (hCG) and luteinizing hormone (hLH) are two glycoproteins which share a common alpha and a unique beta subunit joined non covalently. Since they bind the same receptor (LHCGR) located at the cell membrane as dimer/oligomer, it is traditionally assumed that hLH and hCG are biologically equivalent. However, there are some hints that LHCGR mediates different responses depending on ligand; stimulation by hLH and hCG results in different signaling, in terms of signal transduction, gene expression and steroid synthesis, in primary granulosa cells.

Objectives

The aim of this study is to compare the action of hLH and hCG, in terms of cAMP/PKA- and ERK1/2-pathways activation in primary mouse Leydig cells, naturally expressing the murine lhcgr.

Methods

Testis from four 3-5 months C57BL6 mices were collected, mechanically and enzimatically digested, then isolated from the interstitial cell suspension by stratification in Percoll gradient. Dose-response experiments were performed by stimulating the cells with increasing hLH or hCG molar doses (1 pM-100 nM range), then total cAMP production was evaluated after three hours by ELISA, and ERK1/2 phosphorylation after fifteen minutes by Western blotting. To evaluate cAMP accumulation, the experiments were performed in the presence of 500 µM of the phosphodiesterases inhibitor 3-isobutyl-1-methylxanthine (IBMX).

Results

Upon hCG stimulation, the total cAMP production is about 10-fold more potent than hLH (hCG EC_{50}: 18.64±10.14 pM; hLH EC_{50}: 192.00±53.96 pM; Mann-Whitney's U-test, $p<0.05$, $n=4$) in mouse Leydig cells. Conversely, hLH appears more effective than hCG in inducing ERK1/2 phosphorylation (n=1), although more repetitions are needed to confirm the result.

Conclusions

This study demonstrates that lhcgr mediates a differential cAMP accumulation and phospho-ERK1/2 activation depending on hLH and hCG stimulation *in vitro*, in primary murine Leydig cells, bearing previous observation in human granulosa cells. The evaluation of the kinetics of cAMP and testosterone production, and gene expression will be performed in the presence or absence of specific inhibitors, to further dissect the different action of hLH and hCG at molecular level.

Dr. Marta RIGONI

CEM Curriculum: Public Health
Tutor: Prof. Marco Vinceti

BASICS AND METHODS FOR CLINICAL OUTCOME EVALUATIONS

Background

The evaluation of the performance of health care is essential for healthcare policy making. The national outcomes evaluation program (PNE) offers a systematic assessment of clinical interventions and treatments. The PNE poses several problems about transfer and handling of the datasets, and is not able to give a comprehensive assessment of the clinical pathway, beyond hospital care and mortality. Conversely, care processes, also when extended to the non-hospital services and organizational models can more easily evaluated at local level.

Aims

Starting from the validation of the PNE results, the aim is to implement a provincial program for clinical outcome evaluations (PPE). As a case study, we are structuring an evaluation framework for the orthogeriatric pathway (OCP) in elderly patients after hip fracture.

Results

The early results indicated that the introduction of a new care model (OCP) brings better outcomes in terms of survival, percentage of operated within 48 hours from the admission, waiting time for surgery, and average length of stay. However, these outcomes are raw proxy of the effectiveness of the treatment. A more accurate outcomes evaluation of the entire care process needs to include other relevant outcomes such as post-discharge care (at home, rehabilitation, short-term or permanent stay in a nursing home), functional assessment (ADL - Activities of Daily Living through Barthel Index), services use (emergency department accesses, readmissions) and costs.

Conclusions

In conclusion, we are building up a provincial model for clinical outcome and care process evaluations. Methods and instruments are being settled on a specific case study of major clinical relevance.

Dr. Chiara STENTARELLI

CEM Curriculum: Cellular and Molecular Pathophysiology
Tutor: Dr. Giovanni Guaraldi

MAGNETIC RESONANCE SPECTROSCOPY, DUAL-PHASE AND MULTI-ECHO GRADIENT-ECHO MRI IN THE QUANTITATIVE ASSESSMENT OF LIVER STEATOSIS IN HIV INFECTED PATIENTS

PLEASE ASK TO THE PhD STUDENT OR TO THE SUPERVISOR FOR THE FULL ABSTRACT

Dr. Alberto VAONA

CEM Curriculum: *Health Sciences*
Tutor: Dr. Roberto D'Amico

COMPARATIVE EFFICACY OF INTERFERON β VERSUS GLATIRAMER ACETATE FOR RELAPSING-REMITTING MULTIPLE SCLEROSIS

Background

Interferons (IFNs)-beta and glatiramer acetate (GA) were the first two disease-modifying therapies (DMTs) approved 15 years ago for the treatment of multiple sclerosis (MS). DMTs prescription rates as first or switching therapies and their costs have increased substantially over the past decade. As more DMTs become available, the choice of a specific DMT should reflect the risk/benefit profile, as well as the impact on quality profile. As MS cohorts enrolled in different studies can vary significantly, head-to-head trials are considered the best approach for gaining objective reliable data when two different drugs are compared. The purpose of this study is to summarize available evidence on the comparative effectiveness of IFNs-beta and GA on disease course through a systematic review of head-to-head trials.

Objectives

To assess whether IFNs-beta and GA differ in terms of safety and efficacy in the treatment of patients with relapsing-remitting MS (RRMS).

Methods

Randomized controlled trials (RCTs) comparing directly IFNs-beta versus GA in study participants affected by RRMS. We used standard methodological procedures as expected by The Cochrane Collaboration.
We searched the Trials Specialized Register of the Cochrane Multiple Sclerosis and Rare Diseases of the Central Nervous System Group (29 October 2013) and the reference lists of retrieved articles. We contacted authors and pharmaceutical companies.

Results

Five trials contributed to this review. A total of 2858 participants were randomly assigned to IFNs (1679) and GA (1179). The treatment duration was three years for one study and two years for the other four RCTs. The IFNs analyzed in comparison with GA were IFN-beta 1b 250 mcg (two trials, 933 participants), IFN-beta 1a 44 mcg (two trials, 441 participants) and IFNbeta 1a 30 mcg (two trials, 305 participants). Enrolled participants were affected by active RRMS. All studies were at high risk for attrition bias.

Both therapies showed similar clinical efficacy at 24 months, given the primary outcome variables (number of participants with relapse (risk ratio (RR) 1.04, 95% confidence interval (CI) 0.87 to 1.24) or progression (RR 1.11, 95% CI 0.91 to 1.35).

However at 36 months, evidence from a single study suggests that relapse rates were higher in the group given IFNs than in the GA group (RR 1.40, 95% CI 1.13 to 1.7, P value 0.002).

Secondary magnetic resonance imaging (MRI) outcomes analysis showed that effects on new or enlarging T2- or gadolinium(Gd)-enhancing lesions at 24 months were similar (mean difference (MD) -0.01, 95% CI -0.28 to 0.26, and MD -0.14, 95% CI -0.30 to 0.02, respectively). However, the reduction in T2- and T1-weighted lesion volume was significantly greater in the groups given IFNs than in the GA groups (MD -0.58, 95% CI -0.99 to -0.18, P value 0.004, and MD -0.20, 95% CI -0.33 to -0.07, P value 0.003, respectively).

The number of participants who dropped out of the study because of adverse events was similar in the two groups (RR 0.95, 95% CI 0.64 to 1.40).

The quality of evidence for primary outcomes was judged as moderate for clinical end points, but for safety and some MRI outcomes (number of active T2 lesions), quality was judged as low.

Conclusions

The effects of IFNs-beta and GA in the treatment of patients with RRMS, including clinical (e.g. patients with relapse, risk to progression) and MRI (Gd-enhancing lesions) activity measures, seem to be similar or to show only small differences. When MRI lesion load accrual is considered, the effect of the two treatments differs, in that IFNs-beta were found to limit the increase in lesion burden as compared with GA. Evidence was insufficient for a comparison of the effects of the two treatments on patient-reported outcomes, such as quality of life measures.

Dr. Alexander Michael WITKOWSKI

CEM Curriculum: Clinical, Genetic and Molecular Medicine
Tutor: Prof. Giovanni Pellacani

INTEGRATED IMAGING, MOLECULAR, AND GENETIC TECHNOLOGIES FOR THE CHARACTERIZATION OF MELANOMA SUBTYPES: ESTIMATION OF BIOLOGICAL AGGRESSIVENESS, IDENTIFICATION OF PREDICTIVE FACTORS, AND DIAGNOSTIC IMPROVEMENT WITH REFLECTANCE CONFOCAL MICROSCOPY

Background

Reflectance confocal microscopy (RCM) can improve diagnostic accuracy of dermoscopically equivocal cutaneous lesions by providing cellular information about skin lesions via near-histologic resolution images. Reliability of RCM image evaluation has been tested significantly showing the capability of an accurate diagnosis. To this day the use of RCM to estimate biological aggressiveness and further improve triage of melanoma patients has not been tested

Objectives

To determine the improvement of diagnostic accuracy and management using combined dermoscopy-RCM to evaluate equivocal cutaneous lesions in differential with melanoma; to identify correlations between RCM, molecular biology, and genetic analysis.

Methods

This is a retrospective study of 300 lesions. All lesions were evaluated and imaged clinically, dermoscopically, and with RCM, followed by biopsy for histopathological analysis. Phase 1 of the study is to categorize melanoma subtypes with the first 100 cutaneous lesions. Phase 2 is an additional evaluation of 200 cases to validate Phase 1.

Results

Final results expected in July 2015.

Conclusions

RCM allows for the further subclassification of melanoma subtypes at the bedside allowing for improved early diagnosis and triage of patients who can benefit from targeted therapies.

cycle XXIX

Dr. Elena BIANCHINI

CEM Curriculum: Translational Medicine
Tutor: Prof. Marcello Pinti

INVARIANT NATURAL KILLER T CELLS AND CD8+CD161^{++} T CELLS IN DIFFERENT FORMS AND TREATMENTS OF MULTIPLE SCLEROSIS

Background

Multiple Sclerosis (MS) is a T cell-mediated autoimmune disease affecting the central nervous system, characterized by inflammation, demyelination and neurodegeneration. Associations between MS and defects in different subsets of T cells, such as invariant Natural Killer T (iNKT) and CD8+CD161^{++} T cells, have been reported. However, only a few studies investigated iNKT and CD8+CD161^{++} T cells in MS, and conclusive data are still lacking.

Objectives

Our aim was to understand if iNKT and CD8+CD161^{++} T cells could have a pathogenetic role in MS, by analyzing their frequency in different forms of MS and during different treatments of the disease.

Methods

We studied a total of 111 patients followed by the MS Center (NOCSAE, Modena, Italy). Seventy-nine patients suffered from Relapsing Remitting (RR) MS: 16 were assuming interferon 1-β (IFN), 15 natalizumab (Nat), 14 glatiramer acetate, and 34 had no treatment. Fifteen out of 34 non treated RR patients were classified as Benign RRMS. We also studied 32 patients with Progressive (PR) MS: 16 with Primary Progressive and 16 with Secondary Progressive form. Thirty-two age- and sex-matched subjects were used as healthy controls (CTR). CD3+ T cells were volumetrically counted using a CyFlow Counter (Partec, Germany). Peripheral blood mononuclear cells were isolated from fresh blood and stained with the following monoclonal antibodies: anti-Vα24Jα18 T cell receptor, -CD4, -CD8, -CD161, -CD3, -CD19 and -CD14. Up to 20 million cells per sample were acquired for the single cell analysis on an acoustic focusing flow cytometer (Attune NxT, Thermo Fisher, USA).

Results

iNKT cells did not vary in Benign RR patients and in PR patients compared to CTR, but PR patients displayed a higher percentage of CD4+ iNKT cells and a lower percentage of CD4+CD161+ iNKT cells in comparison to CTR. PR patients displayed less CD8+ and CD8+CD161^{++} T cells compared to CTR. RR patients assuming Nat

had increased levels of iNKT and CD8+CD161+ iNKT cells in comparison to those treated with other drugs or without therapy, and higher CD3+, CD8+ and CD8+CD161^{++} T cell counts compared to those receiving IFN.

Conclusions

The dominant role assigned to CD4+ T helper 1 cells in MS is an historic paradigm, but the importance of other immune compartments in the disease development is emerging, including iNKT cells and non-cytotoxic, pro-inflammatory CD8+CD161^{++} T cells. Our data suggest that iNKT and CD8+CD161^{++} T cells might have a pathogenetic role in MS, but further studies are needed. *In vitro* functional studies are ongoing to better understand the activity of such cells and their role in MS pathogenesis.

Dr. Chiara BORSARI

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FLAVONOLS AND FLAVONOL-LIKE COMPOUNDS AS ANTIPARASITIC CANDIDATES

Background

The group of infections known as neglected tropical diseases (NTDs) collectively affects one billion people worldwide and represents an important burden in terms of human suffering. Parasites of the family of Trypanosomatidae are agents of serious human diseases, including African sleeping sickness, Chagas disease and Leishmaniasis. Drugs currently in use against *Leishmania* and *Trypanosoma* infections have limitations in terms of efficacy, safety, duration of treatment, toxicity and resistance. For these reasons, there is an urgent requirement for new effective drugs.

The folate pathway is a successful target for the treatment of bacterial infections and some parasitic diseases, such as malaria. In theory, folate-dependent enzymes targeting drugs should provide useful candidates to face trypanosomatidic infections. However, the classical inhibitors of dihydrofolate reductase (DHFR) are ineffective against *Leishmania* and *Trypanosoma* because of Pteridin Reductase 1 (PTR1). PTR1 is an enzyme responsible for the salvage of pterins in parasitic trypanosomatids and it overlaps the activity of DHFR providing a metabolic bypass to alleviate DHFR inhibition[1],[2]. Therefore, PTR1 is considered a promising target for the development of improved therapies. PTR1 is a validated drug target in *Trypanosoma brucei*. However, the correlation between *Tb*PTR1 inhibition and growth inhibition of the parasite has not been demonstrated yet [3].

Aiming to develop new PTR1 inhibitors and antiparasitic candidates, computational studies were performed to screen a library of 90 natural compounds from plants of different origins. Flavonoids turned out to be an interesting class to be explored as PTR1 inhibitors. Even though flavonoids have been widely explored and often pleiotropic properties have been observed, they represent an interesting starting point for our work.

Objectives

The general aim of the project is to identify novel compounds able to inhibit PTR1 and with suitable properties in order to be further developed for antiparasitic and animal testing.

The specific aim of the first year of my PhD project was to identify flavonoid derivatives that are able to inhibit PTR1 and provide compounds with the suitable properties to be further developed for antiparasitic testing and eventually to start the early phase of animal testing, e.g. pharmacokinetic studies.

Methods

Compounds synthesis

In general, flavonols were synthesized by Claisen Schmidt condensation followed by oxidative cyclization (Algar–Flynn–Oyamada method). Cleavage of methoxy protecting groups using boron tribromide gave the hydroxylated compounds. Different synthetic procedures were followed for the synthesis of flavanones. The majority of the compounds were purified by recrystallization from ethanol or column chromatography. The chromatography was carried out with ISOLERA (Biotage), an advanced and innovative automatic flash purification system. The compounds were fully characterized through Nuclear Magnetic Resonance (NMR) spectroscopy, mass spectral techniques (Ion Trap LC-MS) and elemental analysis.

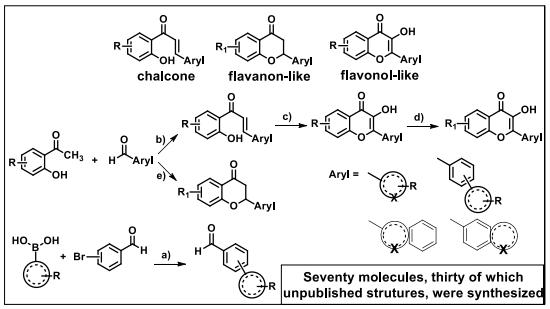
NMR spectra (1D-NMR: ^{1}H and ^{13}C-NMR; 2D-NMR: COSY, HSQC, and HMQC) were recorded on a *Bruker Advance 400 MHz WB* spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. Deuterated chloroform ($CDCl_3$), methanol (CD_3OD) and dimethyl sulfoxide (DMSO) were used as solvents.

Biological evaluation

1. Enzymatic inhibition assays towards the target (PTR1) and towards cytochromes, hERG and Aurora B kinase were performed at Fraunhofer-IME, Hamburg (Germany).
2. Antiparasitic assays towards *Trypanosoma brucei* and *Leishmania major* were performed at IBMC - Instituto de Biologia Molecular e Celular, Porto (Portugal).
3. Pharmacokinetic properties (e.g. solubility in water, PBS and PBS-10%DMSO and PBS-50%DMSO) were evaluated at the University of Modena and Reggio Emilia (Italy), as part of my PhD project.

Results

Aiming to explore the classical flavonoid scaffold and to understand the structure-activity relationship, I have synthesized 37 molecules maintaining the classical flavonoid scaffold. In details, 13 chalcones (flavonoids open-chain), 18 flavonols and 6 flavanones were synthesized. Only flavonols and flavanones bearing hydroxyl groups turned out to inhibit PTR1, with the former being more active than the latter. Aiming to achieve chemical diversity, we shifted from the synthesis of classical flavonoids to the synthesis of flavonoid-like compounds. We have synthesized 38 molecules: 21 flavonol-like compounds, 3 flavanon-like compounds and 14 chalcones (flavonols intermediates). The phenyl ring of the classical flavonol scaffold was replaced with heterocyclic rings. The introduction of heteroaromatic rings led to an increase of the compounds solubility and heteroatoms, such as N and O, could form interactions with the amino acids of PTR1 active site. Moreover, compounds with a biphenyl ring were synthesized aiming to increase the inhibitory activity



towards PTR1 and the potency towards the parasites. Among the 75 synthesized molecules, 32 are unpublished compounds.

Among the 75 synthesized compounds, fifty-two were investigated for their activity towards *Trypanosoma brucei* and *Leishmania major* PTR1. Only hydroxylated flavanones and flavonols showed significant enzyme inhibitory activity (> 50% at 50 µM). The most potent compound presented a *Tb*PTR1 IC_{50} equal to 4.3 µM. Eleven compounds were assessed for their antiparasitic activity and two of them turned out to be potent antitrypanosoma candidates (IC_{50} towards *Tb* = 4.2 ± 0.2 µM and 7.6 ± 0.5 µM, respectively). Moreover, the most active compound (IC_{50} towards Tb = 4.2 ± 0.2 µM) showed a good selectivity (SI = 12). None of the 11 compounds tested showed antileishmanial activity. Perhaps this is due to a permeability problem, since *Leishmania* is not a bloodstream form like *T. brucei*. As a matter of fact, *Leishmania* parasites live within macrophages.

The fifty-two compounds were assessed for their inhibitory activity towards hERG and cytochromes (CYP1A2, CYP2C9,CYP2C19, CYP2D6 and CYP3A4). According to the data, few compounds decrease hERG activity by more than 10%. Most of the compounds turned out to inhibit only few isoforms of cytochrome (i.e. CYP1A2). Only two compounds inhibit Aurora B kinase activity at a concentration of 10 µM.

For all these compounds the percentage of A549/W1-38 cell growth was evaluated and no molecule showed cytotoxicity. Moreover, the compounds were assessed for mitochondria toxicity and none of the molecules turned out to be mitotoxic.

Two compounds have been selected for starting the animal study track against *Trypanosoma brucei* infection. For these compounds the solubility properties in different solvents and at the different concentrations needed for intravenous (IV) and oral administration (OS) have been evaluated.

Conclusions and perspectives

All the 75 synthesized compounds will be tested both towards the enzymes and the parasites. Among the eleven compounds already tested, some flavonoids proved to have a good *in vitro* antiparasitic activity with no cytotoxicity. We have selected the most promising antitrypanosoma compounds for pharmacokinetic studies in order to assess their stability in blood and to understand whether the quantity of compound that remains unchanged in blood is enough to kill the parasite. According to literature, natural flavonoids show antiparasitic activity *in vitro*, but there is an absence of notable *in vivo* activity due to the inability of the compounds to reach the target in a sufficient concentration and for a sufficient duration (*D.Tasdemir, 2006*). For this reason, the compounds will be included in a site-directed drug formulation in order to release the compound directly in the brain. Our aim is to point out whether an efficient drug delivery strategy is able to overcome the flavonoids instability problems.

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ACKNOWLEDGEMENT

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CEM Curriculum: Translational Medicine
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BIOLOGICAL ACTIVITY OF NOVEL THYROID HORMONE ANALOGUES: ROLE OF Na^+ TAUROCHOLATE COTRANSPORTING POLYPEPTIDE IN LIVER SELECTIVITY

Background

The interest in the potential effect of thyromimetics in lowering serum cholesterol is growing. Thyroid hormones actions on lipids metabolism are exerted in the liver and mediated by TRβ1. The creation of molecules transported into hepatocytes by liver-specific transporters can increase the liver selectivity of thyromimetics. Sodium taurocholate cotransporting polypeptide (NTCP), a solute carrier protein primarily expressed on the basolateral membrane of hepatocytes, is particularly interesting.

Objectives

The role of NTCP in the liver preferential uptake of a series of new thyromimetics was analyzed.

Methods

The experiments were performed in the the Laboratory of Endocrinology, Thyroid Laboratory, Department of Internal Medicine, Erasmus MC in Rotterdam, Netherlands, under the supervision of Professor Theo Visser.

The compounds to test (KB141, KB5588, KB6628, KB6823, KB3488, KB3493, KB3495, KB4933, KB4956, KB5035, KB5160, KB5359, KB5525, KB5526, KB5866, KB6594, KB8038) were obtained from Karo Bio AB. To explore the effect of NTCP on the nuclear availability of each compound, COS1 cells were co-transfected with TRβ1, NTCP, a construct coding for a TRE-dependent luciferase reporter and a control renilla reporter. Two days after transfection, cells were incubated for 24h with 0, 0.1, 1, 10, 100, 1000nM of each compound. Incubation with the same concentrations of T3 was added as a control. The luciferase/renilla ratio was the measure of the compound transcriptional activity.

Results

KB141, KB5588, KB3488 and KB6823 demonstrated no differences in transcriptional activity of the compounds in presence or without NTCP. KB6628, KB5035, KB5866, KB5160 and KB4956 showed a higher activity in cells transfected with NTCP compared to cells transfected with empty pcDNA3 vector. KB3493, KB3495, KB5359, KB5525, KB5526, KB4933, KB6594 and KB8038 showed a greater difference: no activity if NTCP was absent and high activity when it was expressed.

Conclusions

NTCP has a role in the transport of some thyromimetics and can have a role in their liver-selectivity.

Dr. Anna Rita DOMINGUES DA SILVA

CEM Curriculum: Translational Medicine
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LATE PRESENTATION INCREASES RISK AND COSTS OF HANA CONDITIONS IN PATIENTS WITH HIV

Background

Late Presentation (LP) at HIV diagnosis has been associated with shorter life expectancy and impaired ART efficacy and tolerability. No data exists exploring the association between LP and HIV-Associated Non-AIDS conditions (HANA) conditions that aggregate in complex multi-morbidity patterns (Mm).

Objectives

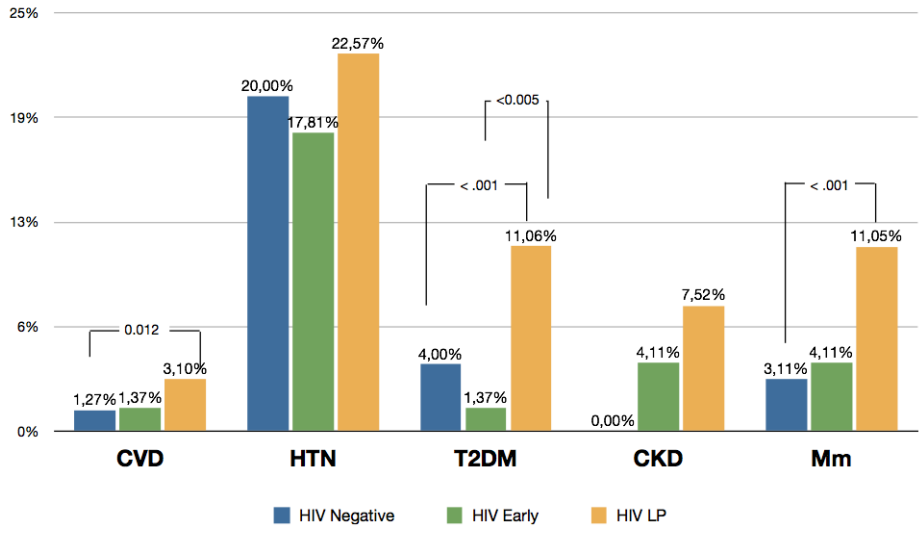
To assess the LP impact on HANA conditions and Mm, comparing them to matched seronegative controls.
The secondary objective was to provide estimates and determinants of direct cost of medical care in LP patients.

Methods

Cross-sectional study performed in 2013 involving 2 groups of patients attending the Modena HIV Metabolic Clinic divided into LP or NonLP, matched in 1:3 ratio, with age, sex, race and geographical origin controls from the CINECA ARNO database.
HANA conditions were cardiovascular disease, hypertension, diabetes mellitus, chronic kidney disease, osteopenia/osteoporosis and non-AIDS cancers. Life-style, viro-immunological and metabolic parameters were collected.
Direct health care cost were retrospectively analysed using hospital cost (HANA conditions ICD-9 codes from hospital admission), outpatients HIV medical care cost (most recent reference CD4-strata adjusted value), HANA conditions and ART medication costs (pharmaceutical tracing). Total costs were defined as the sum of the mean costs of HANA, hospitalization and drug costs.
Groups were compared using χ^2 test, *t* test or Mann-Whitney test. Probability for Mm was drawn across age distribution using a logistic model. Sex and age-adjusted uni- and multivariable logistic regression models were constructed to determine factors associated with LP. Generalized linear models were constructed to evaluate factors associated with total cost in the groups.

Results

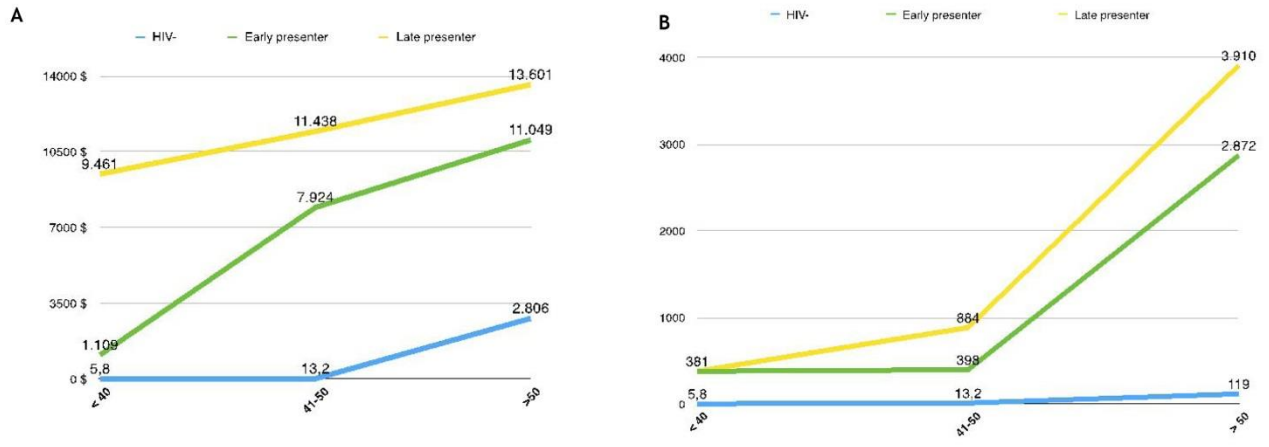
404 HIV NonLP and 404 HIV LP patients were compared with 2424 controls. Overall mean age was 46.7 years (±6.2) and 28.9% were women. HANA and Mm prevalence are shown in figure 1.



A significant OR difference in the HANA and Mm risk was found comparing NonLP, LP and controls after sex and age adjustment. There was a 5 fold increased Mm risk (OR=5.0, 95%CI 3.3-7.6, p<0.01) in NonLP pts vs controls and almost 4 fold increased Mm risk (OR=3.8, 95%CI 2.5-6.0, p<0.01) in LP vs controls.

In a sub-analysis of HIV pts only, factors significantly associated with Mm were age (per 1 year of increase: OR 1.1; 95%CI 1.04–1.13; p<.001), male sex (OR 4.7; 95%CI 1.8–12.2; p<.001), lipohypertrophy (OR 2.1; 95%CI 0.9–4.5; p=0.06), and NonLP vs LP group (OR 1.7, 95%CI 1.01–3.0, p=0.04). No association with ART classes was found.

Total costs of medical care and HANA related costs for the groups are shown in figure2.



Independent predictors of total costs in the whole population were being HIV+ (NonLP and LP), presenting Mm and taking ART.

At the subanalysis in HIV patients, predictors were: being LPvsNonLP, presenting Mm, being older, having <200CD4/mm^3 and having a longer exposure.

Conclusions

At any age the risk for individual HANA conditions plus Mm was 5 fold higher in patients NonLP compared to controls, while LP patients had an intermediate risk. Host factors and duration of HIV exposure were associated with increased risk of Mm compared to the general population.

Dr. Riccardo FANTINI

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ROLE OF DIAPHRAGMATIC ULTRASOUND IN THE RESPIRATORY FUNCTION ASSESSMENT OF PATIENTS WITH AMYOTROPHIC LATERAL SCLEROSIS

Background

Amyotrophic lateral sclerosis is the most common motor neuron disease, characterized by the gradual degeneration of upper and lower motor neurons in the motor cortex, brainstem and at the level of the spinal cord, with progressive loss of muscle function.

With the progression of the disease the respiratory muscles are affected with the final result of the emergence of a diaphragmatic dysfunction with hypercapnic respiratory failure which is the leading cause of death.

Some more recent studies suggest that the early use of noninvasive mechanical ventilation, when functional tests show initial involvement of the respiratory muscles or nocturnal hypoventilation, is able to increase life expectancy.

Currently the use of mechanical ventilation is recommended when the patient has respiratory symptoms and lung function abnormalities suggestive of dysfunction of the diaphragm. Functional tests most commonly used are the FVC (Forced Vital Capacity), sniff nasal pressure and nocturnal oximetry, which also showed a significant correlation with prognosis. Unfortunately most of the respiratory function tests require an important patient's ability to collaborate and therefore are not always able to provide reliable parameters.

Ultrasonography of the diaphragm is instead a non-invasive technique that has proven to be able to evaluate the performance of the diaphragm, so it might be an ideal test for the evaluation of the function of the diaphragm in patients with amyotrophic lateral sclerosis. Ultrasound study of the diaphragm in the zone of apposition is able to discriminate between diaphragm with normal function and paralysis with high sensitivity and specificity. More recent studies have shown the clinical utility of this technique in predicting the success of extubation in patients undergoing mechanical ventilation and in the diagnosis and monitoring of paralysis of the diaphragm.

Objectives

1. To study a possible role of ultrasound as a diagnostic test of diaphragmatic ventilatory insufficiency in patients with ALS.

2. Study the presence of correlation between the measurement of ΔTdi and ΔTmax, compared to the appearance of daytime hypercapnia (EGA), nocturnal hypoventilation (the nocturnal oximetry) and modification of the parameters of muscle strength (MIP, MEP, SNIP, FVC) in patients with ALS.
3. Observe if changes in the values of ΔTdi and ΔTmax correlate with survival, time free to ventilation and time free to tracheotomy of patients.
4. Compare the values of ΔTdi and ΔTmax in ALS patients with a group of healthy volunteers matched for sex and age.

Methods

Inclusion criteria:

1) Diagnosis of ALS possible, probable or defined according to the criteria of El Escorial Revised

2) Date of diagnosis <24 months at enrollment and without Tracheotomy or need for noninvasive mechanical ventilation

3) Age> 18 years

5) Capacity performing pulmonary function tests

50 healthy subject, without signs of respiratory and neurological disease and with normal respiratory function test will be enrolled and submitted to respiratory function test and to ultrasound examination.

ΔTdi measurement

The ΔTdi was calculated as:

(Tdi inspiratory - Tdi expiratory / Tdi expiratory) X 100

For the statistical analysis we used the best value obtained for ΔTdiVt and ΔTdiTLC in each hemidiaphragm.

ΔTmax measurement

The determination of ΔTmax was performed with the same methods and probe used for the determination of ΔTdi. ΔTmax values were calculated as:

$$\boldsymbol{\Delta Tmax =} \; Td\, i_{inspiratory\ vt} / Td\, i_{inspiratory\ TLC}$$

Tdi *inspiratory* Vt refers to the thickness of the diaphragm after an inspiration to tidal volume, and Tdi *inspiratory* TLC refers to the thickness of the diaphragm after a maximal inspiration to TLC.

Results

Good correlations were found between measures of functionality diaphragmatic ultrasound and the obtained values of FVC and SNIP test. Particularly good correlation between FVC and ΔTdiTLC p = 0.003; ΔTdiTLC and SNIP, p = 0.006; ΔTdiTLC and the presence of hypercapnia p = 0.028. Among ΔTmax and p = 0.001; ΔTmax SNIP and p = 0.003; ΔTmax and presence of hypercapnia p = 0.058.

The comparison between the measurements of the ΔTdiTLC and ΔTmax, performed with the determination of the ROC curves determined for individual comparison test, shows the best values of area for Δtmax.

Were estimated values of sensitivity and specificity in ΔTmax to predict functional respiratory changes alterations which require to start noninvasive mechanical ventilation. Values of ΔTmax of 0.75 show sensitivity of 75% and specificity of 85% for the prediction values of FVC less than 50%, while values of 0.71 show sensitivity and specificity of 71 and 81% respectively in predicting SNIP test below 30 cmH2O.

Conclusions

In conclusion our study shows how the ultrasound of the diaphragm is able to predict functional alterations secondary to respiratory neuromuscular disease with greater specificity for the diaphragm muscle. The introduction of ΔTmax could increase the ability of ultrasound to identify early when the threshold of muscle fatigue is almost reached, so this index may help the clinician in the decision to start noninvasive ventilation.

Dr. Gaia GOZZI

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Tutor: Prof. Gaetano Marverti
CoTutor: Prof. Maria Paola Costi

TARGETING HUMAN OVARIAN CANCER CELLS WITH NOVEL INTERFACE BINDING THYMIDILATE SYNTHASE INHIBITORS

Background

Ovarian cancer (OC) is the fifth most common cause of death by cancer in women. The standard first-line treatment is a combination of paclitaxel and cisplatin (cDDP) or carboplatin alone. In the case of progressive disease or drug resistance to treatment with platinum drug, either alone or in combination, investigational compounds should be used. The mechanisms behind acquired resistance to cDDP and its derivatives are not clear yet, although it is evident that the process is multifactorial and always includes enhanced DNA repair and synthesis. The enhanced expression of the folate cycle enzymes, thymidylate synthase (TS) and dihydrofolate reductase (DHFR), strongly accounts for this mechanism. TS has been shown to regulate its protein synthesis by interacting with its own mRNA as well as the mRNAs of several other proteins. Active-site inhibitors of TS are widely used in chemotherapy, such as Raltitrexed (RTX), Pemetrexed (PMX), and 5-fluorouracil (5-FU), but they induce an over-expression of the protein, considered a mechanism of resistance, due to the loss of RNA regulatory capacity when the hTS is bound to its inhibitors. It is important to identify TS inhibitors that act through new mechanisms that do not alter RNA regulation or increase protein levels. As a strategy to inhibit this mechanism avoiding induction of TS expression, some peptides (LR and [DGln^4]LR), small molecules (LC compounds) and peptide-folic acid bioconjugates have been designed and synthesized as dimer interface binding inhibitors (protein-protein interaction inhibitors-PPI) of the human TS and proved to be effective anticancer agents against sensitive and resistant OC cells (1). Unlike classical TS inhibitors such as 5-FU, RTX and PMX, the two compound classes inhibit TS and cancer cells growth without causing TS over-expression (1,2). Thus these novel TS inhibitors promise to substitute classical ones in therapeutic strategy aiming to overcome resistance associated to TS over-expression and to be used in combination to enhance the efficacy of drugs otherwise limited by the increased levels of TS.

My PhD is focused on the study of the biological effects of protein-protein interaction inhibitors at the cellular level.

In particular two peptides were conjugated with folic acid (FA) carrier to target the folate receptor (FR) membrane protein, allowing internalization via FR-mediated endocytosis in an attempt to improve the selectivity issue. This strategy is based on the observation that the α isoform of FR is expressed in more than 90% of non-mucinous ovarian carcinomas (OCs), while is almost absent in normal cells.

Objectives

The final aim of the planned research is to test whether these new drug strategies may be effective inhibitors of TS expression and may have therapeutic potential by themselves or as chemosensitizers in combination with cDDP and/or TS inhibitors compounds.

Specific aims of the past year are:

1. evaluation of the effects of **LC compounds** on cell growth, enzyme expression and cell cycle phase distribution in human ovarian cancer cell lines.

2. study the nature of the combination of the peptides with currently used anticancer drugs against human ovarian cancer cell lines.

3. evaluation of the interaction of FA-peptides bioconjugates with FR on human ovarian cancer cells and of the consequent biological effects.

Methods

In this research, we have used five human ovarian cancer cell lines, including cisplatin-sensitive, 2008 and A2780, and their –resistant counterparts, C13* and A2780/CP, respectively, and IGROV-1 cells.

The effects on cell growth of all compounds was determined using a crystal violet dye assay in which the dye extracted from the samples is proportional to the cellular biomass (3). Peptides were delivered into cells by means of the SAINT-PhD peptide delivery system (Synvolux Therapeutics, NL). Scheduling experiments to determine the optimum conditions for combining [DGln^4]LR with chemotherapeutic (CT) drugs included: (a) adding [DGln^4]LR and CT drugs simultaneously for 72 h, (b) adding [DGln^4]LR 24 h before addition of CT drugs for a further 48 h. The effects of drug combination were quantified by synergism quotient (4,5).

In order to verify the correlation between cell growth inhibition and the decrease of enzyme activity and protein level after treatment with LC compounds, the hTS catalytic assay by [^{3}H]dUMP intracellular incorporation and western blot analysis by anti-human TS mouse TS106 monoclonal primary antibody were performed (6).

Quantitative measures of the cell cycle phase distribution were performed by flow cytometry (7).

Results

LC compounds study. Almost all LC compounds tested showed a scant cytotoxic effect since they did not reach the 50% inhibition of cell growth even at 100 μM, both in cDDP-sensitive and in -resistant cell lines. However, LC1296, LC1297 and LC1343 were identified to have an IC_{50} value <50 μM in 4 ovarian cancer cell lines and showed to be more effective in A2780 and A2780/CP. In addition, LC1296 showed to decrease both hTS levels and catalytic activity in 2008 and C13* cells. Perturbation of cell cycle phases was observed

particularly in A2780 cells, with a reduction of cells in the G0/G1 phase and a corresponding increase of the percentage of apoptotic cells.

Peptides studies. Most of the combinations of the lead LR peptide derivative, [DGln^4]LR, with cDDP, Paclitaxel, 5-FU and RTX at 72h produced additive or supra-additive effects in A2780 and A2780/CP cell lines. In A2780 cells, sequential combinations increased cell killing in comparison to concurrent exposure to cDDP, Paclitaxel and RTX, whereas in the resistant counterpart A2780/CP cells improvements were observed for combinations with 5-FU and RTX. In 2008 cells, sequential exposure to [DGln^4]LR and then to the drugs does not cause supra-additive effects but a little reduction of the effectiveness for cDDP and RTX. On the contrary, in the resistant C13* cells, all drugs except Paclitaxel, increased their cytotoxicity when administered after [DGln^4]LR, in particular RTX. This is also further evident in Igrov-1 cells, which did not display significant increases in SQ values for cDDP and Paclitaxel between concurrent and sequential combinations, but supra-additive or synergistic SQ values were evident for 5-FU and RTX.

Bioconjugates compounds studies. The effect of the bio-conjugates was tested on IGROV-1 cells, which express high level of folate receptor α. After evaluation of the relative binding affinity of the engineered conjugated system for the FR, we have tested the effect of peptides internalized via FR-mediated endocytosis on cell growth. The previously estimated intracellular concentrations of the bioconjugate FA-LR caused a cell growth inhibition of about 20-25%. Among the LR derivatives obtained by SAR studies, the chirality inversion at position 4 of the lead LR peptide was the most prominent modification, yielding [DGln^4]LR that increased the potency relative to LR and the other derivatives, when transfected into cells by means of a specific peptide delivery system (2). Accordingly, [DGln^4]LR also showed slightly higher efficacy when internalized via FR-mediated endocytosis as bioconjugate with FA and more active than the reference drug, 5-FU, when compared at the concentration of 1.25 μM. Owing to the increased sensitivity to [DGln^4]LR peptide in comparison to the lead LR peptide of IGROV-1 cells, we have tested FA-[DGln^4]LR also against other two human cancer cell lines. The bioconjugate FA-[DGln^4]LR showed higher activity at 1.25 μM than at 2.5 μM in all three cell lines. Of note, at the higher concentration, both A2780 and 2008 cell lines, expressing low levels of FR on their cellular surface, were less responsive to FA-[DGln^4]LR than the FR-overexpressing IGROV-1 cells, but about 3-fold more responsive to 5-FU.

Conclusions

LC1296, 1297 and 1343 actually displayed anti-TS activity and cell growth inhibition probably by exploiting a novel TS dissociative mechanism which allows stabilization of the inactive form of the enzyme maintaining the ability to bind its mRNA.

The combinations of most of the drugs with [DGln^4]LR showed additive cell killing when concurrently administered and potentiate their effectiveness when sequentially combined to the peptide, in particular the drugs that target folate cycle enzymes, such as 5-FU and RTX.

The folate-peptide conjugates have the potential to be developed into a new tool for cancer chemotherapy following focusing on lead optimization of both cellular pharmacokinetic properties and biological activity.
In addition, we will investigate the mechanism leading to the decrease of TS levels by LC compounds verifying the protein stability and its capacity to bind its mRNA.
We will verify the nature of the combination of the LC compounds with currently used anticancer drugs against human ovarian cancer cell lines with the aim to potentiate effectiveness by overcoming TS over-expression.

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CEM Curriculum: Translational Medicine
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MITOSTATIN: A POSSIBLE TUMOR SUPPRESSOR GENE IN PROSTATE CANCER

Background

Prostate cancer is a pathology that represents the second cause of male death for cancer in Western countries. The numbers of patients are rapidly growing both for increase of males over 50 years of age and continuous improvement in the diagnostic techniques sensitivity. The prostate specific antigen (PSA) test allows to diagnose patients with clinically localized carcinomas. These kind of carcinomas can be managed conservatively with ''Active Surveillance", but a small fraction of them will progress rapidly and require an immediate patients treatment. As a consequence, a major clinical challenge is posed by the inability to readily distinguish indolent from aggressive tumor forms and to avoid a significant patients ''overtreatment''. The challenge could be addressed by studying the molecular basis of cancer initiation and progression and by identifying new biomarkers to distinguish the two prostate cancer forms.

We recently reported the cloning and partial characterization of a novel protein designated Mitostatin, endowed with tumor suppressor activity, whose gene is located on chromosome 12q24, frequently deleted in a variety of malignant neoplasms. *MITOSTATIN* gene mutation and downregulation have been observed in multiple cancers (reduced in 22% of advanced bladder cancer, in 23% of breast carcinomas and in 35% of prostate cancer analyzed) and its potential as a tumor suppressor has been reported recently. In various cancer cell lines we have demonstrated that ectopically expressed Mitostatin inhibited cell growth, migration, invasion, adhesion and tumor formation *in vivo.* We investigated the molecular mechanisms through which Mitostatin affects inflammation processes, and the related hypoxic status, during prostate cancer progression. The inflammation processes, that usually cause a tissue hypoxic condition, can induce reactive oxygen species (ROS) production, promote neo-vascularization and cell proliferation. ROS, in turn, stimulate p38-MK2-HSP27 signaling cascade that usually controls cancer cells migration and survival. Mitostatin expression is down-regulated by hypoxia both *in vitro* (HUVEC and PC3 cells) and *in vivo* (prostate tissue). Mitostatin is strongly down-regulated by IL-6, a crucial chemokine in prostate cancer initiation and/or progression and IL-6 up-regulates microRNA-503, whose over-expression (by using pre-miR-503) reduces Mitostatin protein levels. MicroRNA-503 plays a determinant role in endothelial cell behavior and angiogenesis process. Mitostatin level determination, in association with IL-6 and micro-503 level determination, might allow to stratify patients for treatment management into "high-risk" and "low-risk" patients groups, avoiding to overtreat patients who would require only conservative management. Recently has been reported that Mitostatin binds centrosomal proteins Odf2 and ninein, and its depletion

causes an alteration of the anchorage of microtubules to the centrosome. Centrosomes are responsible for proper cell cycle progression, controlling both the transition G1/S and G2/M.

Objectives

Recently, has been shown that Mitostatin binds centrosomal proteins and mediates the anchorage of microtubules (MT) to the centrosome. These results suggest that Mitostatin may play an important role in the proper anchoring process between MT and centrosome in proliferating cells. Centrosomes are responsible for proper cell cycle progression, controlling both the transition G1/S and G2/M. We hypothesize that this protein may play an important role in proper cell cycle progression, and its depletion could lead to an incorrect chromosome segregation during mitosis, followed by aneuploidy and chromosomal aberrations that are often observed in many cancers. In fact, in depleted-Mitostatin cells we observed defects that were consistent with faulty activation of the spindle checkpoint, such as shortened mitosis, premature sister-chromatid separation, chromosome bridges and mis-segregation in anaphase, and faster mitotic slippage in the presence of a spindle inhibitor.

1) We will deeply analyze the molecular mechanisms through which Mitostatin interacts with the p38MAPkinase-Hsp27 signaling pathway that is usually activated during the oxidative stress processes. The results obtained would represent a link between inflammation and prostate cancer onset and progression.

2) We will investigate the chromosomal instability, the activity of mitochondrial complexes and glucose metabolism in cells in which Mitostatin is knocked-down. High superoxide levels are known to affect cellular transformation andinduce genetic instability. 3) We will perform a retrospective analysis of biopsy specimens from patients who had been monitored by “Active Surveillance” over a 10-year period to check whether the new biomarkers panel (Mitostatin together with IL-6, IL-1β, microRNA-503, p38-MK2-Hsp27) we propose, will provide additional prognostic information regarding the outcome (i.e. patient that are distinguished on the basis of the parameter “Gleason 6” and had failed surveillance). 4) In order to expand the panel of biomarkers we will perform (on the same biopsy specimens) a proteomic analysis: whole proteome will be analyzed by Mass Spectrometric/bioinformatic method to identify the differentially expressed proteins (DEPs) between patients who failed surveillance or not.

Methods

HeLa cells and HeLa depleted of Mitostatin were synchronized in G1/S by using Aphydicolin, and released into nocodazole-containing medium (prometaphase arrest). The cells were harvested at different times (0,6,12,18,22 hours) and analyzed by flow cytometry (PI staining). The same samples were collected to analyze some of the key regulators of the cell cycle, including Cdh1, Cdc20, cyclin B1, Aurora a, cyclin A, phospho Histone-H3 (pHH3), using western-blot analysis.

Also, to count mitotic cells, HeLa control cells and HeLa depleted of MITOSTATIN, are stained with antibody anti- pHH3 present only in mitotic cells, combined with propidium iodide (PI) to stain DNA and analyzed by FACS at different times (0,6,12,18,22,30 hours).

We analyzed chromosome bridges in anaphase, in HeLa cells (control and depleted of Mitostatin). In this regard, the Histone2B-GFP plasmid was transfected in phoenix-ampho cells using Lipofectamine2000-Reagent. At 48 h after transfection, virus-containing medium was collected and supplemented with 8 µg/ml Polybrene. Hela cells were then infected with the viral supernatant for 5 hours. Finally, we performed metaphase spreads and 5% Giemsa staining to chromosome analysis.

Results

We observed that in Hela cells Mitostatin depletion produces a deficient spindle checkpoint, indicating a mitotic slippage and adaptation to the spindle checkpoint and consequent chromosome instability. HeLa cells were synchronized by aphidicolin (G1/S) block and released into nocodazole-containing medium. The cells, after have been collected at different times, were analyzed by cytofluorometry and lysed for immunoblotting. Cells depleted of Mitostatin showed a decreased percentage in G2-M phase (about 20%) respect to control cells. This finding was confirmed by the levels of several cell cycle markers; phospho Hystone H3 (pHH3), a G2/M marker, was found reduced in cell depleted of Mitostatin respect to the control cells; protein levels of Cyclin B1 were found early degraded (at 18 hr from G1/S block) in depleted Mitostatin cells. Since the spindle assembly checkpoint (SAC) delays cell exit from mitosis by preventing B1 cyclin proteolysis, the B1 cyclin early degradation leads to mitosis escaping. Moreover, Mitostatin depletion causes increased number of lagging chromosomes and chromosome bridges in anaphase, suggesting a premature sister-chromatid separation respect to control cells. Finally, Mitostatin-depleted cells showed a mitotic index decrease in the presence of a spindle poison, despite that cytofluorimetric analysis showed the cells entered into mitosis following a normal kinetic. The overall observations suggest that an increased rate of mitotic slippage and adaptation to the mitotic checkpoint occur in our experimental model.

Conclusions

1) Mitostatin exhibit many of the hallmarks of a typical tumor suppressor gene, and could be a novel putative tumor suppressor gene at 12q24.1. 2) The distinctive features of this protein, suggest it might represent a crucial player for linking prostate inflammation and hypoxia to the progression of chronic prostatic diseases. 3) Mitostatin binds centrosomal proteins and mediates the anchorage of microtubules (MT) to the centrosome during mitotic spindle formation. Mitostatin loss leads to aberrant activation of the spindle checkpoint, and consequently it is required for fidelity of mitosis. Mitostatin reduced levels that are found in certain human tumours might contribute to cellular transformation by promoting genomic instability.

Dr. Stefano MANCINI

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INCREASED EXPRESSION OF AUTOPHAGY-RELATED PROTEINS IN HUMAN COLORECTAL CANCER DEVELOPMENT, AND CORRELATION WITH DNA MICROSATELLITE STATUS

Background

Recent research indicates that autophagy plays a crucial role in many physiological and pathological conditions. The role of autophagy in tumours remains controversial, especially in malignant colorectal tissues. One of the most accredited hypothesis, however, poses autophagic processes as crucial regulators of the balance between cancer promotion/suppression regulating inflammatory pathways, immune responses, metabolic pathways, and even gene expression inside the cell. Our research group has already demonstrated that obesity is a risk factor for the development of a proinflammatory status in colon tissues, but the molecular mechanism has not yet been clarified.

Objectives

The aim of this study is to investigate the cellular mechanisms implicated in colorectal carcinogenesis, with a focus on the role of autophagy in regulating systemic and local (colonic) inflammation in our cohort of patients. The expression of autophagy has been investigated in normal colorectal mucosa and along colorectal carcinogenesis, in order to find a trend in autophagy regulation at different stages in the adenoma-carcinoma sequence.

Methods

The expression pattern of DNA cytosine-5-methyltransferase 1 (DNMT1) was assessed at different steps in colorectal tumour development, and in carcinomas classified as DNA microsatellite stable (MSS) and unstable (MSI). Immunofluorescence techniques coupled with confocal microscopy and immunoblot experiments were performed on 15 samples of normal mucosa (NM), 15 microadenomas (MA) and 15 carcinomas (C) obtained from patients who underwent colonoscopy or surgical resection for colorectal cancer. Moreover, 23 MSS colorectal carcinomas, and 26 MSIs were analysed by immunofluorescence experiments.

Results

Currently, our results clearly showed a significant increasing expression of autophagic factors in the normal mucosa (lower expression) – microadenoma (mid expression) - carcinoma (higher espression) sequence. DNMT1 was higher expressed in microadenomas and carcinomas than in normal mucosa. In MSS

carcinomas, the level of autophagic factors and DNMT1 expression was higher than in MSI carcinomas. All these results achieved statistical significance.

Conclusions

These data support the hypothesis that an upregulated autophagy represents an advantage for cancer cell survival, thus promoting colorectal cancer development and progression. Autophagy is higher in those colorectal cancers expressing factors related to a worst prognosis, such as MSS carcinomas. The next steps of the project consist in elucidating the connection between different proinflammatory factors (such as preclinical markers of atherogenesis, humoral immune responses, psychometric assessment, apoptosis/autophagy balance, and oxidative stress), with the development of colorectal cancer in our cohort of patients.

Dr. Martina MANNI

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STUDY OF "RISK AND RESPONSE-ADAPTED" THERAPIES IN PATIENTS WITH LYMPHOPROLIFERATIVE DISORDERS

Background

During the first year of my PhD I was involved in clinical studies conducted to evaluate the efficacy of "risk and response-adapted" therapies in patients with lymphoproliferative disorders. In particular I worked on the definition of the rationale for a response adapted therapy in patients with follicular lymphoma (FL) and on its practical application with the multi-center, randomized, phase III FOLL12 trial. FL is one of the most common subtypes of lymphoma in Western countries and accounts for 10-20% of all newly diagnosed non-Hodgkin's lymphomas. Clinical course is typically indolent with impressive responses to initial treatments but with frequent relapses, with the need for recurrent therapeutic interventions. Responses to salvage treatment are of shorter duration after every relapse, and most patients ultimately die of their disease or of treatment-related toxicity, with a median survival of 6-10 years. Biologically, the neoplastic clone of the great majority (up to 80%) of FL patients carries the t(14;18) translocation in which the Bcl2 proto-oncogene on chromosome 18 is translocated to the immunoglobulin heavy chain (IgH) region on chromosome 14, thus creating a hybrid Bcl2/IgH gene. This translocation leads to an over expression of the Bcl2 protein, which inhibits apoptosis of lymphoid cancer cells.

The standard of care for patients diagnosed with FL is based on the use of RCHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone) chemotherapy followed by two-year maintenance with R in all responding patients. Responding patients to initial therapy however show a heterogeneous behavior ranging from long lasting remissions to early relapses. The identification of patients at different risk of progression using accurate techniques for response definition makes it reasonable to question if standard treatment and in particular if maintenance is really needed for all patients: on the one hand some patients at low risk of progression could benefit from a reduced intensity treatment avoiding costs and toxicity; conversely on the other hand patients at high risk could need an intensified approach to achieve the same results in terms of outcome and progression free survival (PFS).

Objectives

This project was conducted with the aim of identifying prognostic factors and to translate them into clinically useful tools to improve the outcome of patients with FL. In particular the project was focused on the use of FDG-PET and of minimal residual disease (MRD) that were separately shown to have a both high prognostic power to predict the risk of progression in FL patients.

Methods

In a first phase of this project both FDG-PET and MRD were tested in a retrospective series of patients with FL. For this part of the study, patients were identified from the FOLL05 trial, a phase III, multicenter, randomized study in patients with stage II-IV untreated FL. The study planned investigations included the Bcl2/IgH rearrangement analysis at baseline, at 6 weeks after the end of treatment, and then every 6 months during the second and third year of follow up. However, for different reasons, of all 504 patients enrolled in the study, only 415 were assessed for t(14;18) translocation by PCR at baseline and consequently used to perform a retrospective analysis of the correlation between the presence/absence of molecular marker and PFS.

The predictive value of PET-scan was evaluated in 155 patients who had performed the exam from 10 days to 3 months after the last cycle of induction treatment. The PET scanning was not a mandatory FOLL05 study procedure and this is the reason of the low number of PET available for the retrospective analysis.

The second part of the project that is still ongoing used results of the first part to define the rationale to design a new response adapted clinical trial, the FOLL12 trial. So the FOLL12 study was born with the objective of evaluating if after an initial induction RCHOP therapy, combining clinical response assessed on FDG-PET scan and molecular response measured through MRD detection, is possible to identify patients subgroups at different risk of progression and to consequently modulate the maintenance therapy. If successful this trial will show the advantages achieved with a response adapted treatment strategy vs a standard approach in patient with untreated FL. Once informed consent is obtained, patients are assessed for eligibility to the trial by standard procedures (bone marrow biopsy, histology, CT-scan and FDG-PET) which include the sending of bone marrow aspirate and peripheral blood samples to centralized laboratories for qualitative and quantitative Bcl2/IgH rearrangement analysis. Enrolled patients are randomly assigned in a 1:1 ratio to either standard arm or experimental arm. Initially, all patients receive the same induction therapy with 6 cycles of RCHOP and 2 additional doses of R. At the end of chemo-immunotherapy they are reassessed for disease response by CT-scan and FDG-PET and for molecular response by MRD detection. All responding patients in the standard arm receive standard maintenance therapy with R (every 2 months for 2 years), while patients in the experimental arm are subdivided into risk groups and assigned to different post induction treatment according to PET results and MRD status. Six-hundred and two (602) patients satisfying inclusion and exclusion criteria will be enrolled in a planned period of 4 years from different Italian Centers. Considering four years for accrual completion and 3 years of follow up, the overall duration of the study is planned to be approximately 7 years.

Results

The molecular analysis in FOLL05 documented that the percentage of cases not achieving the complete response at the end of therapy was higher for patients Bcl2/IgH+ in respect of cases without molecular

marker. In particular patients without molecular marker or with a low molecular tumor burden, $<1 \times 10^{-4}$ copies, showed higher complete remission (CR) rate and longer PFS: the 3-year PFS was 80% vs 59% for cases with higher molecular tumor burden (p=0.015). So the different observed rates of response to chemo-immunotherapy and outcome between different patients subgroups could be related to the presence or not at molecular level of t(14;18) chromosomal translocation.

With regard to the PET retrospective analysis, it showed a significantly superior PFS in post induction PET negative patients. In fact with a median follow up of 34 months, the 3-year PFS for this subgroup was 66% while in the PET positive patients was 35% (p<0.001). As a result PET could be considered as strongly predictive tool for the risk of progression and this support its inclusion in response criteria for FL.

Results obtained with MRD and PET study have led to the definition of the FOLL12 trial rationale based on combination of the two techniques described above to determine the individual risk profile and consequently adapt the intensity of the treatment. The FOLL12 study started less than two years ago and until now 312 patients have been enrolled. Of these patients 20 are screening failure for different reasons, mainly for non-compliance with the inclusion criteria and for the informed consent withdrawal; 9 patients are still in the screening phase; 283 patients were actually randomized (140 in the standard arm and 143 in the experimental arm). Of all randomized patients 19 dropped out of the study during or at the end of induction treatment (2 due to deaths, 6 for toxicity reasons, 7 for disease progression and 4 due to patient choice), 128 are in the induction phase, while 136 are in the maintenance phase. Considering the last subgroup of patients, at the end of induction treatment 119 had a negative PET (low risk of progression), 16 had a positive PET (high risk of progression) and for one patient PET result was doubtful. Of the 16 PET+ patients, 6 were negative for the research of molecular marker, 4 were positive and 6 were not reassessed for MRD status at the end of induction because considered “no molecular marker” at baseline; on the other side of all 119 PET- patients, 67 were negative for the research of Bcl2/IgH rearrangement, 12 were positive and 40 were “no molecular marker”. The above PET and MRD results obtained were used to divide patients in risk subgroups and to modulate the subsequent course of treatment.

Conclusions

FOLL05 study allowed us to define the possible prognostic factors in the treatment of FL; the results obtained were used in the ongoing FOLL12 trial with the aim to provide clinicians a more rational use of the available diagnostic and therapeutic resources. However, data currently available are still not enough to perform a preliminary analysis of the primary and secondary trial endpoints. A first interim analysis of PFS (primary endpoint) will be performed after the 40% of planned full information has occurred.

Dr. Francesca MANTOVANI

CEM Curriculum: Translational Medicine
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PRAGMATIC ECHOCARDIOGRAPHIC APPROACH ACCORDING TO THE EUROPEAN SOCIETY OF CARDIOLOGY PROPOSED ALGORITHM IN ELECTIVE PATIENTS WITH CLINICAL SUSPICION OF INFECTIVE ENDOCARDITIS: DIAGNOSTIC YIELD AND PROGNOSTIC IMPLICATIONS IN CLINICAL PRACTICE

Background

Echocardiography plays a central role in diagnosing of infective endocarditis (IE). Accordingly, the European Society of Cardiology (ESC) has proposed a diagnostic echocardiographic algorithm. However, to date its effectiveness in routine practice has not been verified.

Objectives

To investigate the diagnostic yield and prognostic implications of ESC proposed algorithm for clinical suspicion of IE in clinical practice.

Methods

Retrospective analysis of a series of patients undergoing ESC proposed algorithm for clinical suspicion of IE at Our institution. We also examined the association among echocardiographic results and clinical outcomes.

Results

Between January 2009 and June 2013, 325 cases were managed by a multidisciplinary team for clinical suspicion of IE. Following the ESC proposed algorithm, 27 (8%) patients were diagnosed as positive for IE and 298 (92%) patients were diagnosed as negative for IE (Figure 1). In almost 80% of cases, if a good-quality negative transthoracic echocardiography was associated with low level of clinical suspicion, transthoracic echocardiography was considered sufficient. During a mean follow-up of 2.3±1.4 years, these subgroups of patients showed same low rates of combined endpoint (death, stroke, atrio-ventricular block, heart failure, relapsing endocarditis) (Figure 2).

Conclusions

In our experience, only a minority of patients with clinical suspicion of IE undergoing the ESC proposed algorithm had a final IE diagnosis. Therefore, in the current cost-conscious era, echocardiography seems to

be used as a screening test with low diagnostic yield. However, this pragmatic echocardiographic diagnostic approach allows the detection of a low-risk group of patients for whom TTE is adequate.

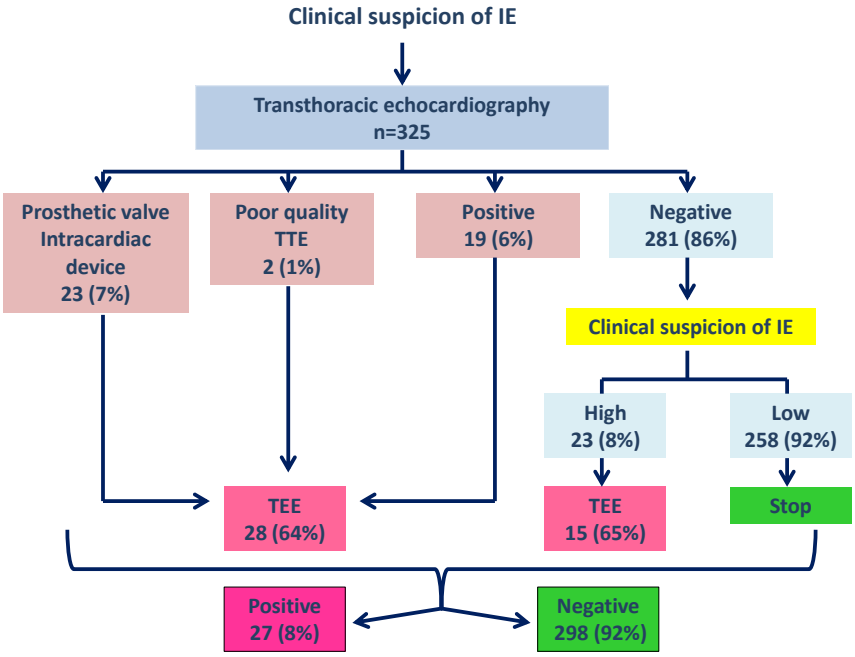
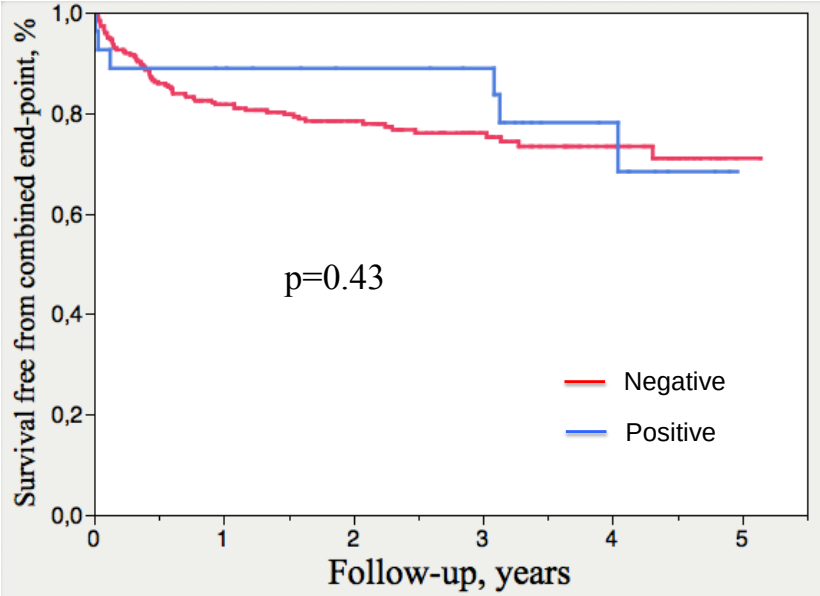


Figure 1 - Diagnostic congruity between 2012 ESC guidelines flow chart and our echo-lab activity in patients with clinical suspicion of infective endocarditis (IE) (from 2009 to June 2013).



Patients at risk

Negative for IE	298	216	150	94	45	3
Positive for IE	27	24	20	18	9	1

Figure 2 - Survival free from combined endpoint (death, stroke, BAV, HF, relapse endocarditis) in patients with and without definite infective endocarditis diagnosis .

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SEIZURES ARE BETTER CONTROLLED BY ADAPTING THE FATS- TO-PROTEINS + CARBOHYDRATES RATIO TO KETONE BODY LEVELS

Background

The ketogenic diet (KD) is an effective antiepileptic treatment. Although its mechanism of action is still unclear, the KD is based on a marked limitation of carbohydrate intake counterbalanced by selection of fatty food, dietary options both aimed at inducing a state of chronic ketosis. The classical KD is calculated using method of the ratio, which was first described more than 80 years ago: today the KD is generally proposed with fixed ratios chosen on empirical basis. A ketogenic ratio tell us the proportion (in grams) of fat in the diet as compared with carbohydrates and proteins.

Objectives

The aim of our research is to evaluate the effectiveness of the diet by defining a ketogenic ratio able to modulate blood ketosis within a subjective range, in which patients might achieve the desired outcome. In particular, crisis reduction, fewer severe side effect and, as a consequence, improvement in quality of life were considered goals of the project. A personalized fats-to-proteins+carbohydrates ratio was established for each patient to improve KD acceptability without altering the efficacy of the proposed diet. To investigate this approach, we designed a small pilot trial to test the optimal fats-to-proteins+carbohydrates ratios by monitoring seizure frequency as a function of ketone bodies levels.

Methods

We considered eight patients (age: 2-12 years) affected by drug-resistant epilepsy, maintained on the classical KD protocol at the Neuropediatric Unit of the University Hospital (Policlinico, Modena). The KD was started during hospitalization as a 1:1 ratio, which was progressively increased until the achievement of an effective level of ketosis. After hospitalization, during which glucose and ketone basal values were obtained, parents monitored glycaemia and β-hydroxybutyrate levels 3 times/day/week up to 16 weeks by using the *Precision Extra Blood Ketone Mether* (Abbot)**.** Seizures were recorded by parents one month before and 16 weeks after the KD onset, and values were used to calculate the seizure frequency. Statistical analysis was performed using Sigma Plot.

Results

Fats-to-proteins+carbohydrates ratios were adjusted to obtain >50% seizure reduction. Ketone body (or glucose) levels were plotted against seizure frequency and a regression analysis was performed. More than 80% seizure reduction was obtained in all patients. KD was started as 2:1 ratio, then increased up to 3:1 (n=3) or 4:1 in four patients until a ketosis larger than 4.0 mg/dl was obtained. Two patients decided to stop the study for reasons unrelated to KD efficacy. Regression analysis was not significant in one patient affected by Lennox-Gastaut syndrome. Conversely, significant findings were found in all other cases. One patient affected by respiratory chain complex I-III defect, and a further one with Glut-1 deficiency both presented a highly significant ($P<0.001$) relationship ($R^2=0.38$ and, respectively, $R^2=0.46$) between seizure reduction and ketosis. Data were also meaningful in presence of a chromosome defect ($R^2=0.70$; $P<0.001$), or in presence of pyruvate dehydrogenase deficiency ($R^2=0.66$; $P<0.001$). No relationship was found between glucose plasma levels and seizure reduction.

Conclusions

Our preliminary results suggest that patients could benefit from a personalized KD ratio able to maintain blood ketosis with in a subjective range. The adaptation of the the fats-to-proteins+carbohydrates ratio to ketone body levels proved to be meaningful in terms of crisis reduction. Furthermore a significant reduction of side effects was observed in the analysed group of paediatric patients. Despite the study should be integrated with more substantial quantitative data (i.e., *n* of patients), it suggested the usefulness of a more accurate and frequent monitoring of ketone bodies in patients treated with classical KD.

Dr. Alberto MODENESE

CEM Curriculum: Health Sciences
Tutor: Prof. Fabriziomaria Gobba

A METHOD FOR THE EVALUATION OF CUMULATIVE SOLAR RADIATION EXPOSURE IN OUTOOR WORKERS

Background

The health risk related to an excessive exposure to solar radiation (SR) is well known. The Sun represents the main exposure source for all the frequency bands of optical radiation (OR): infrared (IR), ultraviolet (UV) and visible radiation. UV radiation (UVR), mainly from the Sun, is able to induce the most severe biological effects: both acute and chronic diseases, particularly to the skin and the eyes. According to recent studies, outdoor workers (OW) have a relevant exposure to SR, largely exceeding the limit of 30 Joule / m2 - effective radiant exposure (Heff) referred to 8 working hours - for artificial UV (European Directive 2006/25/EC). However, few studies have attempted to retrace the history of a chronic exposure to SR in groups of OW.

Objectives

To develop a method for assessing occupational and environmental cumulative exposure to SR, applicable for epidemiological studies. The instrument should be useful to allow a better comparison between exposure levels and early biological skin and eye effects, and to study the role of the protective factors.

Methods

The instrument proposed integrates subjective and objective data. Subjective data are collected with a questionnaire, composed by three sections, dedicated respectively to outdoor work activities, leisure activities and vacation periods. The items have been elaborated by a team of occupational physicians and experts in OR and industrial hygiene. The respondent has to answer considering only the months of the year between March and October (except for vacations on the snow). At the beginning of each section, the interviewer has to define the period of life the section refers to. In each section, the 12 items investigate the type of outdoor activity, the total time people spend outside during the activity and main personal habits that may influence SR exposure. The second part of the method includes the collecting of two types of objective data: climate data and individual exposure measures. Climate data are available since a long time for different geographical regions and they provide an estimate of the SR to the Earth's surface (e.g. clear sky UV index, daily erythemal UV dose). To validate the method taking into consideration environmental and individual factors (posture, personal habits; etc.), two experts in OR and industrial

hygiene have collected several measures of effective radiant exposure (Heff) with individual dosimeters in a group of 6 fishermen.

Results

Regarding subjective data, the preliminary results in a group of 11 men and 3 women aged between 40 and 79 (mean 55,6) show that workers, mostly farmers and construction workers, spend outside an average 5h 50' per day between 9 am and 5 pm and 2h15' between 11 am and 3 pm. More than the 40% of the outdoor workers regularly use protecting clothes (pants and sweater), but they only sometimes use hat. For the section that investigates leisure activities, the respondents declare that they spend outdoor an average time of 4h 50' between 9 am and 5 pm and 1h 45' between 11 am and 3 pm, during the weekend between March and October. Over 25% of the sample affirm to perform leisure activities often/always close to reflecting surfaces. Regarding the protective habits, 21% and 37 % of the subjects report that they always wear, respectively, hat and sunglasses; only 16 % usually wears protective clothes. Sunscreens are often used by the 10% of the sample. For the section investigating the vacation periods, the respondents declare they spend outdoor on average 6h 50' per day between 9 and 17, and 1 h 50 ' between 11 and 15. They are close to reflecting surfaces (mostly sea water, almost never snow) for 3h 15' on average. Over 35% of the subjects declare they often/always experience sunburns during holidays. The 48% of the sample always uses sunglasses; the 47% never wears hat and the 59% never uses sunscreens.

Regarding objective data, the results of the on-field individual measures show that the highest exposure to solar UVR have been measured for the nose, ear and upper shoulder of the fishermen with a dosimeter placed on the cap's peak of the men: 0.4- 0.90 kJ / m^2 - effective radiant energy (h_{eff}). Than a high exposure was found for the back 0.04 - 0.68 kJ / m^2. Lower exposure were found for the chest, 0.15 - 0.28 kJ / m^2, and for the external part of the arm, that due to the "Coroneo's effect" represents the exposure of the lateral face and of the eye: 0.05 - 0.12 kJ / m^2.

Conclusions

Summarizing the results of the questionnaire, we can affirm that the highest sun exposure, in terms of hours spent outside during a single day, can be found during the vacation periods; but if we consider the central hours of the day, when the SR is more intense, the longest outdoor periods are referred during work activities. Regarding objective measurements, the highest exposure to solar UVR have been measured for the nose, ear and upper shoulder of the outdoor workers. The method proposed seems to be appropriate to characterize the relationships between working postures, protective equipment and reflecting / refracting phenomena and the exposure of different parts of the body, allowing us to integrate subjective and objective data to obtain an esteem of the cumulative SR exposure of a specific tissue, with a mathematic model. The proposed instrument is aimed to provide a detailed estimate of lifetime exposure

to SR in groups of outdoor workers, for epidemiological studies, in order to better compare the exposure levels and early biological skin and eye effects, considering also the role of risk and protective factors for the onset of SR-related diseases.

Dr. Claudia ROMANELLI

CEM Curriculum: Translational Medicine
Tutor: Prof. Anna Iannone
CoTutor: Prof. Massimo Federico

PROTEOMIC ANALYSIS OF EXOSOMES DERIVED FROM BREAST CANCER CELLS

Background

Extracellular vesicles (EVs) are a heterogeneous population of membrane-surrounded structures released by cells into interstitial space (both *in vivo* and *in vitro*) and into body fluids. They differ in size, biogenesis and biological function. Although a well-defined terminology for the different subtypes has not been standardized, a distinction can be made between microvesicles and exosomes. Microvesicles (MVs; 100-1000 nm) are released into the extracellular space by outward budding of the cell membrane, while exosomes (30-100 nm) are released after the fusion of multivesicular bodies (MVBs) with the cytoplasmatic membrane [1]. EVs have been initially considered as membrane debris with no real biological significance. Nowadays, it is known that EVs provide local and distant signals via the transfer of their content (proteins, lipids and nucleic acids): thus they play a key role in intercellular communication. EVs regulate not only normal physiological processes (cell maintenance, tissue repair, immune surveillance and blood coagulation), but they are also involved in the pathology. Like most cellular types, neoplastic cells release vesicles, but with a higher rate and with alterations in cargo molecules [2]. There are many well characterized examples of EVs functions in cancer, which may be considered as a coordinated set of mechanisms that promote disease: 1) evasion from immunity surveillance; 2) generation of fertile environments that support malignant lesions and stimulate angiogenesis; 3) induction of molecular features that cause malignant cells migration from the primary tumor; 4) the same pathway used to export less-needed molecules is also used to export chemotherapeutic drugs, determining cellular chemoresistance [3]. Because of all these functions, EVs are becoming reservoirs of tumor-specific biomarkers for cancer detection and progression.

Objectives

Our main purpose is to develop a method for exosomes isolation, pure enough to allow a proteomic analysis of vesicles content, in the search of diagnostic markers of breast cancer. Direct search for markers in the plasma of women with cancer, by proteomic analysis, turned out to be complicated by the abundance of plasma proteins, therefore it was decided to use a two-step approach: 1) *in vitro* investigation to identify differentially expressed proteins in exosomes derived from breast cancer cell lines with different malignancy: MDA-MB-231 and MDA-MB-231 Slug-shRNA, the latter genetically modified to

lose invasiveness; 2) detection of differentially expressed proteins in the plasma of patients with breast cancer.

Methods

First, an attempt was made to setup an exosomes isolation method (ultracentrifugation) from plasma samples [4]. Afterwards, the focus was shifted on exosomes purification from cell cultures: cells were grown under usual conditions until they reached confluence, then in a medium previously depleted of FBS-exosomes. Exosomes were purified from this conditioned medium, through differential centrifugation [5]. To test the efficacy of the isolation method, obtained exosomes, were viewed under a transmission electron microscopy (Nova NanoSem450 FEI) [6]. They were loaded on formvar carbon-coated grids and negatively stained with different colorants (1-2% uranyl acetate, 3% sodium phosphotungstate and 2,4% ammonium molybdate) solved in distilled water, at different pHs: acid (4-5) and physiological (7,4). The colorants precipitate around the particles and between their surface structures, making the morphological characteristics recognizable. The mean diameter of vesicles in each sample was extrapolated after the analysis of four images; all analysis and processing functions are available at any magnification factor by "Image J" (Wayne Rasband), nevertheless it was used the average of fields with the same magnification to quantify the number of vesicles. Exosomes pellets underwent lysis for protein extraction and total protein concentration was determined by Bradford total protein assay. This was followed by a preliminary proteomic analysis. For each cell line, the extraction was performed in quadruplicate to exclude experimental variation and ensure the reliability of the results obtained.

Results

The first idea to search for markers directly in the plasma of women with breast cancer turned out to be complicated. The use of only ultracentrifugation method, proposed in other works, was not adequate to obtain a pure sample of exosomes: electron microscopy analysis showed the presence of two vesicles populations, the most abundant of which was the one in the range size of 21-40 nm, compatible with the one reported in other studies; thus, the isolation was successful, but it was necessary a high dilution of the sample to obtain a reliable image, because there were a lot of protein aggregates in addition to exosomes. Moreover, the proteomic maps showed such a high abundance of plasma proteins, that a real analysis of the vesicles protein content was not possible. The attention was then shifted on exosomes isolation from cell culture. In this case, the ultracentrifugation method allowed us to obtain a protein yield, from both cell lines, of about 1 µg/µl. Electron microscopy revealed that the isolated particles consisted primarily of rounded vesicles of variable size; quantitative analysis indicated that the samples contained a majority of vesicles with a mean diameter between 30 and 80 nm, included in the range 30-100 nm: this allowed us to

classify vesicles as exosomes. Moreover, a proteomic analysis was performed and the resulting proteomic profiles showed a high number of protein spots, which were well separated and with a good resolution.

Conclusions

The growth conditions on both the tested cell lines allowed us to obtain high levels of vesicles. The exosomes purification method turned out to be efficient and pure enough to perform a proteomic analysis. In fact, the proteomic maps showed a lot of spots suitable for a qualitative and quantitative analysis. After this preliminary proteomic analysis, the proteomic profiles of the vesicles already pointed out differences in expression between the two cell lines: in future we will perform the proteomic analysis in quadruplicate to find statistically significant differences. The 2DE of the samples is going to be performed by the use of an electrophoretic chamber which allows to run 12 gels simultaneously, so that the experimental variability will be reduced. The protein maps derived from the exosomes of the two cell lines will be compared and the differentially expressed proteins, whose changes will be statistically significant, will be processed and sent to the analysis in mass spectrometry (MS) for identification [7]. The results obtained with the proteomic analysis will be confirmed by western blotting method.

This first step of *in vitro* investigations will be followed by a second one, where we will search those proteins, found as differentially expressed in 2DE analysis of cultured cells, in the plasma of patients with breast cancer. This could allow the identification of cancer biomarkers.

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Dr. Navneet SAINI

CEM Curriculum: Health Sciences
Tutor: Prof. Paola Borella

THE EFFECT OF ENVIRONMENTAL FACTORS ON HUMAN HEALTH AND POSSIBLE PREVENTIVE MEASURES

Background

The environment can influence human health in important ways, and exposure to hazardous pollutants in the environment is also implicated in chronic human diseases. Association between environmental hazards and health outcome are, however, complex and not well defined. Therefore, there is an urgent need to evaluate possible links between environmental exposure and effects on health.

Objectives

In this first year of doctorate course I approached the following research themes: The objectives were 1) to promote physical activity as a basic measure able to reduce the risk of chronic degenerative diseases; 2) to compare traditional and molecular methods for detection and quantification of *Legionella spp* and 3) to measure the concentration of essential (Zn, Mn, Fe, Cu, Se) and toxic (Pb, Cd) elements in infant formula, to better understand the role of trace elements on infant health.

Methods

Study I: We organized a race of 4.5 km, to which 113 students of our University participated. Eight volunteers (4 active and 4 sedentary) were chosen, to establish their energy consumption and complete a detailed questionnaire on general characteristics, lifestyle, and sports activities. A latest-generation accelerometer was worn by participants in order to assess the time, energy expenditure (EE), the number of steps and intensity of physical activity (METS).

Study II: Sixty-five water samples were collected and simultaneously analyzed by culture method (ISO 11731:1998), qPCR by using a new *Legionella spp* Quantitative kit, and EMA-qPCR that selectively quantifies viable cells including non-culturable (VBNC).

Study III: Aliquots of 2 ml of infant formula were subjected to an acid mineralization controlled by microwave system, and, subsequently trace elements were measured by flame and graphite furnace atomic absorption spectroscopy.

Results

Study I: The questionnaires revealed that the sedentary subjects (activity <10 h/month) were a minority of the sample, those with regular physical activity (10-30 h/month) were prevalent, particularly among females, while a few were hyperactive (>30 h/month). Lifestyle factors that influenced the race participation were: low body mass index (BMI) ($p<0.01$), habitual physical activity ($p<0.05$), and no smoke (non-significant, $p<0.092$). Comparing the data obtained from the accelerometer, the time spent to run (25.2±1.2 vs 33.5±6.9 min) and the number of steps (3572±768 vs 4463±768) were lower in active subjects than in sedentary ones. The intensity of physical activity was higher in active participants (8.7 vs 7.0 ml O2/kg), whereas energy expenditure was equivalent (258±55 vs 256±26 kcal/day).

Study II: In this study, 33 out of 65 (50.8%) water samples were *Legionella spp* positive with culture. The EMA-qPCR was positive in 78.5% of samples and the qPCR in almost all samples (62/65; 95.4%). On the other hand, 32 samples negative by culture, 18 were positive with both molecular methods indicating the presence of potentially infectious VBNC cells, and 11 were positive only with qPCR, suggesting the presence of dead cells.

Study III: The concentration of all essential elements were within the limit set by the European legislation for infant formula. However, Fe, Zn and Mn were significantly higher than of breast milk, particularly in infant formula with specific clinical indications (Fe about 25 times and Mn 15-40 times). Toxic elements Pb and Cd, absent in maternal milk, were found in infant formula, albeit in low concentrations.

Conclusions

Study I: The portable accelerometer showed that sedentary subjects consume less energy than those trained, and they perceive less fatigue, demonstrating that they have addressed the performance with lower competitive spirit. Interestingly, the students had good lifestyles, as only a few were smokers, overweight, and not conducting regular physical activity.

Study II: In order to isolate *Legionella* from environmental samples, culture is the gold standard worldwide. A negative culture, however, can cause a false sense of security. In this context, we stress the usefulness of EMA-qPCR as a rapid tool for detection and monitoring contamination of viable *Legionella* in water systems.

Study III: The results of this study highlight two important aspects: the presence of toxic elements (Pb,Cd) in infant formula, albeit at low concentrations, and high levels of essential elements such as Fe, Zn and Mn added in milk for specific clinical indications. In our opinion, supported by other researchers, Mn has a documented neurotoxic effect at doses above 2 mg/day in infants. Our results raise the question about the need for fortification of these elements to such high concentrations, which contribute an unnecessary burden on metabolic and other physiologic functions of the infant.

Dr. Daniele SANTI

CEM Curriculum: Translational Medicine
Tutor: Prof. Manuela Simoni

CHRONIC, LONG TERM ADMINISTRATION OF VARDENAFIL IMPROVES ENDOTHELIAL FUNCTION AND CORRECTS HYPOGONADISM IN PATIENTS WITH TYPE 2 DIABETES MELLITUS. A LONGITUDINAL, PROSPECTIVE, RANDOMIZED, PLACEBO-CONTROLLED, DOUBLE BLIND, CLINICAL TRIAL

Background

Endothelial dysfunction leads to cardiovascular complications in type 2 diabetes mellitus (T2DM), through a reduction of nitric oxide (NO)-mediated relaxation. Phosphodiesterase-5 inhibitors (PDE5i) have hemodynamic effects, improving NO levels.

Objectives

To investigate if long term, chronic treatment with the PDE5i Vardenafil improves systemic endothelial function in men with T2DM.

Methods

A longitudinal, prospective, investigator-started, randomized, placebo-controlled, double-blind, clinical-trial was carried out. 54 male patients affected by T2DM diagnosed within the last 5 years were enrolled. 26 and 28 patients were assigned by permuted block randomization to the verum and placebo-group, respectively. The study consisted of an enrollment phase, a treatment phase (24 weeks) (Vardenafil/placebo 10mg twice-daily), and a follow-up phase (24 weeks). Parameters evaluated: International Index of Erectile Function (IIEF)-15, flow mediated dilation (FMD), intima media thickness (IMT), routine hematologic analyses. Serum testosterone (T) and its precursors were measured by liquid-chromatography tandem mass-spectrometry (LC-MS/MS). Gonadotropins were evaluated by ELISA.

Results

Only one serious adverse event was registered in the placebo group. The erectile function domain of IIEF-15 ($p=0.049$) improved after treatment. At the end of the treatment phase FMD significantly increased ($p=0.002$) while IMT ($p=0.003$), fibrinogen ($p=0.005$), white blood cells count ($p=0.018$) and Red cells Distribution Width ($p=0.028$) significantly decreased. FMD was significantly related to T serum levels ($p=0.002$), which significantly improved after Vardenafil treatment only in hypogonadal men ($T<10.4$ nmol/L) ($p=0.023$), without changes in gonadotropin serum levels. Smoking-habits, hypertension and glycemic control influenced the hemodynamic and inflammatory parameters.

Conclusions

This is the first double-blind, placebo-controlled clinical-trial in which T2DM men are chronically treated with Vardenafil for 6 months, and followed-up for 6 months after therapy-withdrawal. Chronically administered Vardenafil is safe and effective in T2DM patients and improves both tissue oxygenation and inflammatory markers, but this effect is lost after therapy withdrawal. For the first time, we demonstrate that chronic Vardenafil therapy improves T (measured by LC-MS/MS) in diabetic, hypogonadal men, an effect possibly due to improved microcirculation in the testis. [EudraCT number 2009-014137-25].

Dr. Davide SOLOPERTO

CEM Curriculum: Translational Medicine
Tutor: Prof. Livio Presutti

ENDOSCOPIC EXCLUSIVE TRANSCANAL APPROACH TO THE TYMPANIC CAVITY CHOLESTEATOMA IN PEDIATRIC PATIENTS

Background

Surgical management of pediatric cholesteatoma is currently very controversial. Traditionally, canal wall up (CWU) and canal wall down (CWD) techniques are the most used approaches and several studies have focused on the well-known advantages and disadvantages of both techniques, reporting the personal experiences of skilled surgeons. These concepts are, of course, based on microscopic surgical management. Endoscopic ear surgery, recently adopted and developed in our department, has brought new anatomic, physiologic and surgical concepts, and could also be performed successfully in children. The endoscopic approach gives some real advantages, such as ‘‘looking around the corner’’ vision, with the magnification of middle ear structures and is minimally invasive.

Objectives

The aim of the present study is to describe our experience in the management of tympanic cavity cholesteatoma in pediatric patients, treated with endoscopic exclusive transcanal approach.

Methods

A chart review of clinical data and videos from the operations of 54 pediatric patients, undergoing surgery between January 2007 and December 2013, was made. Patients presenting with cholesteatoma involving the tympanic cavity (mesotympanum, epitympanum, protympanum and/or hypotympanum), with no mastoid involvement, were included in the first group and underwent an exclusive transcanalar endoscopic approach (TEA). In case of mastoid extension of the pathology, patients were included in the control group and underwent a canal wall up microscopic technique (CWU).

Results

In this study, 34 males and 20 females, including 5 bilateral cases, giving a total of 59 ears, were reviewed. Median age was 9.6 years (range 4–16 years). 31 cholesteatomas underwent a TEA approach, while 28 underwent a CWU approach, based on inclusion criteria. No differences from congenital vs acquired form was made, due to the difficult to correctly distinguish always the two forms. Recurrence rate was 12.9% (4 ears) for the transcanal endoscopic approach group and 17.2% (5 ears) for the canal wall up microscopic approach. Residual disease was present in 26.6%: 19.3% (6 ears) for the transcanal endoscopic approach

group and 34.4% (10 ears) for the canal wall up microscopic approach. The mean follow up was 36 months (range 8–88). Kaplan–Meier analysis at 36 months showed a lower recurrence risk for the transcanal endoscopic approach compared with the canal wall up microscopic approach, but this data was not statistically significant.

Conclusions

The transcanal endoscopic approach represents a feasible, minimally invasive and conservative technique for the management of pediatric middle ear cholesteatoma. Certainly, a long-term follow up and more patients are necessary to definitively validate the procedure and related studies on QofL should be added.

Dr. Natalia Genowefa STASIAK

CEM Curriculum: Medicinal and Pharmaceutical Sciences
Tutor: Dr. Giuseppe Cannazza

DEVELOPMENT OF A METHOD FOR SIMULTANEOUS MEASUREMENT OF NEUROTRANSMITTERS AND NEUROMODULATORS IN RODENT BRAIN BY MICRODIALYSIS AND CHROMATOGRAPHY/TANDEM MASS SPECTROMETRY (LC-MS/MS)

Background

The study of brain metabolism and its disorders challenges clinicians and researchers as the current understanding of these processes is still limited. The focus on development of the method to comprehensively study levels of particular neurotransmitters and neuromodulators present in the specific brain areas, may be crucial to understand brain metabolism and how it can be manipulated. The data and tools provided by methods which are applied allow studying in deep and understanding not only the changes of brain metabolism in its basal state, but also modifications occurring after the administration of exogenous biologically active substances. Thus, the study of metabolic signatures in brain enables looking for and identification of novel biomarkers for neurodiseases, what makes the methods being significant for drug development and discovery.

Objectives

The aim is to develop a method which will allow simultaneous evaluation of neurotransmitters, like dopamine (DA), acetylcholine (Ach), 5-hydroxytryptamine (5-HT) or neuromodulators such as for example adenosine (ADE) in the rodent brain area of Nucleus Accumbens (nAc) and Hippocampus (Hip).

The first step of the study involves adjustment of microdialysis in order to collect samples with brain dialysate. Apart from choosing the best tubing, perfusate flow rate, etc. the adjustment includes collection of the microdialysate in the best possible conditions to avoid decomposition of the substances after leaving the brain area (e.g. absence of natural light, presence of ice around the vial of dialysate).

The second step is a development of a method for simultaneous detection and measurement of levels of bioamines, what still remains an analytical and technical challenge due to the low basal levels of the substances (fmol range) in the small sample size collected during microdialysis (10-20 µl). The method used to analyse microdialysates is liquid chromatography coupled with tandem mass spectrometry (LC/MS-MS). The equipment is proved to show a high sensitivity and quality in the evaluation of bioamines from brain dialysates.

Methods

Before the examination anesthetized mice (gaseous isoflurane) is placed in a Kopf stereotaxic apparatus and operated in order to insert Guide Cannula in two different brain areas: Nucleus Accumbens (nAc) and Hippocampus (Hip).

The first method applied is microdialysis – widely employed technique to monitor the chemical constituents of extracellular space of living tissue. It enables sampling and collecting small-weighing substances, in this case from specific brain areas. The principal element is a microdialysis probe which is inserted in the brain area from which a dialysate is to be collected. It is composed of a semipermeable dialysis membrane that permits a flow of solutes (material used, length and size of membrane pores depends on the area to which it is inserted and on analyte to be collected). Its permeability is limited typically to compounds with a size of less than 20.000 Da.

Once, the probe is inserted in the tissue and perfusate is administrated continuously by inlet tubing (in this case 1.2-1.5 µl/min), the dialysate with neurotransmitters and neuromodulators is flowing out by outlet tubing along their concentration gradient and reaches a vial, where it is collected. The collection of samples is every 20 min (20-30 µl) and then they undergo an LC/MS-MS analysis. What allows a simultaneous detection and measurement of neurotransmitters and neuromodulators of interest. At the end, data received from LC/MS-MS is statistically calculated, so as the real substance concentration is known. Apart from evaluation of the neurotransmitters and neuromodulators present in the brain in the basal state, microdialysis is conducted after administration of various substances, like nicotine, cocaine, etc. what allows a measurement of their effect on brain metabolism.

Results

Microdialysis and the LC–MS/MS method is being developed and applied to evaluate the basal levels and effects of acute systemic administration of cocaine and amphetamine on the release of biogenic amines such as DA and 5-HT as well as ACh and ADE in the mouse nucleus accumbens.

The administration of amphetamine and cocaine were followed by long-lasting, approximately 60-fold and 5-fold increase in DA release which reached a peak 40 min after the administration and slowly decreased until the end of experiment. 5-HT levels increased approximately 6-fold and 2-fold after administration of cocaine and amphetamine reaching a peak 40 min after drugs administration and slowly decreased until the end of experiment. ACh release remained constant over a 2h period in amphetamine and cocaine treated mice. ADE levels increased 4-fold in 40 minutes after amphetamine administration while it remained constant after cocaine administration.

Conclusions

Application of microdialysis technique in order to collect samples with neurotransmitters and neuromodulators from specific brain areas together with the following analysis by liquid chromatography coupled with tandem mass spectrometry (LC-MS-MS) may be successfully applied to evaluate the levels of bioamines and brain metabolism.

Dr. Sofia TADDEI

CEM Curriculum: Translational Medicine
Tutor: Prof. Leonardo Michele Fabbri
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DEVELOPMENT AND VALIDATION OF A COMPUTED ALGORITHM FOR AUTOMATIC RECOGNITION OF VELCRO CRACKLES IN PATIENTS WITH SUSPECTED ILD

Background

Idiopathic Pulmonary Fibrosis (IPF) is a rare but fatal disease of unknown cause causing a progressive scarring in the lungs. Diagnosis often represents a medical challenge, and it is not possible to know how fast the disease will progress, making its management an issue. Few therapeutic options are currently available, that are able to slow disease progression at best. Thus, there is a definite need for better tools for anticipating the diagnosis and improving the management of IPF.

The fibrotic process in the lungs of patients affected by IPF generates typical lung sounds (so called “velcro type" crackles), easily revealed by chest auscultation. Nevertheless, the accuracy of this finding for making early diagnosis and for inform the monitoring the disease progression has never been assessed. Our hypothesis is that lung sounds may reflect the presence and the severity of the scarring, and therefore the progression of the disease over time. Thus, we aim to assess the value of lung sounds, recorded by means of an electronic stethoscope, as a low-cost, reliable tool for monitoring the disease. In particular, in this first phase, we would like to develop and validate a computed algorithm for automatic recognition of velcro crackles in patients with suspected ILD.

Objectives

Primary objective of this study is to develop and validate a computed algorithm for automatic recognition of velcro crackles in patients with suspected ILD and then compare the sensitivity and the specificity of automatic algorithm results and those of traditional auscultation of the chest.

Secondary objectives are at first to assess the feasibility of electronic recording of lung sounds in patients who undergo high resolution computed tomography (HRCT) of the chest for suspected ILD, then to create predictive clinical risk algorithm, comprehensive of demographics, medical history data (smoking, exposures, drugs) and electronic recordings of lung sounds, that could address patients to a earlier chest HRCT scan.

Methods

Partcipants: Center for Rare Diseases of the Lung, Clinic of Respiratory Diseases (University Hospital Policlinico of Modena), Department of Science and Methods of Engeneering (University of Modena and

Reggio Emilia), Service of Radiology (University Hospital Policlinico of Modena) and Service of Radiology (NOCSAE of Baggiovara, Modena).

We first analyzed lung sounds of ten healthy volunteers, recorded by electronic stethoscope Littmann 3200^{TM} (3M, USA), and those of ten patients with diagnosis of idiopathic pulmonary fibrosis (according to current guidelines). Then sounds were processed and compared (by a chest physician), identifying a frequency of normal breath sounds and the one of pathological "velcro crackles". In the healthy control's spectrum almost all power spectral density is concentrated between 500-700 Hz. In the IPF patient's spectrum the intensity of sound at frequencies between 600 and 800 Hz is significantly more represented. These values would be used as references for the next step. In the next step patients undergoing chest HRCT for any indication at the University Hospital Policlinico of Modena and NOCSAE of Baggiovara are enrolled. We collect data into a web-based database, using a questionnaire (a modified version of the questionnaire ILD IQ 8.1 in use at the University of California, San Francisco - UCSF Medical Center - courtesy of dr. Harold Collard, Director of Interstitial Lung Disease Program) in which we report patient medical history data (in particular history of smoke, environmental, working and drugs exposures); respiratory signs and symptoms and physical examination. Chest auscultation is performed with a mechanical stethoscope followed by registration of lung sounds with electronic stethoscope, in six spots of auscultation on the chest, three for hemithorax. In correspondence of sites of registration we apply radiopaque metal markers. Then recorded files are transferred from electronic stethoscope to PC via a bluetooth wireless connection and extracted in extensive way, using the Software Zargis® StethAssist ™ Heart and Lung Sound Visualization. Automatic recognition of lung sounds provides at first artefact elimination, then recognition of inspiratory phase (the power associated with the sound signal and its harmonic content are higher during inspiration). After this the extraction of the frequency band corresponding to the sound is made and then we compare the value of this analysed band and the one of normal breath sounds (500-700 Hz) and finally the recognition of pathological "velcro crackles" sound for every patient (in this first phase we analysed all the six registrations for patients together). The results of both lung sounds analysis (with mechanical and electronic stethoscope) are compared with radiological pattern (actual gold standard for diagnosis of fibrotic interstitial lung diseases) of each patient. Analysis of radiological pattern is performed by radiologists and pulmonologists with expertise in rare diseases of the lung. The statistical assessment provides sample analysis with descriptive statistics in terms of averages and percentage frequencies and the analysis of algorithm success rates, its sensitivity and specificity in recognizing pathological pattern. These result s are compared with those of traditional auscultation.

Results

We have actually enrolled 212 patients, who underwent to a high-resolution CT scan of the chest , were enrolled, 52% male and 48% female with a mean age of 66 years, 19% active smokers, 45% former, 11%

passive, 25% no smokers. 45% had exposure to environmental factors implicated in the pathogenesis of some interstitial lung diseases, 14.7% to occupational pollutants, 11.8% to potentially pneumotoxic drugs. Radiological pattern was consistent with diffuse infiltrative pulmonary diseases in 31% of sample, 71.2% of these typical for usual interstitial pneumonia. Results of pulmonary auscultation are that computed algorithm has a sensitivity not inferior than an expert physician in recognizing pathological velcro crackles , while it is less specific.

Conclusions

Computed algorithm recognizes a higher number of false positives, probably because it is not selective in detecting pathological lung sounds or artefacts at the same frequency of crackles (600-800 Hz). False negatives, instead, similar between auscultation with mechanical stethoscope and automated analysis of sounds, fundamentally depend on some characteristics both of the patient and of the electronic stethoscope. In order to avoid these limits the sound engineers are still working to create a supervised algorithm by neural networks, which can report only velcro crackles, identifies lung sounds even finer that ear does not distinguish (very early stages of the disease), and which can also pointing, during registration, the number of breaths needed to analyse the data.

We are also trying to identify if there is any correspondence between each of the six lung sounds recorded in the landmarks identified by metal markers and single image HRCT for each patient.

Work in progress: enrollment is still ongoing in order to assess the feasibility of electronic recording of lung sounds in patients who undergo HRCT of the chest for suspected ILD, and to create a predictive clinical risk algorithm, comprehensive of demographics, medical history data (smoking, exposures, drugs) and electronic recordings of lung sounds, to have an INDEX of probability of fibrotic ILD, that could address patients to an earlier chest HRCT scan.

Dr. Roberta VALSECCHI

CEM Curriculum: Translational Medicine
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ROLE AND REGULATION OF HIF-1α TRANSCRIPTION FACTOR IN CHRONIC LYMPHOCITYC LEUKEMIA

Background

Hypoxia-inducible transcription factors (HIFs) regulate a wide array of adaptive responses to hypoxia that are often activated in cancer. HIF transcription factors consist of α and β subunits, both belonging to the basic helix-loop-helix family. While β subunit is constitutively expressed, HIF-1α is remarkably high during hypoxia whereas it is maintained at low levels under normoxic condition because this subunit is degraded via pVHL-mediated degradation. In hypoxic conditions HIF-1α is stabilized, moves in the nucleus, heterodimerizes with HIF-1β and forms an active transcription factor recognizing specific "Hypoxia Response Elements" in the promoter of several genes that mediate tissue and cellular adaptation to hypoxia. HIF-1α has emerged as a critical player in the pathogenesis of solid tumours and recently is beginning to be appreciated as an important regulator of haematological malignancies such as Chronic Lymphocytic Leukemia (CLL). CLL, the most common leukemia in adults, is a clinically and biologically heterogeneous disease characterized by the accumulation of mature $CD5^+$ B cells both in peripheral blood (PB), in the bone marrow (BM) and secondary lymphoid organs. It has been recently demonstrated that HIF-1α is aberrantly expressed in neoplastic cells from CLL patients, due to normoxic miRNA-directed downregulation of pVHL. Abnormal upregulation of HIF-1α was suggested to lead to high VEGF production, thus causing the increased BM and lymphoid tissues neoangiogenesis observed in CLL patients.

Objectives

Because preliminary data of gene expression profile revealed that in CLL cells HIF-1α regulates the expression of a number of chemokine receptors and cell adhesion molecules known to promote the interaction of leukemic B cells with protective stromal niches in bone marrow and spleen. We hypothesize that HIF-1α factor carry out other important functions in the pathogenesis of CLL, in particular it may regulate homing and adhesion of CLL cells to bone marrow niches, where it is known that CLL cells acquire anti-apoptotic properties and resistance to chemotherapy.

Methods

The role of HIF-1α factor is investigated by using *in vitro* migration and adhesion assays in MEC-1 and patients' cell upon silencing by using different strategies either by shRNA-mediated interference or,

oligonucleotides or pharmacological inhibition (using EZN-2208, a compound with HIF-1a inhibitory activity) and in vivo by using mouse models for CLL.

Results

We found that inactivation of HIF-1α impairs cell adhesion to stroma and chemotaxis *in vitro*, and reduces bone marrow and spleen colonization *in vivo* in xenograft and allograft CLL mouse models, thus resulting in prolonged mice survival. We also found that in CLL cells HIF-1α is itself transcriptionally regulated upon co-cultures of CLL cells with stromal cells. Finally, mRNA levels of HIF-1α vary significantly in CLL patients, and correlate with important prognostic markers such as CD38 e ZAP70, this suggesting that HIF-1α could be a new therapeutic target for CLL treatment.

Conclusions

Taken together these results suggest that HIF-1α is a key element in governing CLL cell survival and resistance to chemotherapy by regulating and being regulated by the interaction of CLL cells with protective microenvironments.

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Dr. Federico ANTENORA

CEM Curriculum: Translational Medicine
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INCIDENCE AND PROGNOSTIC VALUE OF DIAPHRAGM DYSFUNCTION EVALUATED WITH TRANS-THORACIC ULTRASOUND IN ACUTE RESPIRATORY FAILURE SECONDARY TO ACUTE EXACERBATION OF CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Background

Diaphragm dysfunction (DD) in critically ill patients who received mechanical ventilation patients has been the focus of many recent studies and was found to be related not only with an higher rate of weaning failure and length of mechanical ventilation, but also with sepsis, disease severity and ICU mortality.

COPD patients with acute hypercapnic respiratory failure have decreased diaphragm mobility due to the presence of dynamic hyperinflation when compared with healthy, age matched individuals.

There are no large studies evaluating incidence of diaphragm dysfunction in AECOPD patients with acute respiratory failure and its role in NIV failure and prognosis.

Ultrasonographic evaluation of the diaphragm with measurement of diaphragm thickening (DTdi) was used to determinate the presence of diaphragm dysfunction in invasively ventilated patients and was found to be related with weaning failure.

Diaphragm ultrasound with determination of DTdi could be a useful tool for the diagnosis of DD in AECOPD patients and to predict NIV failure.

Dtdi is calculated as the ratio between the maximum thickness, in inspiratory, and the minimum thickness detected in expiratory.

Objectives

Primary outcome is evaluate DD incidence in AECOPD patients admitted in Respiratory Intensive Care Unit for acute hypercapnic respiratory failure

Secondary objectives are:

-evaluate differences in incidence of DD between AECOPD patients with acute respiratory failure successfully treated with NIV and patients who underwent NIV failure.

-evaluate impact of DD on mortality and ICU-length of stay in AECOPD patient admitted for acute respiratory failure.

Methods

This prospective monocentric cohort study aims to enroll150 consecutive patients with COPD and acute hypercapnic respiratory failure. Arterial blood gas, respiratory and cardiac rate will be recorded before starting NIV and 2 hours later. Lactate dosage will be performed at admission and repeated if needed. A chest radiogram and blood sampling including haemachrome assay with WBC count and dosage of CRP and procalcitonin. APACHE II and SAPS II scores will be performed at admission, such as GCS , P/F ratio will be recorded at admission and 2 hours after NIV starting. Comorbidities (diabetes mellitus and kidney failure in particular) and presence of concomitant pneumonia and/or sepsis will be recorded.

An operator will perform a diaphragmatic ultrasound with measurement of at admission, at discharge and in case of late failure of NIV (at least 48 hours after NIV starting).

Medical treatment will be started according to the normal standard of care.

After 12 and 24 hours arterial blood gas will be repeated in accordance with hospital procedures.

Expected Results

We expect to observe a correlation between diaphragm dysfunction diagnosed with diaphragm ultrasound and NIV failure in patients treated with NIV for AECOPD . We also expect to notice an increase in mortality and ICU length of stay in patients with diaphragm dysfunction compared with patients with normal diaphragm motility.

Conclusions

Diaphragm ultrasound is a completely non-invasive technique, available in spontaneously breathing patients, which recent studies have found to be reliable and comparable to other invasive measures in detecting DD. DD is emerging as an independent negative prognostic factor for ICU patients receiving mechanical ventilation, increasing mortality and length of mechanical ventilation. This finding suggests that DD might be a strong predictor for NIV failure in a group of patients usually responsive to NIV, influencing prognosis and clinical management of these patients.There is no literature available on DD in non-invasively ventilated COPD patients. Proving that diaphragm ultrasound is effective in this setting could provide a non-invasive, safe and reproducible tool to stratify prognosis and guide treatment, avoiding delayed intubation and providing appropriate monitoring.

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RISK FACTORS ASSOCIATED TO ADVERSE PREGNANCY OUTCOME IN DIABETES MELLITUS: A RETROSPECTIVE COHORT STUDY IN TWO PROVINCES OF THE EMILIA ROMAGNA REGION

Background

Epidemiologic studies have indicated that pregnancy in women with diabetes is associated with increased risk of neonatal and maternal complications[1]. The most common fetal adverse outcomes investigated in pregnancies of women with diabetes are congenital abnormalities and malformations, premature delivery, miscarriage and macrosomia, and maternal complications are pregnancy induced hypertension, pre-eclampsia, hemolysis, Cesarean section, hypoglycemia and the worsening of any degree of a pre-existing renal insufficiency and retinopathy[2]. It turned out that most of these outcomes increase in diabetic pregnancies, though some inconsistencies between epidemiologic studies make their relation with pregestational diabetes not entirely clear. For example, a recent population-based cohort study carried out in the Emilia Romagna region by Vinceti et al[3] found an excess prevalence of birth defects associated with pregestational type 2 diabetes, confirming previous observations[4], but no excess teratogenic risk emerged in type-1 diabetic women. Most studies have been based on administrative data, which lacked important clinical information, such as metabolic status, concomitant obesity, folic acid consumption and comorbidity.

Objectives

The primary objective of my study is to assess the risk factors that mainly influence adverse pregnancy outcomes (birth defects, macrosomia, miscarriage and maternal complications) in women affected by pregestational diabetes. The secondary objective is to assess changes in risk of adverse pregnancy outcomes among diabetic pregnancies in the 1997 – 2014 period.

Methods

Study population

We will carry out a retrospective cohort study to identify all pregnant women with pregestational diabetes residing and delivering in the province of Modena and Reggio Emilia in the period 1997 – 2014. To define this cohort we plan to use two methods:

1. Cohort 1997 – 2009: this cohort is composed by all deliveries recorded in the National Health Service database, identified through the analysis of the Hospital Discharge (HD) record carrying a diagnostic code of pregestational diabetes (ICD IX 250.0 and 648.0). These HD records are matched

with the Birth Certificates Archive (CEDAP) through a joint code, thus allowing to obtain clinical data for both newborn and mother (gestational age, nationality, education, 5 year-pre pregnancy smoking habits, mode of delivery.

Limitation of this method:

Hospital discharge record could be an inadequate methodology to ascertain cases of chronic disease, in particular when – as in the present study - we want to distinguish between gestational and pregestational diabetes. Therefore cases of pregestational diabetes identified through HD must be validated using a diabetes register (implemented in Modena and Reggio from 2010) and contacting the general practitioners (GP) contacted by e-mail or phone.

2. Cohort 2010 – 2014: : this cohort will be created using as only source of data the Diabetes Register (which is created by linkage of six routinely collected data sources using an algorithm able to ascertain cases or through clinical diagnosis in diabetologists or GP medical records). Only women aged less than 45 years will be selected from register. Cases selected from register will be matched with Birth Certificates Archive (CEDAP) using a joint code.

Newborn and maternal adverse outcomes

We plan to investigate birth defects, macrosomia (defined as a birth weight above 4 kg and/or >90th percentile weight for gestational age or large for gestational age), premature delivery (delivery occurring before 37 weeks' gestation), miscarriage, caesarian section delivery, hypoglycemia during pregnancy and blood pressure alterations (hypertension, eclampsia).

Risk factors to be taken into account are smoke habits, nationality, mother's age, type of diabetes, blood glucose control before and during pregnancy, Body Mass Index before pregnancy and weight increasing during pregnancy, folic acid prescription (including dosage), therapies for diabetes and concomitant diseases. Hospital Discharge records, Birth Certificates Archive (CEDAP), Registry of Birth Defects (IMER) and Diabetes register are the sources of data. We plan to compare pregnancy with and without adverse outcomes, and to analyze which risk factors are mainly associated to the outcome.

Expected Results

Through diabetes register we will be able to take into account several risk factors associated with adverse pregnancy outcomes in women with diabetes mellitus. We would like to create a multivariate model to assess the weight of each potential risk factor in influencing the outcome.

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CEM Curriculum: Medicinal and Pharmaceutical Sciences
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NEW METHODS FOR THE STUDY AND CHARACTERIZATION OF NATURAL PRODUCTS AS SOURCES OF BIOACTIVE COMPOUNDS

Background

Natural products are wide spread throughout the world and herbal supplement companies have expanded considerably into the market [1]. This increasing interest has been triggered by more and more scientific results, which demonstrate that these products are effective for the prevention of several diseases and for the maintenance of health conditions [1,2]. This has resulted in the wide use of natural products as supplements or substitutes to conventional drugs in the developed countries [2]. However, natural products are often categorized as "dietary supplements" and, therefore, not regulated as drugs; thus, the main concern consists in their efficacy, safety and quality [3]. In addition, plant-based products continue to play an essential role for human health in the developing countries [4]: in this ambit, the World Health Organization (WHO) has reported that natural products represent the primary medicinal source for 60% of the world population [5].

Since plant extracts are composed of a complex mixture of different compounds, called secondary metabolites, that often work "synergistically" or can have different biological effects, it is highly recommended to define and measure all the phytochemical constituents of natural products to ensure the reliability, repeatability and safety in their use and enhance product quality control. In this context, the development of advanced analytical methods for the quali- and quantitative analysis of secondary metabolites in natural products is extremely important [3]. To achieve these goals, several methodologies are available in phytochemical analysis: the *metabolite profiling*, based on the study of chemically related compounds or secondary metabolites involved in specific biosynthetic pathways, and the *metabolite fingerprinting*, based on the full characterization of samples by complete analysis of its secondary metabolites, are highly recommended [4].

Moreover, as potential sources of new bioactive molecules, medicinal plants provide also a large number of compounds that can be screened to find new potential leads for drug discovery [4]. In fact, the pharmaceutical history contains several examples of drugs developed starting from medicinal plants [4,5]. In this perspective, the isolation and characterization of new bioactive natural compounds from plant material is highly important.

Objectives

In the light of all above, my PhD project is focused on the development of highly efficient and innovative analytical techniques for the comprehensive multi-component analysis of bioactive secondary metabolites in different natural sources, as well as the validation of the developed methods to show compliance with international requirements for analytical procedures for the quality control of pharmaceuticals (ICH guidelines). A purpose of my PhD project is also the isolation and characterization of new bioactive natural compounds and the biological activity evaluation of crude extracts, fractions and purified components, with particular attention to the antioxidant, antibacterial, antifungal, antiparasitic and antiproliferative activities.

Methods

The metabolite profiling/fingerprinting of natural products can be performed with both separation and non-separation techniques. The first ones include high-performance liquid chromatography (HPLC) and gas chromatography (GC), both hyphenated with mass spectrometry (MS), and require a suitable procedure for the sample preparation. HPLC is the most frequently used technique for both quali- and quantitative analysis of natural products with different detection methods. GC is widely applied for the analysis of complex mixtures of volatile organic compounds produced as secondary metabolites in plants, such as essential oils. As regards the sample preparation, the use of innovative extraction techniques, based on the application of ultrasound (UAE), microwave (MAE), solid-phase extraction (SPE) or solid-phase microextraction (SPME), is highly recommended.

The study of the metabolomic profiles of natural products can also be achieved with non-separative and non-destructive techniques, such as by means of nuclear magnetic resonance (NMR).

Given the complexity and the quantity of the data obtained with the above mentioned methodologies, the use of chemometric tools is usually recommended in order to extract and display the information and use it for pattern classification, regression and calibration. Statistical techniques are also very useful in the development, optimization and validation of new analytical methods.

As regards the isolation and characterization of new bioactive natural compounds from plant material, this is usually performed by means of bio-assay guided fractionation. In view of the biological activity data, the fractionation of the most active extracts is carried out, followed by the final purification of secondary metabolites by means of preparative column chromatographic processes. The structural characterization of newly isolated molecules is done by spectroscopic (UV, IR and NMR) and spectrometric (MS) techniques.

The evaluation of the antioxidant, anti-infectious and antiproliferative activity of crude extracts, fractions and purified compounds can be performed by means of an array of specific *in vitro* assays.

Results

In the last few months, my research has been focused on *Punica granatum* L., commonly known as pomegranate, which has recently gained an increasing interest, due to the health-promoting properties (antioxidant, antibacterial, antiproliferative [6,7,8]) of its fruit extracts. This great variety of positive effects on human health is most likely due to its polyphenolic constituents, mainly hydrolysable tannins and anthocyanins [7].

In particular, my initial work was focused on the validation of a method for the quantification of the main bio-active compounds in several pomegranate cultivars of different origin, in order to select those that could be applied for the preparation of extracts with nutraceutical and pharmaceutical application. To do this, a RP-HPLC method coupled with UV/DAD and ESI-MS^n detection, using a fused-core stationary phase, previously developed in our laboratory, was applied for the metabolite fingerprinting of all pomegranate fruit components in a single chromatographic run. The method was completely validated for linearity, sensitivity, accuracy and precision, according to ICH guidelines. Finally, the validated method was applied to 17 pomegranate cultivars for the quantification of their polyphenols. While pomegranate arils were directly analyzed after squeezing, mesocarp and exocarp samples were extracted by maceration. The quantitative data obtained by HPLC will be further processed by suitable statistical tools (principal component analysis, PCA). Another goal of this study will be the evaluation of the biological activity of the extracts obtained from these fruits, in order to select the most promising sources of polyphenols within the pomegranate cultivars analysed in this study.

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CEM Curriculum: *Translational Medicine*
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IMMUNOLOGICAL PREDICTORS OF SUCCESSFUL AND SAFE ANTI-REJECTION THERAPY

Background

The Liver and Multivisceral Transplant Centre of the Policlinico Modena Hospital started a solid organ transplant (SOT) program for HIV infected patients in 2001 and 41 HIV patients have been transplanted so far. In particular, 36 HIV infected patients underwent a liver transplant and 5 had a combined kidney-liver transplant. 16 HIV transplanted patients out of 41 have died so far.

We know that the risk for infection in the transplant recipient is a function of the interplay among host susceptibility, exposure to an opportunistic microorganism, and the inherent virulence of a given microorganism.

Following SOT, immune responses of the recipient are profoundly altered. Several factors as immunosuppressive agents, donor-recipient mismatch, graft rejection, viral re-activation, transplantation and surgical trauma influence this immunodeficiency.

Immunosuppressive therapy causes a marked suppression of cell-mediated immune responses. The depth of the deficiency of cell-mediated responses is influenced by the number and the type of immunosuppressive agents used, the dose of corticosteroids and other immunosuppressive agents, the use or absence of antithymocyte globulin, and the degree of mismatch between donor and recipient. Moreover, the occurrence of rejection episodes and the need to intensify immunosuppression augment the immunodeficiency[1].

Humoral immunity is also frequently impaired in the post-transplantation setting because of the use of corticosteroids and the deficiency in the cell-mediated responses that are necessary for T-cell dependent humoral responses[1].

Changes in host defences take place over time. Following SOT, the changes in the perisurgical period that are most prominent are those related to the surgery. The surgical incision disrupts the integumentary barrier, rendering the patient susceptible to wound infections. Following the acute postoperative period, integumentary and mucosal barrier deficits become less problematic as the need for nosocomial invasive procedures is reduced and the use of perioperative antibiotics decreases. Increasingly prominent is the suppression of humoral and cell-mediated immunity from the immunosuppressive regimen. Thus, viral and fungal infections are increasingly problematic. Acute rejection episodes are most common during the first 3 months after transplantation. The increased intensity of immunosuppression that is given during a rejection

episode renders the patient more vulnerable to an infectious episode. Invasive fungal infections, CMV infections, and other viral infections are prominent infectious complications during this interval[1].

In HIV infected patients that undergo a SOT, physicians have to consider HIV infection as another important player in patient net state of immunosuppression and as an infection risk factor.

Nowadays, despite the impressive improvement of patient and graft survival, and despite the fact that the life-long, unavoidable anti-rejection therapies are becoming more specific and personalized, infections still remain the leading cause of morbidity and mortality among SOT recipients[2-5]. Thus, it is essential to understand which are the impairments in humoral and cell-mediated immunity after transplant, when such impairments are detectable, and which kind of immunity alterations can lead to a real and clinically significant increase in the risk of infections.

An immunological approach could help clinicians to classify transplanted patients in different infection risk categories, helping in differentiating prophylaxes and therapies on the basis of their infection risk.

Objectives

A successful anti-rejection treatment has, on one side, to block the immune response against the SOT, and, on the other, to allow an efficient immune response against pathogens. Thus, the main aim of my PhD program is to identify which characteristics of the specific immune response can predict the clinical success of such therapy. For this reason, in transplanted HIV-positive patients, transplanted HIV-negative patients, non-transplanted HIV-positive patients and controls (HIV-negative, non-transplanted patients), my PhD project will focus on:

- Comparison of the T cell response to a number of relevant and recall antigens of viral, bacterial or fungal origin (such as CMV, smallpox, *Candida* spp., tetanus toxoid), in terms of the identification of the quantity and of the quality (i.e., number of functions simultaneously performed by a single cell) of the specific response;
- Analysis of molecular and cellular parameters related to the activation or inhibition of either the innate or adaptive response, paying a particular attention for the mechanisms involved in the triggering of inflammation and in the activity of the inflammasome system.

The secondary aim of the project is to find an immunological predictor of vulnerability to infection, that has a large applicability and is able to help clinicians in stratifying the infection risk of transplanted patients, either HIV-positive or negative.

Methods

Case-control single-centre study.

I plan to investigate the aforementioned immune parameters in all alive HIV-positive patients who have received a SOT at the Liver and Multivisceral Transplant Centre (Policlinico Hospital, Modena) from 2001 to 2014.

I will evaluate the same T-cell responses in other three groups (transplanted HIV-negative patients, non-transplanted HIV-positive patients, and HIV-negative, non-transplanted controls) and we will compare the eventual differences among the groups.

Patients will be matched for age, sex, MELD, year of transplant, immunosuppressive regimen, absolute CD4 cell number, and antiretroviral treatment.

All of the immunological studies will be performed in collaboration with the Chair of Pathology and Immunology (Prof. Andrea Cossarizza), where the most sophisticated molecular and cellular technologies (including cell sorting, high-speed acoustic cytometry, digital and real time PCR, etc.) are routinely used and available for this project.

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Dr. Lavinia GIVA

CEM Curriculum: Translational Medicine
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TARGETING INFLAMMATION IN ATHEROSCLEROSIS: ROLE AND THERAPEUTIC POTENTIAL OF SPHINGOSINE-1-PHOSPHATE (S1P) AND ITS RECEPTORS

Background

Sphingosine-1-phosphate (S1P) is a lysosphingolipid which regulates many important biological functions through the interaction with its specific sphingosine 1-phosphate receptors (S1PRs). The S1PR family is composed of five isoforms (S1P1-5) belonging to the G-protein coupled receptor superfamily which is involved in many intracellular signal transduction pathways, such as cellular proliferation, survival, migration, differentiation, control of immune cell trafficking, angiogenesis and vascular integrity. So, it is not surprising that S1P affects the immune system, the central nervous system and the cardiovascular system; therefore this lysosphingolipid is implicated in a huge range of diseases, including atherosclerosis, diabetes, cancer and inflammatory disorders.

It has been demonstrated that, in plasma, S1P is associated with the high density lipoproteins (HDL). Also, several epidemiological studies pointed out to HDL as the most potent plasma-born atheroprotective factor and they documented an inverse relationship between HDL cholesterol levels and the extent of atherosclerotic disease; these evidences suggested that HDL atheroprotective effects may be in part attributed to S1P through the stimulation of its receptors, mainly type 1 and/or 3 (S1P1, S1P3), on atherosclerosis-relevant cells like macrophages or endothelium. Nevertheless S1P anti-atherogenic effects derived primarily from experiments in vitro or in vivo utilizing synthetic S1P analogues with poorly defined side effects, further investigations are necessary to demonstrate the protective effects of endogenous S1P and to explore underlying mechanisms in vivo.

Animal models designed to amplify S1P signaling may help to show protective effects of endogenous S1P against atherosclerosis. To this purpose, our research group recently generated mice, basing on the Cre-LoxP technology, able to overexpress S1PR1 or S1PR3 in specific target tissues (S1P1-Lyz and S1P3-Lyz mouse models).

Objectives

The several S1P tasks in the immune and cardiovascular system may be relevant to atheroprotection, but the S1P accurate molecular mechanisms are still unknown. In order to illustrate that endogenous S1P plays a key role in the modulation of inflammatory responses associated to the atherogenesis, we planned to investigate the S1P effects on the development of atherosclerosis in mice overexpressing S1P1 or S1P3

receptors, in particular in primary cells involved in the atherogenic processes such as macrophages, endothelial cells (EC) and vascular smooth muscle cells (VSMC). The tissue-specific approach designed in this project selectively targets S1P pathways, gaining insights into the underlying pathophysiological mechanisms. These worldwide unique mouse models will be exploited to investigate the effects of endogenous S1P on:

1. the development of atherosclerosis and the associated inflammatory responses
2. the cellular and systemic lipid homeostasis, with particular attention to lipid efflux and reverse cholesterol transport (RCT)
3. the signaling downstream of S1P-receptors (S1PRs), important to define pharmacological targeting

Finally, by clarifying the role of S1P in the atherogenesis, our findings could get the potential to formulate new strategies for therapeutical intervention in the diseases linked to atherosclerotic background such as coronary heart diseases and stroke.

Methods

For the molecular and functional characterization we collect the peritoneal macrophages from mice (MPM) that are previously injected with thioglycolate to induce a mild local inflammatory response. Macrophages are cultured in multiwells plates under appropriate conditions, according to the protocol guidelines. After that, nucleic acids and proteins are extracted and quantified for downstream applications, such as Real Time PCR, Western Blot and Immunofluorescence in order to detect receptors, cytokines or cholesterol transporters.

Since the S1P1-Lyz and S1P3-Lyz mouse models are on C57BL6 background which is resistant to atherosclerosis development, we are currently crossbreeding S1P1-Lyz and S1P3-Lyz mice with LDLR-/- mice to obtain athero-prone strains. Atherosclerotic lesions are induced by feeding mice with Western diet (0.5% cholesterol, 16.5% fat; w/w) for 12-20 weeks. We aim to evaluate the extension of the atherosclerotic plaques to verify if the amplification of S1P signaling, due to the overexpression of S1P receptors, may modulate the atherogenic processes.

Expected Results

Worldwide unique mouse models were obtained in our laboratory. These models, based on the Cre-Lox technology, permit the tissue-specific overexpression of S1P1 or S1P3 receptors causing the amplification of endogenous S1P signaling. Mouse models were fully validated for tissue specific S1PRs overexpression at gene, protein and functional level by performing quantitative Real Time PCR, Western Blot and intracellular cAMP or Ca++ handling assays. Furthermore, S1P analogues, characterized by a selective agonist activity towards S1P1, execute potent anti-inflammatory effects in macrophages both in vitro and in vivo and reduce the development of murine atherosclerosis.

In case of successful completion the project I will:

1. establish beyond reasonable doubt S1P as an anti-atherogenic constituent of plasma and HDL
2. identify S1P receptor responsible for anti-atherogenic effects and thereby carry out new target for pharmacological therapies of atherosclerosis
3. discover key cell targets of atheroprotective effects of S1P and allow discriminating between mechanisms underlying these effects (anti-inflammatory effects, effects on cholesterol efflux and transport).

We hope that the project outcomes will open new ways of pharmacological research in order to establish S1P mimetics as beneficial therapy for atherosclerotic vascular diseases.

Dr. Maurizio GRECO

CEM Curriculum: Translational Medicine
Tutor: Prof. Giovanni Pellacani

THE DIFFERENT WAYS SKIN REPAIR DUE TO DIFFERENT SOURCES OF DAMAGE: MORPHOLOGICAL STUDY IN VIVO AND BIOLOGICAL CHARACTERIZATION OF TISSUE

Background

The understanding of the mechanisms of laser tissue interaction is still not completely clarified. The majority of the data available in literature regard the histopathological analysis of the skin following laser treatment. To obtain cellular details of the changes occurring during laser therapy, several skin biopsies were performed, often on the face or other sensitive areas. The need to take invasive biopsies limited the study of laser effects in complex tissues. Moreover, histopathology exam provides only a "static one point-in-time picture" of the tissue while leading to the inevitable scar formation.

In general, lasers are considered safe interventions with an associated rapid healing time. Post-treatment side events may occurs, but they are usually limited to pigmentation disorders. However, at my best knowledge, there are not consistent data concerning long term effects of lasers, in particular on their potential cancerogenic effects.

Objectives

The main purpose of my research is to explore laser effects in different skin conditions and to study the biological effects on different tissues in order to evaluate potential cancerogenic effects of different wavelenghts and energies, and to discover the optimal light source to target a specific tissue or a disease.

Methods

My research interest is in the application of confocal microscopy to in vivo assess the laser-tissue interactions and the biologic effects of different laser sources.

It is my intention to investigate the biologic effects of distinct laser sources (CO2, fraxel CO2, NdYag 532/1064, CW 532/1064,) on the skin tissues and in the treatment of different pathologies (Epithelioma, melasma, sarcoidosis, ect.). Since different laser sources may differently interact with different tissues, my research will investigated the effects on the different cell component in ex-vivo cell cultures and in parallel in in-vivo settings.

The first disease studied was the BCC (basal cell carcinoma) treated with CO2 laser and PDT.

Two patients with a nodular basal cell carcinoma and two patients with an infiltrative basal cell carcinoma localized on the face, histologically proven, were treated with combined modality.

We performed a long-term follow-up using clinical assessment, clinical pictures, digital dermatoscope examination and reflectance confocal microscopy.

Results

The results of this study will impact the current applications of laser in inflammatory and tumoral skin diseases and the management of these entities that cannot be treated efficaciously with other treatments. Moreover, a profile of safety and efficacy could be outlined basing on different laser and tissue characteristics. I hope that this research will improve the knowledge on this topic and will help to offer better, less invasive and more safe treatments to the patients. Furthermore, a more in depth knowledge of in-vivo and ex-vivo effects of laser on different tissues may open new applications of laser technology in Medicine.

Preliminary results obtained in the treatment of BCC have shown that the mean age was 48 years with a range of 32 to 80. Follow-up period after 3 years is still ongoing. Recurrence was noted in one patient with infiltrative basal cell carcinoma at 6 months follow-up and repeated combined modality was performed obtaining a complete response. No significant complications were observed.

Conclusions

Combined modality demonstrated a good efficacy and better cosmetic outcome than surgery in the treatment of nodular and infiltrative basal cell carcinomas.

Dr. Eleonora MARETTI

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LIPID- AND POLYSACCHARIDE-BASED MICROCARRIERS FOR TUBERCULOSIS INHALED THERAPY BY DPI DEVICE

Background

Tuberculosis (TB) disease is caused by *Mycobacterium tuberculosis* that survives and replicates within human alveolar macrophages. It is characterized by a long chronic stage of infection and progressive pathology that mainly compromises (90% of cases) the respiratory system. More than nine million people still develop active TB each year and nearly two million die. TB occurs all over the world with the highest burden in Asia and Africa. Since 1995, over 51 million people have been treated and an estimated twenty million lives saved through use of Directly Observed Treatment, Short (DOTS) course. The recommended TB chemotherapy includes a multi-drug regimen with four first-line drugs (isoniazid, rifampicin, ethambutol and pyrazinamide) administered for two months and a subsequent phase of four months with rifampicin and isoniazid. However, poor adherence to administration schedules, several side effects and multi-drug resistant TB infections are chiefly responsible for chemotherapy failure. Current TB therapies have exploited conventional routes of administration, such as oral or intramuscular, based on high and frequent dosages to maintain the drug therapeutic concentration in infection site because of poor drug ability to cross the cell membrane, poor drug bioavailability and pre-systemic clearance. An alternative acceptable therapy to systemic treatments involves inhalation route delivering the drug directly to the desired site, enabling a rapid onset of the action and avoiding the long period of the current treatment and the first-pass metabolism, as well as the use of high doses of drug resulting in drug resistance onset and in severe side effects on other organs. Since 75-80% of TB cases remain localized in the lungs, inhalation therapy could also arrest TB dissemination to other organs by maximizing drug concentration at the infected sites in the lungs, achieving therapeutic but nontoxic systemic levels of drugs. Inhaled TB therapy could presuppose the development of micro- or nanoparticles acting as drug carriers toward the alveolar region in the deepest lung so inducing the endocytosis process of alveolar macrophages being many antimicrobials difficult to transport through cell membranes

Concerning inhaled anti-TB therapy, very limited marketed products, pre-clinical and clinical trials are available, although successful results of few research studies on volunteers. Only kanamicin is approved for anti-TB aerosol but seldom used, and capreomycin loaded into collapsed hollow spheres received FDA approval and completed phase I in USA. Recently, the scientific research has revived an interest in the

administration of anti-TB drugs by inhalation especially due to the advent of multi-drug-resistance (MDR-TB) and extensively drug resistant (XDR) strains, even if it is still in its infancy.

In a previous research study, lipid microparticles (SLM) loaded with rifampicin were designed in perspective of an inhaled therapy for the treatment of TB infection by means of Dry Powder Inhaler (DPI). Studies with dried powder formulations are relatively scarce although the advantages of a DPI device compared with MDI or nebulizers: no propellants, no coordination between the patient and the device, drug stability owing to its dried state which makes DPIs suitable for developing countries in warm climates, higher drug payload delivery, portability, and patient compliance. Rifampicin loaded SLM composed of stearic acid and sodium taurocholate were characterized for aerodynamic diameter, surface charge, physical state of the components, drug loading and release as well as drug biological activity on *Bacillus subtilis* strain. Moreover, SLM cytotoxicity and cell internalization ability were evaluated on murine macrophages J774 cell lines. SLM exhibited aerodynamic diameter proper to be transported up to the alveolar epithelium, negative charged surface able to promote uptake by the macrophages and preserved drug antimicrobial activity. The negligible *in vitro* release of rifampicin indicated the capacity of the microparticle matrix to entrap the drug preventing its spreading over the lung fluid. *In vitro* studies on J774 cell line demonstrated SLM low cytotoxicity and ability to be taken up by cell cytoplasm. The microparticulate carrier, showing features suitable for the inhaled therapy and for inducing endocytosis by alveolar macrophages, could be considered promising in a perspective of an efficacious TB inhaled therapy by means of a Dry Powder Inhaler (DPI) device [Maretti, 2014]. Subsequently, on the basis of these preliminary results, SLM formulation was optimized in order to improve powder respirable fraction preventing particle aggregation during the freeze-drying process by adding cryoprotectants, sample dilution before freezing and freezing at lower temperatures.

Objectives

The research project aims to develop biocompatible, biodegradable and eco-friendly processed microparticles, lipid- or polysaccharide-based, loaded with anti-TB drugs able to be taken up by AM and induce proper bactericidal effect on infected cells in a perspective of an inhaled therapy by means a DPI device for the treatment of primary TB infection.

Lipid- or polysaccharide-based microparticulate systems are biodegradable and biocompatible, poor liable to swell upon contact with the moisture located into the lungs and, consequently, to release the drug before the target site. Solid Lipid Microparticles (SLM), constituted by a solid lipid core stabilized by a surfactant at the surface and polysaccharide microparticles (PM), represent a promising approach focusing to improve TB management by DPI devices. These microparticulate Drug Delivery Systems (DDS) exhibit several favourable properties as production without organic solvents, high drug loading levels and scale-up

feasibility. Furthermore, they could be considered proper to provide powder aerodynamic features essential for the particle deposition into the deep lung.

The research intends to implements two work steps.

1) Microparticle formulation optimization and characterization by evaluating:

- influence of the substrate materials and production methods
- identification of the production parameter role on powder breathability by means of Design Of Experiments (DOE) analysis
- drug entrapment and *in vitro* drug release
- physical properties (aerodynamic diameter, morphology, size, surface charge, circularity, density, porosity)
- breathability behaviour
- antimicrobial activity

2) Microparticle behaviour on cell lines:

- cytotoxicity and internalization on macrophage cell lines
- quantitative determination of intracellular drug on macrophage cell lines
- cytotoxicity and internalization on lung primary cell lines
- drug biological activity on infected cell lines

Methods

SLM will be produced by the melt emulsifying technique through sonication and freeze-drying to obtain a final dried powder. Polysaccharide microparticles (PM) will be developed by both crosslinking interfacial reaction and spray-drying process. The obtained microparticles will be subjected to purification procedures and physical characterization by means of Differential Scanning Calorimetry, Scanning Electron Microscopy, Transmission Electron Microscopy, Photon Correlation Spectroscopy. Drug loading levels and release will be determined by HPLC and UV-VIS spectrophotometry. Fast Screening Impactor and Cascade Impactor were applied to evaluate microparticle breathability properties. A statistical Design of Experiments (DOE) will applied to evaluate the formulation factors that mainly control the inhalation parameters. Drug biological activity will be determined by microbiological agar well diffusion method on sensitive microorganisms. Murine macrophage J774 cell line and lung primary cell lines will be used to assess cytotoxicity by MTT test and internalization capacity by flow-cytometry and confocal laser scanning microscopy.

Expected results

Pulmonary tuberculosis is characterized by alveolar macrophages that host the bacillus of tuberculosis. Many antibiotics are unable to cross the cell membrane and hardly reach sufficient concentration at site of infection. The project is intended to develop microparticles able to transport anti-TB drugs upon contact

with the alveolar epithelium, to be phagocytised by alveolar macrophages, and to induce an effective intracellular biological activity in a perspective of a more efficacious and sustainable TB treatment. Moreover, the use of proven drugs and biomaterials already accepted by the regulations, as proposed in this research, may represent an opportunity in designing novel dosage forms.

Dr. Gloria MONTANARI

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MESENCHYMAL PROGENITOR CELLS AS BIOMARKERS IN IDIOPATHIC PULMONARY FIBROSIS

Background

Idiopathic pulmonary fibrosis (IPF) is a specific form of chronic, progressive fibrosing interstitial pneumonia of unknown cause, occurring primarily in older adults, limited to the lungs and associated with the histopathological and/or radiological pattern of usual interstitial pneumonia (UIP). IPF is characterized by the accumulation of fibroblasts/myofibroblasts and aberrant remodeling of the lung parenchyma. However, the sources of fibroblasts in IPF lungs are unclear. Fibrocytes are bone marrow-derived, circulating mesenchymal progenitor cells that play a role in several fibrotic disorders, including lung fibrosis. They are attracted to injured tissue by various chemokines. It is likely that fibrocytes play a detrimental role in tissue homeostasis and promote fibrosis. This would make fibrocytes a possible novel treatment target for fibrotic disorders. Fibrocytes also have some potential as a biomarker for idiopathic pulmonary fibrosis and other diseases, but the promising preliminary data still require independent and large scale validation. Despite several, as yet, unresolved issues, it has become clear that fibrocytes are more than an incidental finding in lung injury and repair, and may hold great promise for the future of IPF management.

Objectives

The aim of the present study is to evaluate the relationship between circulating fibrocytes and clinical and functional parameters of disease activity and progression in patients with IPF and to test the hypothesis that assay of these cells may provide a biomarker for activity and progression of IPF.

Methods

IPF patients are enrolled at different referral Centers across Italy (Modena, Naples, Reggio Emilia, Bologna, Parma, Catania and Siena, Italy). Patients are followed for 2 years. Progression is defined as a significant decline in lung function or death. 88 patients with IPF have been included to date, 51 from Modena, 7 from Reggio Emilia, 4 from Bologna, 2 from Parma, 21 from Naples, 1 from Catania and 2 from Siena. Every diagnosis of IPF has been confirmed by multidisciplinary review as recommended by current ATS/ERS/JRS/ALAT 2011 guidelines. All patients agreed to participate to the study by signing a written informed consent.

Clinical and anamnestic data and pulmonary function test are obtained at the enrollment and during follow up. Circulating progenitor cells in blood samples are measured at the enrollment and then every 6 months. Laboratory specimens are centralized and flow cytometry analysis for all patients is performed in the Immunology Laboratory of the University of Modena (directed by Professor Andrea Cossarizza). All data are recorded on an electronic web-based database available to all Centers. Plasma samples of all patients are centrally stored for future studies.

Fibrocytes are identified on the basis of the co-expression of the mesenchymal marker collagen-1, the leukocyte marker CD45 ± the hematopoietic stem cell marker CD34.

Conclusions

The clinical management of IPF remains a major challenge not only due to limited drugs available to treat the disease, but also because of the lack of good indicators for disease progression. The identification of suitable biomarkers is an important task; these biomarkers would help act as predictors of prognosis and possibly treatment response. Exciting developments in molecular biology have opened up new approaches for biomarker discovery, but circulating blood cells remain also an attractive target. For fibrotic disorders, these include circulating fibrocytes. The present study aims to assess whether circulating fibrocytes may have utility as: (1) prognostic biomarkers that are correlated with disease progression or mortality, and/or (2) biomarkers that can be used as tools for serial monitoring of disease severity, which might be helpful in driving patients' management in the future. In the future, potential role of cytokines in the recruitment of fibrocytes from the bone marrow will be addressed in future studies. As such, next step will include the measurement of cytokines levels (including interleukin (IL)-6, IL-10, interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), transforming growth factor-beta 1 (TGF-β1), CXCL12, GM-CSF, IL-8 and KL-6) on stored and frozen plasma samples.

Dr. Elvira MOSCARELLA

CEM Curriculum: Translational Medicine
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CoTutor: Dr. Giuseppe Albertini

CUTANEOUS SIDE-EFFECTS OF SELECTIVE BRAF INHIBITORS IN METASTATIC MELANOMA PATIENTS

Background

New therapeutic options have been recently introduced in the treatment of metastatic melanoma. Selective BRAF Inhibitors (SBI) are BRAF mutated kinase inhibitors that were recently shown to induce a substantial effect on melanoma metastatic patients. However, in contrast to the initial enthusiasm about the drug efficacy, some concerns were raised especially because of the cutaneous side effects of the treatment. In several studies, a large proportion of patients developed squamous cell carcinomas (SCC) and keratocanthomas (KAs). Furthermore, a paradoxical activating effect of the drugs on BRAF wild-type cells has been described, which may lead to the development of second primary melanomas.

Objectives

To determine whether selective SBI have a truly oncogenic effect, thus promoting the development of second primary melanomas in treated patients.

To get insights into the mechanisms that are triggered by the selective SBI in melanocytic lesions, which may provide a rationale for re-designing the use of these drugs in a more effective way accompanied by less adverse effects.

Methods and Expected results

Patients undergoing treatment with SBI, will be visited before initiation and ones per month during therapy. A control group of patients not undergoing SBI treatment will be also enrolled. We expect to include 70 patients. Each patient will be subject to clinical and dermoscopic examination. All lesions will be monitored and digitally documented every month for 12 months or more. Morphological changes in each of the monitored lesions, as well as the appearance of new melanocytic lesions, will be recorded. All changing lesions will be additionally analyzed by reflectance confocal microscopy, which allows the in vivo visualization of subclinical features at a quasi-histologic resolution. Those melanocytic lesions that have acquired atypical features suggestive of melanoma will be surgically excised. A board-certified pathologist will examine all excised lesions and define the histologic diagnosis (melanoma or benign lesion). Lesions that will be classified based on histological examination as malignant will be morphologically and histologically compared with the original primary melanoma and, where available, with histology and

morphology of secondary metastases, and with melanomas arising in patients not undergoing SBI treatment. The appearance of non-melanocytic skin cancer, i.e. squamous cell carcinomas (SCC) and keratoacanthomas (KA) will be also registered and correlated with the presence of atypical melanocytic lesions. Each of the lesions that will be excised during the SBI treatment will be subject to Single Nucleotide Polymorphisms (SNPs) probe microarray analysis by using OncoScan FFPE Express 2.0 (Affymetrix). DNA will be extracted from Formalin Fixed Paraffin Embedded (FFPE) melanoma slices manually dissected under microscopy guidance. This approach allows the evaluation of chromosomal alterations, including copy number variations (CNV), chromosome gains and losses and copy-neutral loss of heterozygosity, with a whole-genome coverage, by the analysis of variation in SNP loci.

Dr. Simona PAULONE

CEM Curriculum: Health Sciences
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FUNGAL PATHOGENETIC POTENTIAL: STUDIES ON GUT MYCOBIOTA AND HOST CELLS

Background

Human gut is colonized by numerous microbial species that form the so-called microbiota, consisting mainly of bacteria; yet, fungi, protozoa, viruses and archaea may also be present. The microbiota is essential for human health, but sometimes it becomes cause of pathologies, especially when it changes anatomical location or when some species become too numerous because of antimicrobial therapy or change in diet habits. For these reasons, the interest in microbiota by the scientific community is increasing.

Most of the studies performed so far have been focused on bacteria as the major component of human microbiota, while little is known about the presence and the possible role of fungi, the so-called "mycobiota". Initial studies have identified *Candida, Galactomyces* and *Blastocystis* as the prevalent fungal genera in healthy human gut, while, preliminary evidence indicates an increase in variety of fungal species in sick subjects compared to healthy subjects. Little or no information exists on the possible interactions occurring among different components of human gut microbiota or among mycobiota and host cells.

Extensive literature describes biofilm production as a critical virulence factor, through which many microorganisms, including fungal cells such as *Candida spp.*, enhance their pathogenetic potential; in particular, adhesion to abiotic surfaces as well as host cells, resistance to immune mediated-defenses, reduced susceptibility to disinfectants and antifungal drugs appear significantly affected. In this event, also another fungal peculiarity, namely *Candida* transition from yeast-to-hyphal form, happens to play a critical role. Currently, no findings are available on the impact, if any, of dimorphic transition or biofilm formation on gut mycobiota composition and local balance maintenance.

By in vitro studies, our group has recently shown that: a) once *C. albicans* biofilm is formed, fungal resistance to macrophages increases; b) *C. albicans* yeast-to-hyphal cell transition is affected by probiotic Lactobacilli; c) *C. albicans* biofilm may entrap viral particles, that retain infectivity and better resist to disinfectants and antiviral drugs.

Objectives

Based on the above mentioned results, the aims of my PhD project are:

- To investigate the biomolecular peculiarities of gut mycobiota from healthy individuals and its interactions with host cells;

- To study the characteristics and peculiarities of mycobiota from patients with intestinal disorders;
- To study the impact of fungal biofilm on other microbial agents, including probiotic bacteria and viruses.

Methods

In order to realize the project, the following methods will be employed:

1. By microscope-based morphological analysis and quantitative colorimetric assays, the capability of fungal isolates to produce biofilm under basal conditions and upon exposure to several stresses (including other microbial agents) will be assessed;
2. By fluorescence-based protocols, the adhesion of fungal cells to enterocytes will be evaluated;
3. By means of colony forming cells assays, the susceptibility of fungal cells to macrophages will be tested;
4. By means of antibody microarrays and enzyme immunoassays, the secretory response of host cells to fungal cells will be investigated.

Results

The expected results will be:

- By analysing fungal species isolated from faecal samples of healthy subjects, we will provide data on the composition of normal human gut mycobiota and its ability to interact with host cells;
- By analysing fungal species from patients with gut disorders, we will provide data on mycobiota composition and behaviour with respect to host cells;
- By focusing on *C. albicans*, we will provide information on whether some peculiarities (such as biofilm formation, capacity to adhere to epithelial cells or to resist to macrophages) may differ among isolates from patients and healthy donors.

Dr. Luca PINZI

CEM Curriculum: Medicinal and Pharmaceutical Science
Tutor: Prof. Giulio Rastelli

DESIGN OF DUAL INHIBITORS OF HSP90 AND BRAF AS A NOVEL PHARMACOLOGICAL APPROACH AGAINST MELANOMAS

Background

Melanoma is a type of cancer that arises from the uncontrolled growth of melanocytes (cells responsible of pigmentation in the skin). Melanoma is less common than other skin cancers. However, it is much more dangerous if not found in early stages. It causes the majority of deaths related to skin cancer.[1]

Most melanomas start as a new skin growth on unmarked skin and their growth may causes changes of color, shape, or size. The most important warning sign for melanoma is any change in size, shape, or color of a mole or other skin growth, such as a birthmark.[2]

RAF inhibitors recently approved by Food and Drug Administration (FDA), such as Vemurafenib, have therapeutic effects in patients with the BRAF-V600E mutant melanoma. Unfortunately, responses to Vemurafenib are temporary and rarely complete, with a median time to progression of 6 to 7 months.[3]

Recent research and clinical studies have identified two general explanations for resistance to RAF inhibitors and it has been shown that Hsp90 may play an important role in this context. Hsp90 is required for the stability of several of the oncoproteins that mediate RAF inhibitor resistance. Therefore, inhibitors of this cellular chaperone may be effective in patients with intrinsic or acquired resistance to RAF inhibitors.[4]

In the literature the importance of this topic and the possible implication on the development of new strategies to treat the diseases is well documented.[5,6] Remarkably, the combination of Hsp90 inhibitors with B-Raf inhibitors provided significant synergistic effects, and resulted in drug combinations that are currently being evaluated in clinical trials. Given these premises, we were interested in evaluating whether a single molecule with dual inhibitory activity against both Hsp90 and B-Raf could be designed and developed. If feasible, such an approach could provide a valuable therapeutic strategy for melanomas, especially for the drug resistant forms, avoiding the need and the associated cons typically associated with drug combinations.

Objectives

To the best of our knowledge, no B-Raf/Hsp90 dual inhibitor has been identified so far. The objective of this project is to provide proof of principle that such dual inhibitor approach is feasible and that such compounds can indeed be obtained.

Methods

No crystal structure of B-Raf in complex with ATP is currently available. We developed an *in silico* model starting from the chain 'B' of the PDB complex 3SKC.[7] We docked the ligand ATP inside the binding site of B-Raf using FRED.[8] We refined the crystal structure of Hsp90 with ATP (PDB code: 3T0Z), using Maestro of the Schrödinger suite.[9,10] Each complex was prepared by '*Protein preparation wizard'* using Maestro of the Schrödinger suite. Protonation states of residues were assessed according to pH 7.4 and any problem relating to structures such as inconsistent atom types were fixed. Using the TLeap tool of AmberTool12, topology and coordinate files were produced.[11] Systems were relaxed with two cycles of minimization and a molecular dynamic of 20 ns using pmemd tools of the Amber12 suite.[11] For each biological target, the most representative conformations resulting from molecular dynamics were collected after convergence on RMSD values based on backbone.

We superimposed the two structures based on the bound ligand. This approach highlighted some similarities between the interactions of ATP with the two proteins. The most relevant features identified by the superimposition have been used for the development of a pharmacophore query for ROCS.[12] Then, to perform virtual screening and select compounds for biological testing, the Life Chemicals and Asinex databases of commercially-available compounds were filtered based on custom parameters using FILTER.[13,14,15]

Subsequently, tautomers and stereoisomers were produced according to 7.4 pH and MMFF force field, using Ligprep.[16,17] Using ROCS, the databases were pre-screened through the pharmacophore query previously developed. Defaults parameters were used to accomplish that stage of database preparation. Filtered molecules were prepared for next stage of virtual screening, using OMEGA2.[13]

The filtered databases were docked into each target. Default parameters were used to assess the docking with FRED. Results from each screening were subjected to a consensus scoring "rank by rank" approach.

The best poses highlighted in both targets will be rescored with BEAR, the protocol developed in our laboratories.[18]

Results

The starting protein structures used for the analyses were the crystal structure of Hsp90 and B-Raf in complex with the substrate ATP. As reported in the previous section, we developed a reliable model for B-Raf bound to ATP according to the information provided in the literature and we refined the crystal structure of Hsp90 co-crystallized with ATP.

Given the two models, we have superimposed the two structures by superimposing the bound ATP ligand. Then, we developed a pharmacophore query for ROCS based on the ligand-protein interactions in common between Hsp90 and B-Raf.

Life Chemicals and Asinex databases were filtered according to Lipinski's rules, possibility to make aggregates, presence of known reactive groups and other custom parameters. Subsequently, using the previously developed query, the databases were pre-screened in order to remove molecules not likely to form interactions retained interesting to our purpose. This operation was performed in order to enrich the database with molecules possessing anchoring features important for both targets. The filtered molecules were then docked in each target using FRED. The molecules that did not show a plausible binding mode and particular interactions deemed fundamental for binding were removed. Results from the two target were processed and ranked by a consensus scoring "rank by rank" approach. The most interesting compounds possessing novel scaffolds and favourable interactions with both targets will be rescored with the well-known BEAR protocol (work in progress). BEAR rescores the ligand-protein complex using the MM-PBSA method.[11] Pose with the best score and the best binding mode are currently being selected for bioassays.

Conclusions

Today, melanoma is a type of cancer less common than other skin cancers but much more dangerous if not found in early stages. Many studies identify a clear benefit on pharmacological treatment of melanoma and its resistant forms by the simultaneous inhibition of B-Raf and Hsp90. Starting from information available in the literature for each target, we developed and applied a rigorous structure-based method to identify a dual inhibitor for B-Raf and Hsp90. We are in the process of finalizing the selection of chemical compounds (around 30-50) to be submitted to bioassays. If active hit compounds are identified, these will be further developed in hit-to-lead optimization phases.

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ECHOCARDIOGRAPHIC ASSESSMENT OF HEART FUNCTION AND PULMONARY HEMODYNAMICS IN ADULTS REQUIRING NON-INVASIVE VENTILATION FOR RESPIRATORY FAILURE

Background

Ventilation is based on a complex interplay of lung parenchyma, intrathoracic pressure and heart function. Non-invasive ventilation (NIV) induces dynamic changes in intrathoracic pressure, which modify venous return, cardiac preload, pressure and output in both the right and left heart. Since the introduction of NIV in clinical practice, many authors have documented these hemodynamic changes, both with invasive methods such as cardiac catheterization, and with noninvasive measures such as transthoracic echocardiography. However, most studies focus on few selected parameters, and conflicting results have been reported.

Objectives

To describe and quantify differences in echocardiographic measures of chamber size, function, and hemodynamic, caused by positive pressure non-invasive ventilation, and provide evidence for selection of best echocardiographic parameters to be used in clinical practice.

Methods

This is a prospective, single-center, spontaneous, case series study. All consecutive patients admitted to the hospital (Department of Respiratory disease) for acute respiratory failure requiring positive pressure NIV will be evaluated, and patients > 18 years, without significant cardiovascular comorbidities, and willing to comply with study procedure will be enrolled. Data regarding socio-demographic status, medical history, therapy, hospital admission diagnosis and laboratory finding will be collected. Two echocardiographic examination will be performed, the first during NIV therapy, the second shortly after NIV weaning. All echocardiographic examination will be performed according to the most recent guidelines from the American Society of Echocardiography. Outcome of the index hospitalization will be recorded. No changes in patient therapy and no influences on the standard of care will derive from participating in this protocol.

Expected results

The influence of NIV-induced positive intrathoracic pressure on hemodynamics and heart function should induce evident and measurable changes in chamber size and ventricular function. From clinical experience,

we hypothesize that such changes will be more evident in patients with a normal heart function, while subclinical or mild heart dysfunction will result in less evident dynamic adaptation to the sudden change of intrathoracic pressure. This is a pilot study, and if the hypothesis is confirmed, it could provide interesting data for clinical practice, and prompt future research.

Conclusions

This protocol aims to provide up-to-date evidence on the physiopathology of lung-heart interaction, by performing echocardiographic assessments during NIV and shortly after: we expect that positive intrathoracic pressure will induce measurable changes in chamber dimension and ventricular function, more evident in structural normal heart. Given the widespread diffusion of echocardiography in the acute care setting, including ventilated patients, data derived from the present protocol may help understanding which parameters are feasible and more useful in clinical practice.

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PROTEOMICS STUDIES FOR THE IDENTIFICATION AND VALIDATION OF CELLULAR PROFILES OF NEW DRUG CANDIDATES AND DRUGS IN THERAPY

Background

Proteomics has evolved into a very powerful bioanalytical platform for the simultaneous measurement of a large number of expressed proteins, known as proteomic profiling and has become an important tool for the discovery of new biomarkers useful for clinical application. Mass spectrometry proteomic can also be applied to drug discovery for the study of the mechanism of action of new drugs and for tracking the pharmacodynamic of known drugs in model systems and patients samples.

My work is focused on two main aspects: the study of the protein modulations following drug therapy in clinical patients and novel drug development. These are applied to: ì) a translational project focused on the discovery of new leads against ovarian cancer; ìì) discovery of novel drugs against trypanosomatidic infections.

Ovarian cancer (OC) represents the fifth most common cause of death from cancer in women. The standard first-line treatment consists of platinum derivatives plus paclitaxel, that, despite a high initial response, often gives rise to drug resistance onset with still unclear mechanisms. However, there is evidence that the resistance process includes the over-expression of thymidylate synthase (TS), a key enzyme involved in folate metabolism, as well as of other enzymes of the folatome (enzymes involved in the folate metabolism). The known TS targeting drugs do not show the expected efficacy because they rapidly develop drug resistance[1]. A strategy to overcome the resistance process is the identification of novel drugs that could target TS with a mechanism of action different from that of the classical TS-targeted drugs. In our group a novel strategy to inhibit TS has been developed leading to the identification of candidate lead compounds that work as protein-protein interaction PPI inhibitors and do not induce TS overexpression[2]. On these compounds we have developed a proteomic work to study the protein modulation effect on ovarian cancer cells and started a translational research work. The translational research work was conducted on a clinical trial with a randomized phase II study to assess the safety and efficacy of standard versus high-dose of pemetrexed , a known TS targeting drug, on platinum-resistant epithelial ovarian cancer (PR-EOC).

The second area of application of proteomic study is based on the identification of novel drug candidates against trypanosomatidic infections (human african trypanosomiasis - HAT, Chagas diseases, Leishmaniasis) within the NMTrypI project[3]. In particular Leishmaniasis is an infection caused by obligate intracellular

protozoan Leishmania parasites transmitted by the bite of certain sandfly species. There are an estimated 12 million humans infected. It is currently endemic in Africa, Asia, South America), and the population at risk reaches 350 million people. One of the most significant recent advances in this area has been the identification of miltefosine (MIL), an alkylphosphocholine originally developed as an anticancer drug. MIL is far away from the optimum: its long half-life could induce the development of clinical resistance; it has been shown that MIL is teratogenic and abortifacient and this limits its use in pregnancy. The proteomic work has the aim of tracking the proteomic profile modulation due to Miltefosine, its novel derivatives and new candidates on Leishmania species to study the mechanism of action.

Objects

The aims of my PhD work are:

ì) to identify a protein panel that can work as a biomarkers of the pharmacodynamic activity of the drug in therapy, with particular focus on pemetrexed and 5-Fluoro uracil in ovarian cancer.

ì) characterization of the mechanism of action of Miltefosine, its novel derivatives and new candidates emerging from the drug discovery project aginst trypanosomatidic infections.

The objective of the first year is focused ì) on the identification of a protein panel that can work as a biomarkers of the pharmacodynamic activity of the pemetrexed detected in a translational study within a clinical trial on ovarian cancer using a targeted proteomic approach (MRM modality) and quantify the expression of a selected protein panel, consisting of 6 proteins and 7 housekeeping proteins. and on the characterization of a protein panel detected on leishmania infantum infected cell colture using a whole proteomic approach in a differential mass spectrometry analysis.

Methods

Both projects are based to mass spectrometry platforms with bioinformatics tools for the data analysis.

Targeted proteomic approach on ovarian cancer biopsies was developed because multiple reaction monitoring mode (MRM) is a highly selective and sensitive mass spectrometric methodology for precise and accurate quantification of low-abundant proteins in complex mixtures. Moreover MRM is the alternative to antibody-based assays for discovery and validation of clinically relevant biomarkers. The analysis are performed on LC-MS/MS Triple quadrupole 6410B (Agilent Technologies).

For the NMTripI project the quantification of differences between two or more physiological states of a biological system is very important to understand the metabolic pathway involved in MIL's mechanism of action. So label-free proteomic approach was chosen in order to achieve the objectives and mass spectrometry analysis with ESI-Q-TOF 6520 (Agilent Technologies), was employed.

Results

The method of the selected proteins identification has been developed. The 13 proteins are identified in each biological sample. The identification of right internal standard (labeled peptide) is developing because the ionic suppression is shown by the biological samples.

The comparison between biological samples, treated and untreated with MIL, will allow the protein identification of proteins subset modulated by the treatment. The given protein panel will be useful to understand the pathway involved in the MIL's mechanism of action.

In the translational study of Pemetrexed, the quantification through the calibration curve will be performed with proteotypic peptides. The protein panel modulation enable the identification of a typical background protein reported by given outcome.

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THE GROUP PSYCHOTHERAPY PROCESS: CONCURRENT AND PREDICTIVE VALIDITY OF THE FERRARA GROUP EXPERIENCE SCALE (FE-GES)

Background

Group psychotherapy effectiveness, in the treatment of many mental disorders, is supported by empirical evidence, and it is comparable to that found for individual psychotherapy (Burlingame 2004). Similar efficacy has been demonstrated across a range of approaches including dynamic, cognitive-behavioural therapy, psychological therapy, social skills training, and others (Eklund 1997, Bernard 2008). Group treatments are widely provided in Public Mental Health Services and generally considered as a routine therapeutic intervention (Granholm 2012, McGurk 2007). However, group therapeutic processes, defined as the 'mechanisms of action that are considered to be the causal agents that mediate patients improvement' (Garcia-Cabeza 2011) is still being investigated by very few researches. Currently there is a great gap between the number of theoretical work and empirical research carried out regarding psychotherapy group process (Barlow, 2008). The group setting is characterized by relational complexity (member-member, member-group as a whole, leaders and members relationship) that is hypothesized to be an element of great clinical value, but simultaneously presents empirical research with methodological difficulties, due the many variables theoretically interconnected (Gullo, Lo Coco, Giannone & Lo Verso, 2010).

From a theoretic point of view several processes have been proposed to explain the mechanisms of group therapy (Bion 1961). Foulkes is one of the pioneers of the group psychotherapy theory, his principal contributions (*Therapeutic Group Analysis*, 1964) are considered the conceptualization of the Matrix and of the therapeutic power of mirroring. The Matrix is the hypothetical basis of all group transactions that provides the group's capacity for containment and holding. Mirroring and resonance are suggested to be group-specific therapeutic factors. The value of communication is a vital therapeutic factor as well: the ability to translate the language of symptoms into articulate, exchangeable communications. In 1974 Yalom identified eleven Therapeutic Factors (TFs) to describe the essential mechanisms for change, common to diverse group therapy approaches, which employ a variety of techniques, they consist of: instillation of hope, universality, imparting information, altruism, corrective recapitulation of the primary family group, development of socializing techniques, imitative behavior, interpersonal learning, group cohesiveness, catharsis, and existential learning (Yalom & Leszcz, 2005). These interconnected TFs are theorized to serve the therapeutic process and to be related to outcome (symptoms reduction, function improvement) in

similar ways. Some later authors tried to group the eleven factors in higher order constructs to explain therapeutic process in a more parsimonious manner: group climate, cohesion, alliance and empathy. These constructs are associated to positive outcome and low drop-out rate (Lo Coco, et al., 2008). Cohesion is considered the most central: it is a therapeutic mechanism in itself and it facilitates the action of other TFs. There is growing consensus that cohesion greatly influence the establishment of the therapeutic relationship in groups (Burlingame, Earnshaw, et al., 2002; Yalom & Leszcz, 2005), and it may account for up to nine times greater impact on patient improvement than the approach-specific mechanism of action (Martin, Garske, & Davis, 2000, Wampold, 2001).

More precision in both the definition (theoretic) and measurement (empirical) of group process elements is necessary to advance the research and practice of group therapy (Bednar and Kaul 1994).

From a research point of view the need to integrate group psychotherapy studies in routine clinical practice, has been growing in recent years, reducing the gap between research and clinical work (Lambert & Ogles, 2004). For these reason a number of scales have been developed to empirically explore different aspects of group process. Many have been designed to specifically evaluate Yalom's 11 factors (Yalom, 2005), starting from Yalom's Q-sort questionnaire (1966), which measures factors by patient responses to 60 specific statements about the group experience. However, less than half of Yalom's original Q-sort questions demonstrated adequate internal consistency, suggesting the need for more robust direct measures of TFs (Freedman and Hurley, 1980). Another measure of group process is the Group Climate Questionnaire-Short Form (GCQ-S; MacKenzie,1983) that is one of the most widely used measures, in the literature, with a validated Italian version (Costantini, 2002). The GCQ-S consists of three subscales, common to all therapy groups: Engagement reflects a cohesive environment and willingness to participate, Avoidance highlights a reluctance of group members to take responsibility for problems within the group, and Conflict represents the presence of interpersonal friction. TFI-8 is a brief version of the Therapeutic Factor Inventory (Semands, 2000), reliable, and valid measure of a higher-order group therapeutic factor, but an Italian version is not available. It is useful for frequent and repeated administrations and it may function in clinical and research contexts to identify problematic group sessions and to provide continuous feedback to group therapists in training, supplementing supervisory discussions. A recent Italian research (Caruso et al. 2013) described an initial validation of the Ferrara Group Experience Scale (FE-GES), an useful tool in the assessment of group treatment experiences of patients with severe mental illnesses. This scale has good face and content validity, and authors called for further studies needed to confirm concurrent validity. It is a brief scale (20 items) and designed to detect distinct and typical factors of group experiences, and can be used to assess mediating processes and outcomes in research.

Having a valid and reliable measurement tool is useful in both research and clinical work for the evaluation and quality management of group treatments in clinical practice.

Objectives

The present study will further validate the Ferrara Group Experience Scale (FE-GES).

In particular, it will assess the concurrent and predictive validity of the FE-GES. Documenting these aspects of a rating scale validity is important in order to establish its clinical utility. In order to assess the concurrent validity of the FE-GES, we chose to examine its association with another measures of group process, the Group Climate Questionnaire-Short From (GCQ-S; MacKenzie,1983) and the brief version of the Therapeutic Factor Inventory (TFI-8, Tasca et al, 2014). In order to assess the predictive validity of the FE-GES, we will examine its association with change on several outcome measures, in particular level of psychopathology, work and social functioning and quality of life.

Method

The research protocol will be submitted for approval to the provincial ethics committee, a abide by the last version of the Helsinki Declaration reccomendations.

Setting

The study will involve different therapeutic groups in the Emilia Romagna Region. In particular, the study is expected to recruit in the district of Modena, Bologna and Ferrara, where there are community mental health services which deliver group therapy in residential units (providing short-to medium-term care for patients with acute and subacute psychiatric conditions), day-hospitals and outpatients clinics. Various group therapies modalities which employ a variety of techniques are regularly offered within these settings. The researcher will contact the leaders of the groups and Directors of the Services to propose the research.

It is expected recruitement of about 60 patients for each district in six months.

Inclusion and exclusion criteria

Eligible subjects will be patients attending to group psychotherapy in the Emilia Romagna Mental Health Services of Mental Health, with any diagnosis of mental disorder other than mental retardation.

Exclusion criteria are: younger than 18 years old, insufficient knowledge of the Italian language that prevents completion of the questionnaires, cognitive impairment.

Patients evaluation

After a description of the project by the study coordinator, written informed consent will be obtained from subjects choosing to participate, and patients will be asked to fill in:

- a Socio-demographic Schedule (gender, age, education, marital status, employment, house situation);
- a Clinical variables Schedule (descriptive psychiatric diagnosis (ICD-9), age at first contact with mental services, number of hospitalizations, number of sessions expected, number of sessions performed, psychopharmacological treatment;
- Mini Neuropsychiatric Interview Italian Version 5.0.0 (MINI 5.0.0);

- Structured Clinical Interview for DSM-IV Personality Questionnaire (SCID II);
- Ferrara Group Experience Scale (FE-GES),
- Group Climate Questionnaire-Short Form (GCQ-S),
- Therapeutic Factor Inventory-8 (TFI-8),
- Work and Social Adjustement Scale (WSAS),
- Quality of life assesment (WOHQOL brief),
- Brief Symptoms Checklist (BSCL)

At the time of their entry into the group, participants of group therapy complete each of the outcome measures (WOHQOL, WSAS, BSCL). These measures will be repeated again after 6 sessions and at the end of the treatment. GCQ-S, TFI-8 and FE-GES will be administered at the beginning of group therapy and at the follow up.

Statistical Analysis

Data recorded from the scales will be collected in a database for subsequent analysis. Statistical analysis will be performed using STATA software

Expected results

- A measurement tool for the evaluation of the group psychotherapy process, brief, reliable, self-administered and validated, could have a significant impact to the advancement of scientific knowledge of the therapeutic factors of group experiences;

-FE-GES could be a scale easily implemented in public services clinical practice as a process indicator, self-evaluation procedures and support for the clinician;

-Finally, this scale could be used in future research projects to compare treatment outcomes of groups that are based on different theoretical models.

-The analysis of the data could lead to establish factors predictive of positive outcome of group therapy at an early stage of treatment.

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NEXT GENERATION SEQUENCING OF MULTI-GENE PANELS: STRATEGIES TO OVERCOME TREATMENT RESISTANCE IN METASTATIC BREAST CANCER

Background

An increasing number of molecularly targeted drugs are available for the treatment of metastatic breast cancer as approved drugs or in the context of clinical trials. These drugs target specific molecular abnormalities, including mutated protein kinases and amplified or rearranged genes. Cells harboring these driver mutations have a survival advantage; therefore, targeting these genetic alterations is a rational strategy to offer more personalized and effective treatment to metastatic patients.

Recently developed NGS instruments have removed the high cost and complexity of genome-scale sequencing. The ability to perform multi-gene testing for a range of recurrent molecular alterations provides an opportunity to clarify the mechanisms of resistance to endocrine and targeted therapies, to find the strategies to overcome that resistance and thus, to identify patients who may be candidates for clinical trials with matched targeted therapies.

Objectives

The purpose of the current study is to compare the potentially targetable genomic abnormalities in primary HR+ and/or HER2+ breast cancers and, after progression to treatments, in relapsed sites; therefore, at the diagnosis before any treatments and at the metastatization, after the development of resistance to endocrine or targeted treatments. Then, we will compare previous treatment with the development of new genomic abnormalities. Finally, we will compare the genomics alterations detected with response to subsequent treatments.

Methods

In this study, we will perform a panel of 31 genes involved in the pathways of resistance to endocrine and targeted treatments, using a standardized, commercially available next-generation sequencing (NGS)-based genomic profiling assay, the Ion Torrent Personalized Genome Machine (PGM) (Life Technologies, Guilford, CT, USA). The multi-gene panel will be performed on formalin fixed and paraffin embedded samples from primary tumors and metastatic sites in 30 metastatic breast cancer patients.

Results

The protocol of the study is now under evaluation at the Ethical Committee. In spring we would begin to enroll patients and collect their samples. Before the end of the year we would begin to run samples and the first results should be ready in the first half of 2016.

Conclusions

Pending.

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Cycle XXX

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