University of Modena and Reggio Emilia

PhD COURSE OF CLINICAL AND EXPERIMENTAL MEDICINE

PhD DAY 2016

Abstracts

March 22

14:00 p.m., Lecture Room U1.1

Department of Life Sciences

(103 Campi street, Modena)

CRUI
Conferenza dei Rettori
delle Università Italiane

Giulio Regeni: il diritto e il dovere di fare ricerca

- Pubblicato in CRUI NEWS (/per-una-nuova-primavera-delle-universita/itemlist/category/42-crui-news.html)

L'angosciosa vicenda di Giulio Regeni ci interpella come persone e come studiosi.

Sappiamo che l'attività scientifica è fatta di dedizione e sacrificio. Sappiamo che essa ci sospinge per sua stessa natura ai limiti del conosciuto. Sappiamo che essa pone dilemmi morali a volte laceranti. Non possiamo però accettare che essa comporti la morte per mano di altri uomini. Non possiamo accettare che la volontà di conoscere e far conoscere sia frenata dall'intimidazione.

Perciò, come studiosi, ribadiamo, di fronte alla tragedia di Giulio, che il posto di un ricercatore è quello in cui la ricerca lo chiama. Rivendichiamo il diritto e assumiamo il dovere di fare ricerca in ogni contesto e di collaborare fraternamente in ogni contesto con tutte le persone di scienza.

Oggi, riconoscere davvero questo diritto e questo dovere vuol dire impegnarsi con urgenza e sincerità a fare emergere la verità sulla fine di Giulio. La Conferenza dei Rettori delle Università italiane richiede questa verità con forza e intanto manifesta ai familiari di Giulio la profonda simpatia del mondo universitario italiano.

Roma, 12 febbraio 2016

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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 XXIX

Dr. Elena BIANCHINI

CEM Curriculum: Translational Medicine
Tutor: Prof. Marcello Pinti

INVARIANT NATURAL KILLER T CELLS IN SECONDARY PROGRESSIVE MULTIPLE SCLEROSIS PATIENTS DISPLAY PRO-INFLAMMATORY PROFILES

Background

Multiple Sclerosis (MS) is an autoimmune disease caused by an aberrant immune response to environmental triggers in genetically susceptible subjects, but the exact contribution of the adaptive and innate immune system in the disease development has not yet been fully elucidated. Innate-like T lymphocytes, such as invariant natural killer T (iNKT) cells, are unconventional T cells that bridge the innate and adaptive arms of the immune system. Several studies have evaluated the frequency and functions of iNKT cells both in human patients and in experimental mouse models of MS. However, contradictory observations have been made, mainly because of non-stringent methods used for the identification of iNKT cells and limited analysis of the distribution and/or function of iNKT cell subsets.

Objectives

To understand whether iNKT cells exert a pathogenic role in MS, we analysed their frequency and polyfunctional response in MS patients with different forms and treatments of the disease.

Methods

We studied 165 MS patients: 121 with relapsing-remitting (RR) MS (17 untreated with newly-diagnosed active RRMS, 19 untreated with not-active RRMS (NARR), 29 treated with glatiramer acetate (GA), 31 with interferon-beta-1a (IFN), 25 with natalizumab (NAT)) and 44 with progressive MS (24 with secondary progressive (SP) and 20 with primary progressive (PP) form). Fifty-five healthy subjects were recruited as controls (CTR). By high-speed polychromatic flow cytometry, we characterized iNKT cell phenotype in all subjects; polyfunctional response was analysed in 41 RR (11 assuming GA, 12 IFN, 13 NAT, and 5 NARR), 12 SP, 4 PP and 26 CTR.

Results

No main differences were found in the frequency of total iNKT cells and their subsets among patients with different forms of MS or during different treatments. However, in MS patients, iNKT cell polyfunctional response mainly showed Th1 and Th17 profiles. In SP patients, iNKT cells expressing CD4 or CD8 produced the highest levels of IL-17. Concerning IL-4, no significant difference was found between MS patients and

CTR. Among treated RR patients, those assuming NAT displayed lower levels of iNKT cells producing IL-17, TNF-α and IFN-γ compared to other treatments.

Conclusions

The frequency of iNKT cells was mostly unaltered, but changes were observed in their polyfunctional response, with a Th1/Th17 cytokine-bias in MS patients. This was most prominent in patients with SPMS, suggesting that the progressive phase of the disease is characterized by sustained iNKT cell activation and skewing towards a steady pro-inflammatory phenotype. Treatments generally did not affect iNKT cell polyfunctional response, except for NAT, which caused a reduction in iNKT cells producing Th1 and Th17 cytokines, by turning off pro-inflammatory cytokine production and modulating iNKT cell function. Overall, our data suggest that iNKT cells could be involved in the pathogenesis and progression of MS. A better understanding of the network of immune cells contributing to MS could be of great interest for the development of new therapeutic strategies for the treatment of the disease.

Dr. Chiara BORSARI

CEM Curriculum: Medicinal and Pharmaceutical Sciences
Tutor: Prof. Annalisa Tait
CoTutor: Dr. Stefania Ferrari

LEAD OPTIMIZATION FOR NEGLECTED TROPICAL DISEASES: CHROMEN-4-ONE SCAFFOLD AND MILTEFOSINE

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.

Miltefosine (hexadecylphosphocholine) was registered as the first oral treatment for visceral leishmaniasis in India in 2002 and it is considered to be the first effective oral treatment for cutaneous leishmaniasis, with greater accessibility and lower toxicity compared to antimonials. However, it has some drawbacks, such as a long half-life (100–200 h) in humans and a low therapeutic ratio. Moreover, Miltefosine is not suitable for pregnant women because it causes teratogenesis in animals. Considering the potential development of resistance, the side effects, the production costs and the mechanism of action still poorly understood, the field of design and synthesis of novel antileishmanial phospholipid derivatives is an ongoing challenge.

Objectives

The aim of the European project called New Medicines for Trypanosomatidic Infections (NMTrypI, EU FP7) is the pre-clinical development of candidate drugs for Trypanosomiasis (human african trypanosomiasis - HAT, Chagas diseases) and Leishmaniasis.

The specific aim of my second year PhD project was to optimize the chromen-4-one scaffold in order to increase the activity of classical flavonoids, to reduce the toxicity and to gain the suitable ADME-Tox properties.

Moreover, I was awarded a grant by the COST Action CM1307 and I spent three months at the National Hellenic Research Foundation (Athens, Greece) working on the synthesis of Miltefosine derivatives. In details, the aim of my project was to explore the effect on the antilieishmanial activity of 5-membered heteroaromatic rings in the lipid portion of ether phospholipids.

Methods

Compounds synthesis.

I have synthesized fifty-one compounds bearing the chromen-4-one scaffold. In general, flavonol-like compounds 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. Tin(II) Chloride was used to selectively reduce the aromatic nitro groups to amino groups. Pd/C-catalyzed cross coupling reactions gave the biphenyl derivatives. Compounds were purified by recrystallization from ethanol or column chromatography using the advanced and innovative automatic flash purification system (ISOLERA Biotage). The general chemical modifications introduced are depitced in Figure 1.

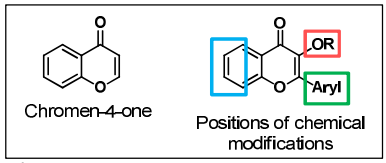


Figure 1.

I have synthesized five new ether phospholipid derivatives bearing 5-membered heteroaromatic rings, and more specifically isoxazole, 1,2,4- and 1,3,4-oxadiazole. The initial step in the synthesis of the phospholipids involved the synthesis of the appropriate alcohols. Afterwards, phosphorylation of the alcohols using $POCl_3$ gave the corresponding phosphoric acid derivatives which were transformed into the pyridinium salts. The reaction of the pyridinium salts with choline tosylate after activation with MSNT (1-(mesitylene-2-sulfonyl)-3-nitro-1H-1,2,4-triazole) yielded the desired phospholipid derivatives. The general chemical modifications introduced are shown in Figure 2.

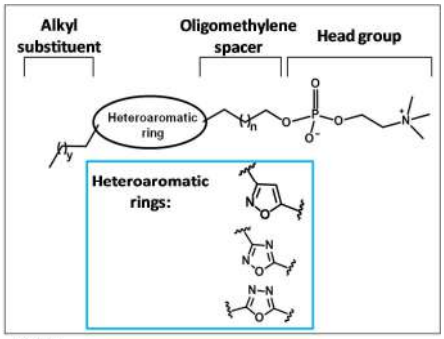


Figure 2.

The compounds were fully characterized through Nuclear Magnetic Resonance (NMR) spectroscopy and mass spectral techniques (Ion Trap LC-MS and ESI-HRMS).

Biological evaluation.

1. ADME-Tox evaluation (Inhibition of cytochromes, hERG and Aurora B kinase. Cytotoxicity and mitotoxicity) were performed at Fraunhofer-IME, Hamburg (Germany).
2. Antiparasitic evaluation towards *Trypanosoma brucei* and *Leishmania infantum* were performed at Instituto de Biologia Molecular e Celular, Porto (Portugal). Antiparasitic assays towards *L. donovani*, *L. major* and *T. cruzi* were carried out at Bernhard Nocht Institute for Tropical Medicine, Hamburg (Germany).
3. *In vivo* stability in BALB/c mice of the selected compounds both alone and solubilized with β-cyclodextrins after oral administration was assessed at Complutense University of Madrid (Spain).

Results

During my second year PhD project, I shifted from the synthesis of classical flavonoids to the synthesis of flavonoid-like compounds, maintaining the chromen-4-one scaffold. I have synthesized fifty-one molecules.

Almost all classical flavonols were active against *T. brucei* with EC_{50}values between 1-10 µM, while they have no activity against *Leishmania infantum.* Classical flavonols showed low selectivity index (SI - less than 10), with the exception of compound **CB12** (SI = 17). The selectivity index is given by the ratio between the EC_{50} towards *T. brucei* and CC_{50} towards THP1 (human cells: human monocytic cell line). Flavonol-like compounds were more active and selective towards *T. brucei*, with compound **CB37** being the best one (EC_{50} = 0.4 µM, SI = 250). Compound **CB37** is almost 15 fold more selective than classical flavonols thus it represents a promising lead for *T. brucei* infections. Moreover, flavonol-like compounds gain activity towards *Leishmania* infections. Compound **CB40** turned out to be an interesting antileishmanial lead. The compound *was active towards T. brucei, T. cruzi and Leishmania infantum with EC_{50} of 0.8, 3 and 1.9 µM, respectively.*The E*C_{50} towards Leishmania infantum is comparable to that of Miltefosine (2.7 µM), one of the drug currently used to face Leishmania infections. Moreover, it has a higher selectivity, thus a lower toxicity towards human cells, with respect to Miltefosine (SI Miltefosine = 3, SI comp.* ***CB40*** *= 17). Compound* ***CB40*** *is more active than Milefosine towards L. donovani (EC_{50} = 4.6 and 10.8 µM, respectively). L. infantum and dovonani are the etiologic agent of Visceral Leishmaniasis. In addition,* ***CB40*** *shows higher activity than Miltefosine towards L. major, the etiologic agent of Cutaneous Leishmaniasis (Figure 3).* ADME-Tox evaluation *is ongoing.*

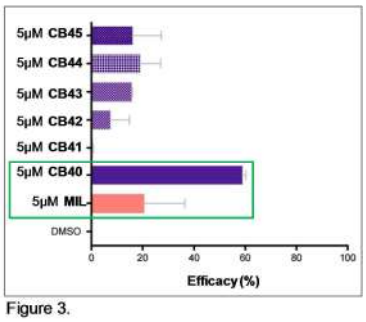


Figure 3.

Regarding Miltefosine derivatives, five novel ether phospholipids bearing 5-membered heteroaromatic rings were synthesized. The initial step in the synthesis of the phospholipids involved the synthesis of the appropriate alcohols. Different procedures were applied in order to optimize the hydrolysis from the bromo derivatives to the corresponding alcohols. We found out that a two steps procedure (bromo deriv. → acetate → alcohol) has many advantages such as short reaction time (3 hours), low temperature, no by-products formation and high yield (70-80%). For the synthesis of the final ether phospholipids, MSNT (1-(mesitylene-2-sulfonyl)-3-nitro-1*H*-1,2,4-triazole) turned out to be a better activating agent than TPS-Cl (2,4,6-Triisopropylbenzenesulfonyl chloride). The compounds were synthesized in good yield (32-46%) and were biologically evaluated. *Compound* ***CB2*** *proved to be more potent and selective than Miltefosine (EC_{50} towards L. infantum: 0.8 and 2.6 µM, SI = 14 and 3, respectively). Moreover, two compounds were active towards T. cruzi. ADME-Tox properties have been evaluated and overall the compounds show a* safe *in vitro* toxicity profile*.*

Conclusions and perspectives

Compound **CB37** represents a promising lead for the treatment of infections caused by *T. brucei*, while compound **CB40** has been selected as lead for Cutaneous and Visceral Leishmaniasis. We are currently evaluating the *in vivo* stability in BALB/c mice of the selected compounds alone and solubilized with β-

cyclodextrins*. Since T. brucei infections reach the central nervous system, the production of solid lipid nanoparticles containing compound* ***CB37*** *is ongoing, aiming to evaluate the blood-brain barrier permeability. We are exploring the structure activity relationship (SAR) of the most promising compounds and we have planned target(s) identification studies.*

The heteroaryl-substituted ether phospholipids show activity both against *L. infantum* and *T. cruzi* and exhibit a safe *in vitro* toxicity profile.

ACKNOWLEDGEMENT

This project has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement n° 603240 (NMTrypI - New Medicine for Trypanosomatidic Infections). http://www.nmtrypi.eu/

COST Action CM1307 funded STSM (Short-term scientific mission; Athens, Greece)

Dr. Giulia BRIGANTE

CEM Curriculum: Translational Medicine
Tutor: Dr. Vincenzo Rochira
CoTutor: Prof. Manuela Simoni

METABOLIC MARKERS OF DIFFERENT FUNCTIONAL THYROID STATES

Background

Hypothyroidism is a common disorder affecting ~4.5% of the population. Despite adequate restoration of biochemical euthyroidism (defined as normal levels of Thyroid Stimulating Hormone (TSH) and thyroxine (T4)), a significant number of patients have persistent symptoms such as fatigue, muscle aches and diminished cognitive and psychological function. Complaints could be due to a change in thyroid hormone metabolism at the peripheral tissues level such as altered deiodinase activity. Alternatively, monitoring of serum T4 and TSH does not reflect tissue thyroid hormone level, which might result in suboptimal substitution therapy.

Objectives

In patients that are going to pass through different thyroid states:

1) to identify changes in TH metabolism, by measuring thyroid hormones metabolites;
2) to investigate how these metabolic changes are related to quality of life;
3) to study the variation of tissue specific markers of thyroid state.

Methods

Single centre, experimental cohort study. We are analyzing changes in thyroid hormone metabolism in 200 patients with differentiated thyroid cancer in various thyroid states. During regular clinical follow-up patients with differentiated thyroid cancer are subject to euthyroidism (before thyroidectomy), hypothyroidism (before I-131 therapy), hyperthyroidism (during suppressive therapy), recombinant TSH stimulation and stable euthyroidism on T4 substitution therapy. Therefore, patients with differentiated thyroid cancer provide an excellent model to study the consequences of variations in thyroid state.

When blood is drawn for routine diagnostic reasons at different thyroid states, two additional tube of blood are drawn to determine a detailed TH profile, needed to study TH metabolism. Moreover, we will measure end-organ markers of response to TH, such as sex hormone binding globulin (SHBG), osteocalcin, urinary telopeptides, total cholesterol, low-density lipoprotein (LDL) cholesterol, lipoprotein(a), creatine kinase, ferritin, myoglobin, and enzymes such as tissue plasminogen activator, angiotensin converting enzyme (ACE), and glucose 6-phosphate dehydrogenase.

We are also collecting PAXgene tubes (containing RNA stabilizer) for gene expression analysis of miRNA that are known to change their expression in response to TH variations. Urine is sampled to measure urine iodine excretion as a marker of iodine state. Serum will be collected and stored in the -80C until samples from all 200 patients are available.

To evaluate quality of life, patients will be asked to fill out a set of self-report questionnaires. The set include the original Italian version of the Multidimensional Fatigue Inventory (MFI-20), the thyroid-specific quality-of-life patient-reported outcome measure (ThyPRO), and the Italian SF-36 Health Survey.

Study population: Patients at the age of 18-80 years who undergo or underwent a thyroidectomy for differentiated thyroid carcinoma.

Main study parameters/endpoints: The main study parameters are changes in thyroid hormone metabolism, changes in serum tissue and urine markers and changes in symptoms evaluated by thyroid specific questionnaires.

Dr. Anna Rita DOMINGUES DA SILVA

CEM Curriculum: Translational Medicine
Tutor: Dr. Giovanni Guaraldi

IMPACT OF POLYPHARMACY ON ANTIRETROVIRAL PRESCRIPTION IN PEOPLE LIVING WITH HIV

Background

The rising prevalence of multimorbidity (MM) as a consequence of prolonged survival of HIV patients has increased the burden of polypharmacy (PP). The interaction between antiretroviral therapy (ART), multiple chronic illnesses and PP is poorly studied. Here, we sought to evaluate the relationship between PP and ART, delivered as conventional multi-tablet three-drug regimens (MTR) or Single Tablet Regimens (STR) as well as Less Drug Regimens (LDR; using simplified mono or dual therapies).

Objective: The aim of our study was to analyse the impact of PP on prescription of ART strategies.

Methods

This is a cross sectional analysis of electronic data from the prospective Metabolic Clinic cohort study at the University of Modena and Reggio Emilia School of Medicine in Modena, Italy. We included last clinical observation for each patient from January 2006 to December 2015. Polypharmacy was defined as the use of 5 or more medications (excluding ART), classified according to high level ATC codes (ie by therapeutic class and subgroups). MM was classified as the presence of 2 or more of Non Infectious Comorbidities (NICM) in the same individual, including cardiovascular disease, end-stage kidney disease, cancer, osteoporosis, hypertension, type 2 diabetes mellitus, liver cirrhosis, and chronic obstructive pulmonary disease. All patients attending for review had a frailty index calculated based on the established deficit accumulation approach: this comprised 37 variables spanning multiple systems but no HIV- or NICM-related factor. Factors associated with different ART regimens were analysed using multivariable multinomial logistic regression analyses with MTR therapy as base outcome.

Results

A total of 2944 patients (33.7% females) were included in the analysis. Median duration of HIV infection was 19 years (IQR 12.5–23.7), median CD4 cell counts was 638/mm3 (460–830), with nadir of 192/mm3 (80–290) and 2,853 patients had undetectable HIV VL (96.9%). MTR was present in 2025 (68.8%) patients, STR in 464 (15.8%) and LDR STR in 455 (15.4%) patients. Within the STR group, 350 patients were on Atripla®, 100 on Eviplera® and 14 on Stribild®.

MM was present in 313 (10.6%) patients and PP was present in 301 (10.2%) patients.

A significant association was found between ART regimens and both FI and PP. To explore their independent contribution to the outcome variable, we built different multinomial logistic regression analysis comparing LDR with standard MTR and STR with MTR.

PP was negatively associated with STR regimen (RRR=0.48, CI: 0.28; 0.81) independently from Frailty (RRR=0.68, CI: 0.59; 0.78), after correction for age, gender, HIV infection duration, CD4 nadir, current CD4 cell count, and calendar year. This association was not found comparing MTR and LDR.

Discussion

This study identified an independent association of PP and FI with regards to STR prescriptions compared to MTR. These results may increase knowledge on ARV treatment with particular regards to older, multimorbid and frail patients more likely to present medication toxicity and benefit from certain organ-sparing treatments. Specifically the role of tenofovir disoproxil fumarate, included in all STR regimens, need to be accurately studied given its renal and bone toxicity.

The balance between treatment tailoring, prevention of drug-associated toxicity and reduction in number of pills need to be prospectively studied in order to provide the best approach to ageing HIV-patients.

Dr. Riccardo FANTINI

CEM Curriculum: Translational Medicine
Tutor: Prof. Enrico Clini

ULTRASOUND ASSESSMENT OF DIAPHRAGM FUNCTION IN ALS PATIENTS

Enrollment: 50 patients and 50 controls (completed). Has been performed 215 visits with enrollment and follow-up.

Background: Evaluation of diaphragm function in Amyotrophic Lateral Sclerosis (ALS) is critical to decide to start non-invasive mechanical ventilation (NIV).
Currently, forced vital capacity (FVC) and sniff nasal inspiratory pressure (SNIP) are volitional means for this evaluation, but require collaboration and are poorly specific. The primary aim of this study was to assess whether the diaphragmatic thickness as measured by ultrasound (US) correlate with lung function impairment in ALS patients. The secondary aim was then to compare US diaphragm thickness index (ΔTdi), with a new parameter (ΔTmax index).

Methods: 41 patients with ALS and 30 healthy subjects were enrolled. All subjects underwent spirometry, SNIP and Diaphragm US, while arterial blood gases were measured in patients only. US diaphragm thickness (Tdi) at Vt, or TLC, and their ratio (ΔTmax) were recorded. Changes (Δ) of the Tdi indices during tidal volume ΔTdiVt) and maximal inspiration (ΔTdiTLC) were also assessed.

Statistical Analysis: Descriptive statistics are presented as median value and interquartile range [IQR].
Group comparisons according to different measures were performed using the $\chi 2$ test or Fisher's exact test for categorical variables, whereas nonparametric Wilcoxon test (Mann-Whitney) was used for continuous variables.
Nonparametric tests were preferred because of the small size of our cohort.
The Spearman's correlation was used to assess association of FVC, SNIP and hypercapnia with ΔTdi and ΔTmax.
Univariate regression models were also graphed.
ROC analyses were performed to assess accuracy of ΔTdi and ΔTmax in identifying patients with altered respiratory tests (FVC and SNIP) and C-statistic was used to evaluate differences in AUCs.
Cox regression test was used to assess the hazard ratio of tests used compared to mortality.
Subject characteristics: 41 patients (30 men and 11 women) out of 180 ALS prevalent cases, followed by the MND Centre met the inclusion criteria of the study and accepted to be enrolled. The median age at baseline was 63 years [IQR 56,73], with 11 patients (26%) presenting with bulbar onset of the disease. The ALSFRS-R

average was 36 [IQR 32, 41]. Four patients (9%) presented with hypercapnia on arterial blood gas measurement. Twelve patients (30%) had SNIP test <40 cmH2O, and 8 patients (18%) had FVC <50% of predicted value.

Results: a good correlation was found between ΔTdi_{TLC} and FVC with rho = 0.52, p<0.001; ΔTdi_{TLC} and Sniph with rho= 0.53, p<0.001; Among ΔTmax FVC and rho= -0.45, p=0.001; ΔTmax and Sniph rho=-0.58, p<0.001 (Table).

ROC curves analysis for comparison of individual tests, showed ΔTmax had high accuracy than ΔTdi_{TLC}. Indeed, the area under the curve (AUC) was wider for ΔTmax when considering both FVC (ΔTmax=0.76 compared with ΔTdi_{TLC}=0.27) and SNIP (ΔTmax=0.71 ΔTdi_{TLC}=0.25)[1].

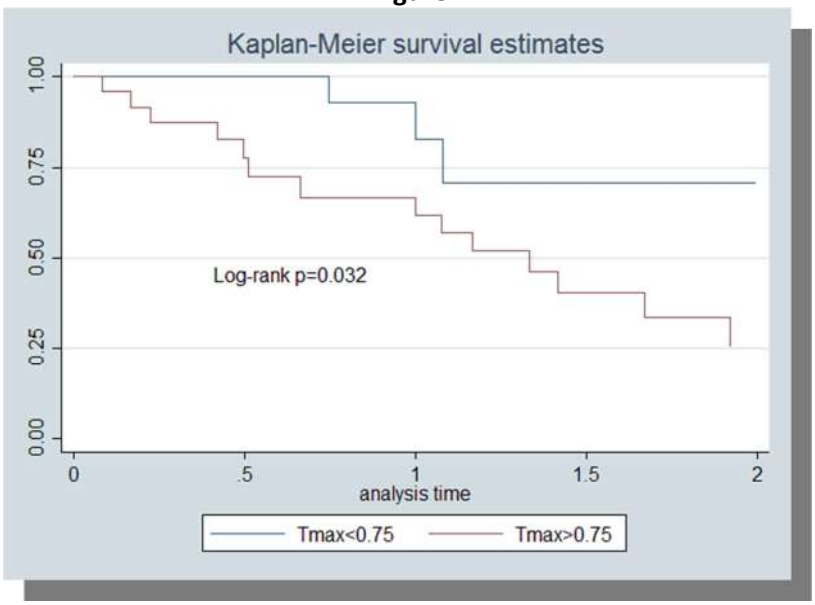
A statistical analysis was conducted longitudinally to assess the prognostic value of ultrasound indices relative to mortality; the hazard ratio for mortality of Δtmax>0.75 is 3,6 (IC 1.1-12) (Figure).

Conclusion: the ultrasound diaphragmatic shows good correlation with the reference parameters and could and can identify patients at increased risk of death that may require mechanical ventilation support guiding the choice on start timing of ventilatory support.

Table

Test	Rho	P Value
ΔTdi_{TLC} vs FVC	0.52	<0.001
ΔTdi_{TLC} vs Sniph	0.53	<0.001
ΔTmax vs FVC	0.45	0.001
ΔTmax vs Sniph	0.58	<0.001

Figure



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Dr. Gaia GOZZI

CEM Curriculum: Medicinal and Pharmaceutical Sciences
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. However, therapy successful is limited by the appearance of acquired resistance to cDDP and its derivatives, a multifactorial process including 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 causing translation repression. 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 (translational de-repression). 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 folic acid-peptide 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. In order to improve the stability, new LR peptides derivatives with cyclic structures were synthesized.

Previous data showed additive and supra-additive effects for most of the combinations of [DGln^4]LR peptide and currently used anticancer drugs in a panel of human OC cell lines. Based on these data, we investigated the effect of same combinations with [DGln^4]LR delivered by pegylated pH sensitive liposomes. Moreover RTX is a selective inhibitor of TS and its mechanism of action offers the potential for synergy with agents that act via other molecular mechanisms, such as oxaliplatin.. Preclinical data suggest that RTX may be successfully combined with 5-FU, or other chemotherapy agents with the aim of improving results in advanced colorectal cancer (CRC) (3). In particular, data on the cytotoxic interactions of RTX and 5-FU in

human colon cancer cell lines suggested that the simultaneous administration of RTX and 5-FU or the sequential administration of RTX followed by 5-FU may be the optimal sequences (4). Based on these data, owing to the over-expression of TS in tissues of the ovary and in particular in OC cells, we have also carried out a study to verify the nature of the combination between RTX and 5-FU in a panel of human OC cell lines.

World Health Organization has included seventeen chronic disabling infections in the category of "Neglected Tropical Diseases" (NTDs), due to the lack of interest by pharmaceutical companies. These diseases include Leishmaniasis (Leishmania) and Human African Trypanosomiasis (Trypanosoma brucei). Nowadays current drugs targeting these NTDs are toxic, expensive, and often difficult to administer; moreover, actually available medicines are outdated and present an increased drug resistance. In infections context, ,the folate pathway, in particular two enzymes that catalyse similar reactions PTR1 and DHFR, plays an important role. PTR1 enzyme is an NADPH-dependent dehydrogenase/reductase only presents in trypanosomatids; it is able to catalyse four reactions, and two of these are the same reactions catalysed by DHFR. The European project called "New Medicines for Trypanosomatidic Infections" (NMTrypI n° 603240) aims to identify new molecules that inhibit both parasitic PTR1 and DHFR (dual inhibitors), or molecules able to selectively inhibit PTR1, to combine them with known drugs acting on parasite's DHFR.

In collaboration with Tydock Pharma, a series of pteridinic molecules, folate analogues that compete with the substrate, has been designed and synthesized to inhibit the enzyme and to develop antiparasitic action. One of the objectives of this project was to test the cytotoxicity of these pteridine compounds against a panel of mammalian cell lines and to compare it with that of reference drugs.

Objectives

Specific aims of the past year were:

1. Evaluation of cell growth inhibitory effect of cyclic peptides, new LR peptide derivatives, against cDDP-sensitive and -resistant human OC cell lines.
2. To study the nature of the combination of the peptide [DGln^4]LR, delivered by pegylated pH sensitive liposomes, with RTX or cDDP in cDDP-sensitive and -resistant OC cell lines.
3. To study the nature of the combinations of RTX and 5-FU on human OC cell lines.
4. Evaluation of cytotoxicity of antiparasitic pteridine compounds against different cell lines: WRL 68 (Human liver embryonic cell line), Hep G2 (Human hepatocellular liver carcinoma cell line), NIH 3T3 (Mouse embryonic fibroblast cell line), A2780 (Human ovarian cancer cell line)

Methods

The effects on cell growth of cyclic peptides was determined using a crystal violet dye assay in which the dye extracted from the samples is proportional to the cellular biomass. Peptides were delivered into cells by means of the peptide delivery system SAINT-PhD (Synvolux Therapeutics, NL).

For combinations of RTX and 5-FU or combinations of peptide-loaded liposomes with RTX and cDDP, cells were simultaneously or sequentially exposed to the drugs and the nature of the combination was evaluated by means of Synergism Quotient method (SQ) (5). Cytotoxicity of compounds alone and in combination was determined by MTT assay. Quantitative measures of the cell cycle phase distribution were performed by flow cytometry.

Results

Cyclic peptides: Peptides were tested against IGROV-1 cell growth up to 100 µM. Among these, two of them showed to inhibit cell growth by the concentration of 25 µM. In particular, PF117 peptide exhibited an IC50 value around 10 µM. This effect was comparable to that of the non-cyclic peptide [DGln^4]LR, when tested up to 25 µM. At least four peptides showed IC50 values lower than 25 µM against the human OC cell line A2780 and its cDDP-resistant counterpart, A2780/CP cells; PF117 especially has confirmed good effectiveness against both cell lines. On the contrary, PF114, was quite active in A2780 cells but scantly in the A2780/CP cells, the opposite for PF116, which was as active in the A2780/CP cells as PF117; likewise PF115. In more detail, the IC50 of PF117 was lower than 10 µM in A2780 cells and A2780/CP cells, whereas, PF116 IC50 value was lower than 10 µM in A2780/CP cells, but higher than 25 µM in A2780 cells. Both peptides resulted more active in cDDP-resistant cells, suggesting a collateral sensitivity of the resistant cells to these peptides.

Combination between [D-Gln4]LR-PEG liposomes and drugs: [D-Gln4]LR_PEG liposomes were employed at the concentration of 0.125mg/mL and drugs at different concentrations. Best results were obtained with concentrations of 10nM RTX and 5µM cDDP for C13* cells, while 10nM RTX and 2.5µM cDDP for IGROV-1 and 2008 cell lines. Peptide-loaded liposomes (PLL) combined with the two anticancer drugs showed greater efficacy against both cDDP-sensitive and -resistant cell lines. The efficacy of cDDP or RTX was increased by the concurrent and PLL-drugs sequential combination, whereas by the other sequence (drugs-PLL sequential combination) an antagonistic effect was observed. This result suggests that in the case of cDDP, for example, the peptide delivered by the liposomes, causing the folate cycle enzymes inhibition, may hamper the repair of DNA damaged by cDDP. Besides, in the case of RTX the potentiated antiproliferative effect could be ascribed to the sum of different mechanisms acting on the same target.

Combination between 5-FU and RTX: We tested different drug concentrations in 4 OC cell lines and the best data were obtained with the 1:250 RTX:5FU ratio in A2780 cell line. In particular, with RTX 4 nM and 5-FU 1µM a synergistic effect was obtained for both the sequential and the concurrent administration at 72 h.

Antiparasitic pteridine compounds: the majority of compounds have shown lower toxicity than the reference drugs towards the different mammalian cell lines, being the WRL 68 cells more sensitive to pteridine compounds than the others. The pteridine derivatives had a different toxic profile depending on their different chemical structure and on the type of cells considered.

Conclusions

Despite no cyclic peptides showed an IC50 value lower than the lead peptide [DGln^4]LR, the PF116 and 117 showed a greater cytotoxicity against resistant than sensitive cells.

The schedule of combination of [DGln^4]LR-loaded liposomes with anticancer drugs confirmed the same synergistic effect previously obtained by means of specific peptide delivery system.

The results of RTX-5FU combinations at cellular level suggest that, as already reported for CRC, the simultaneous administration of RTX and 5-FU or the sequential administration of RTX plus 5-FU may be the optimal sequence also against OC cancer cells.

Pteridine compounds showed promising results in terms of inhibition capacity and selectivity against parasitic proteins and of low cytotoxicity.

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Dr. Angela LAURIOLA

CEM Curriculum: Translational Medicine
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THE KEY ROLE OF MITOSTATIN IN THE MAINTENANCE OF GENOME STABILITY IN PROSTATE CANCER

Background

Prostate cancer represents the second cause of male death for cancer in Western countries. 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 potentiality as a tumor suppressor has been reported recently; in various cancer cell lines we have demonstrated that ectopically expressed Mitostatin inhibits cell growth, migration, invasion, adhesion and tumor formation *in vivo*. Recently it 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. Mitostatin protein level oscillates during these two crucial transitions, suggesting its possible involvement. We hypothesize that Mitostatin may play a critical role in proper cell cycle progression, and its depletion may lead to an incorrect chromosome segregation during mitosis, followed by the aneuploidy and chromosomal aberrations that are often observed in many cancers. In fact, in cells depleted of Mitostatin we observed aberrations that were consistent with defective activation of the spindle checkpoint (SAC) (see results below). Furthermore, we have observed that the overexpression of microRNA-503 in cells, by using pre-miR-503, reduces protein levels of Mitostatin; this microRNA, which belong to the miR-16 family, has been identified as the most down-regulated miRNA during cell cycle re-entry. Rapid degradation during cell cycle re-entry of miR-503 is dependent on its constitutive instability. Thus, miR-503 modulates the cell cycle and it is itself dynamically regulated by the cell cycle, and members of this family are often altered in many types of cancers; in prostate cancer was found up-regulated respect to the Benign Prostatic Hyperplasia (BPH), indicating the

potential of this miRNA as a novel diagnostic and prognostic marker for prostate cancer. Mitostatin in association with micro-503 might be required for the fidelity of cell cycle, and their deregulations may contribute to cellular transformation by promoting genomic instability.

Objectives

Our basal hypothesis is that Mitostatin may play a critic role in guarding the fidelity of genome replication and its deregulations (found in certain human tumors) may contribute to cellular transformation by promoting genomic instability. Through this role, Mitostatin might take part to the switch of low grade prostate tumor (indolent) into the aggressive and lethal form. This hypothesis has been formulated based on the evidence showing that Mitostatin is endowed with tumor suppressor activity and based on our results, which show that depletion of Mitostatin leads to incorrect chromosome segregation during mitosis, followed by aneuploidy and chromosomal aberrations that are hallmarks of genomic instability and cellular transformation. At this stage in the emerging biology of Mitostatin we aim at:

1) Understand the molecular mechanisms by which Mitostatin and mir-503 control the genome stability.

2) Identify the Chromosomal aberrations induced by Mitostatin depletion.

3) Provide Mitostatin as potential biomarker for effectively stratify the low Gleason score tumors into high- and low-risk groups

Methods

HeLa cells depleted of Mitostatin were synchronized in G1/S by using aphydicolin, and released into nocodazole-containing medium (pro-metaphase arrest). The cells will be 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. HeLa cells (control and depleted of Mitostatin) were infected stably with the GFP-histone H2B (retroviral vector) to analyze the presence of chromosomes bridge in anaphase. Also, PNT1A and HCT116 were transfected with siRNA and shRNA for Mitostatin (transient and stable conditions) using Calcium Phosphate transfection method and then the histone H2AX phosphorylation was analyzed using western-blot and immune fluorescence. The phosphorylation of H2AX in Hela cells (depleted of Mitostatin) released for 22hr from G1-S block (Aphidicolin) into nocodazole-containing medium was evaluate using western-blot analysis. To confirm the direct binding of miR-503 to the Mitostatin 3'UTR the 3'UTR of Mitostatin was inserted downstream of a luciferase open reading frame (pLUC) and the vector was co-transfected into HEK293 cells together with control or pre-miR-503 oligos.

Lysates of LNCaP cells (human prostate adenocarcinoma cells) stably expressing V5-tagged full-length Mitostatin (V5-Mitostatin) and empty vector (V5) were immunoprecipitated with antibodies directed against V5 and analyzed by western blotting using anti-V5 and anti- serine antibodies. Mitostatin levels in

low and high Gleason score prostate tumors were evaluated using immunohistochemistry technique (Mitostatin staining).

Results

We observed that Mitostatin depletion in HeLa cells, synchronized by aphidicolin (G1/S) block and released into nocodazole-containing medium, leads to mitotic slippage and adaptation to the spindle checkpoint in the presence of a spindle inhibitor. In the same conditions, the protein levels of Cyclin B1, Mad2 and Cdc20 in Mitostatin-depleted cells resulted early degraded (at 18 hr from G1/S block). Since the activated SAC delays cell exit from mitosis by preventing B1 cyclin proteolysis, the cyclin B1 early degradation, that we have observed, leads to mitotic checkpoint escaping and consequent chromosome instability. Furthermore, in the same cells, Mitostatin depletion provokes increased number of lagging chromosomes and chromosome bridges in anaphase respect to control cells, this suggest a premature sister-chromatid separation as demonstrated by metaphase spreads in HeLa and PNT1A (more than 9% displayed prematurely separated sister chromatids, in contrast with 2% of control cells). Mitostatin depletion induces DNA Damage in PNT1A and HCT116 cells, as demonstrated by increased phosphorylation of Histone H2AX, a well-known marker of DNA damage. Notably, we observed a clear increase of the phosphorylation of H2AX in Hela cells (depleted of Mitostatin) released for 22hr from G1-S block (Aphidicolin) into nocodazole-containing medium, accompanied to increased apoptosis (43% respect to 22% of control cells). Moreover, overexpression of miR-503 using pre-miR-503 reduces protein levels of Mitostatin in the cells. We have confirmed the direct binding of miR-503 to the Mitostatin 3'UTR by 3'UTR luciferase assay. In LnCaP cell line we observed that Mitostatin is phosphorylated at serine residues. In addition, after a retrospective analysis by using immunohistochemistry technique (Mitostatin staining) of biopsy specimens from prostate cancer patients, we have identified decreased levels of Mitostatin in high Gleason score respect to low Gleason score of prostate tumors.

Conclusions

Overall our results showed that Mitostatin is required for the fidelity of mitosis, allowing the optimal activation of the spindle checkpoint (SAC). Mitostatin deregulation produces a deficient spindle checkpoint with consequent chromosome instability and DNA damage that are typical hallmarks of cancer.

Dr. Stefano MANCINI

CEM Curriculum: Translational Medicine
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AUTOIMMUNITY HALLMARKS IN PATIENTS WITH COLORECTAL CANCER AND CANCER AT OTHER SITES ARE RELATED TO POORER PROGNOSIS AND METASTATIC DISEASE: RESULTS FROM A FIVE-YEAR FOLLOW-UP STUDY

Background

Colorectal cancer (CRC) remains one of the most important cause of death and morbidity for cancer worldwide, accounting for an estimated 1.4 million new cases and 693,900 deaths per year [[1]]. Prevention and a precise characterization of the prognostic factors are the fundamental strategies for fighting CRC. Nowadays, autoimmunity is clearly related to an increased risk of malignancies, both hematological and solid [2], but the role of the presence of autoimmunity hallmarks in cancer patients is not univocal [3]. In particular, conflicting evidences demonstrate that the development of tumor associated antibodies (TAABs) could have beneficial effects if directed to few defined antigens (e.g. MUC1 in breast cancer, ENOA 1, and 2 in pancreatic cancer), while on the other side, autoimmunity could have a detrimental role for the increase of systemic inflammation and selecting malignant and potentially metastatic tumor cell clones that lead to a worse prognosis [4,5]. In this context of uncertainty, this study is aimed at investigating the presence, distribution, and significance of autoimmunity in colorectal cancer (CRC) patients and in patients with cancer at other sites.

Materials and methods

128 consecutive cancer patients were enrolled after a written informed consent and in accordance with Ethics Committee approval (Prot. No. 4396/C.E.). Criteria of exclusion: autoimmune diseases, severe kidney/liver impairment, ongoing sepsis, severe prognosis (death within 48 hours), recent infusions of blood or derivatives (within 2 weeks). Clinical data were collected with regards to stage and type of cancer and comorbidities (including Cumulative Illness Rating Scale (CIRS), and Coping Inventory for Stressful Situations (CISS)) and were analyzed in relation to the presence of autoimmunity. Circulating autoantibodies in patient sera were investigated through indirect immunofluorescence analysis by confocal microscopy on rat tissues for non-organ specific autoantibodies (NOSAs: stomach, kidney, liver), and colon-associated autoantibodies (CAAs: rat colon). Anti-nuclear antibodies (ANAs) were evaluated on HEp-2 cells. A titer of 1/40 or above was considered positive. Statistical analysis was performed by SigmaPlot© v.12, Systat Software, Inc. $p<0.05$ was considered significant.

Results

NOSAs resulted higher in the other cancer group than in colorectal cancer group ($p<0.03$), while ANAs and CAAs did not differ significantly between the two groups. Subset analysis for CAAs demonstrated higher rate of staining of colonic glandular epithelium in CRC group than in other cancer group ($p<0.04$), while HEp-2 cytoplasmic staining was significantly higher in other cancer group than in CRC group ($p<0.05$). Considering all 128 cancer patients, survival resulted significantly longer in patients negative for autoimmunity ($p=0.041$), and the presence of a higher autoimmunity was associated to a significant higher probability to have metastases (OR 2.545, CI 95% 1,129-5.739; $p<0.04$). No correlation has been found between CIRS scores and the presence of autoimmunity, as well as CISS scores and autoimmunity. CIRS scores, however, resulted strongly associated with higher values for CISS-Emotion ($p<0.001$).

Discussion and conclusions

This study supports the evidence that autoimmunity tends to differ according to cancer types and stages, in particular few defined immuno-stainings in colorectal cancer patients have been found with respect to patients with other malignancies. Thus, our study demonstrates that characteristic autoimmunity patterns exist for CRC patients, and are worthy to be investigated through a wider analysis for CRC stage, histological types, and oncological therapies already done. This work offers also an important contribution to the evidence that a higher non-specific autoimmunity is overall associated to an advanced oncological disease, presence of metastasis, and poorer prognosis independently from comorbidities. This can have an impact in the clinical setting in order to reconsider a higher expression of TAABs as a negative prognostic factor in the oncological follow-up, and therefore possibly affecting the subsequent management of oncological patients. Further larger prospective studies are necessary for this purpose.

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Dr. Martina MANNI

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STUDY OF "RISK AND RESPONSE-ADAPTED" THERAPIES IN PATIENTS WITH FOLLICULAR LYMPHOMA

Background

My PhD program is focused on clinical studies conducted to evaluate the efficacy of "risk and response-adapted" therapies in patients with lymphoproliferative disorders. In particular I am working on a project involving patients with follicular lymphoma (FL).

FL is one of the most common subtypes of lymphomas in Western countries and accounts for 10-20% of all newly diagnosed non-Hodgkin's lymphomas. The clinical course is typically indolent with impressive responses to initial treatment but with frequent relapse, with the need for recurrent therapeutic interventions. Response to salvage treatment is 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 of FL patients (up to 80%) 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.

At the present 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. Patients responding to initial therapy however show a heterogeneous behavior ranging from long lasting remissions to early relapse.

Objective

The identification of patients at different risk of progression using accurate techniques for response definition makes it reasonable to question if standard maintenance treatment 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 would need an intensified approach to achieve the same results in terms of outcome and progression free survival (PFS).

Recently 18F-fluorodeoxyglucose-positron emission tomography (FDG-PET) scan has been acknowledged as a recommended procedure for FL staging and response assessment. Moreover, the assessment of Minimal Residual Disease (MRD) has been evaluated as a prognostic tool in FL. This two techniques were separately shown to have a high prognostic power to predict the risk of progression in FL patients. Nevertheless, the impact of both PET and MRD performed at the end of treatment (EOT) in prognostic evaluation remains to

be determined. Therefore our study is focused on the ability of the two combined techniques to allow the stratification of patients in different risk subgroups and consequently modulate the maintenance treatment.

Methods

As already mentioned the two techniques investigated in this project are the MRD evaluation and the FDG-PET. The MRD assessment is performed by using a molecular biology technique, the polymerase chain reaction (PCR); in particular in FL, qualitative and quantitative PCR is performed for research of *BCL2/IGH* rearrangement. While the FDG-PET is a technique used in nuclear medicine based on the injection of a radiopharmaceutical associated with fluorodeoxyglucose. Bioimaging is obtained through a scanner marking the reactions occurring in the body. The resulting map shows the tissues in which the sample molecule is more concentrated (abnormal tissue). Therefore the FDG-PET provides information on function, as opposed to CT-scan and magnetic resonance imaging that provide only morphological information.

Results

In the first part of this project both FDG-PET and MRD were tested in a retrospective series of patients with FL. Patients were identified from the FOLL05 trial, a phase III, multicenter, randomized trial in patients with stage II-IV untreated FL. In order to be considered for the study, patients should have had data available on EOT PET, performed up to three months after the last dose of induction rituximab (+/- chemotherapy) and have been assessed for the *BCL2/IGH* at the EOT within 2 months from last dose. The primary endpoint was progression-free survival (PFS), that was calculated as the time from the date of treatment initiation until the date of lymphoma progression, relapse, death from any cause or last follow-up visit.

A total of 41 patients had available data on both PET and *BCL2/IGH* at the EOT. A stratified analysis was performed combining the information of both techniques, in which the 3-yr PFS were 78%, 50% and 27% in PET/MRD -/-, PET/MRD -/+ and PET+ groups, respectively (P=0.015 for all groups, and P=0.067 between PET/MRD -/- and PET/MRD -/+). We also stratified the patients into 2 groups (PET-/MRD- *vs.* PET+ or MRD+), in which the achievement of both PET and MRD negativity was associated to a better outcome (*P*=.012), with a 5-yr PFS of 75% and 35% for PET/MDR -/- and PET+, respectively.

Although conducted on a small set of patients, the strength of this study is the use of a blinded central review of FDG-PET scans and of a dedicated central laboratory for MRD analysis. The results show that combining both EOT FDG-PET and MRD analysis in patients with FL may improve our ability to predict the risk of progression, and provide the rationale to design response adapted trials in FL to tailor post-induction therapy to the real risk of relapse. Based on these results, the Fondazione Italiana Linfomi (FIL) planned the prospective FOLL12 trial to investigate the efficacy of the response-adapted strategy, using EOT PET and MRD studies in patients with FL.

Patients enrolled in this trial 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 assessed 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.

The FOLL12 study started three years ago and until now 525 patients have been enrolled. Of all patients, 39 are screening failures for different reasons, mainly for non-compliance with the inclusion criteria and for the informed consent withdrawal; 8 patients are still in the screening phase; 478 patients are actually randomized (238 in the standard arm and 240 in the experimental arm). Of 478 randomized patients, 50 dropped out of the study during or at the end of induction treatment (due to death, toxicity reasons, disease progression, patient or clinician decision, second malignancies), 159 are in the induction phase, while 269 are in the maintenance phase.

The central review of EOT PET images is available for 303 patients who completed the induction phase. In particular at the end of induction treatment 262 patients had a negative PET (low risk of progression), 40 had a positive PET (high risk of progression) and for one patient PET result was doubtful. Of the 40 PET+ patients, 17 were negative for the research of molecular marker, 7 were positive and 16 were not reassessed for MRD status at the EOT because were considered having “no molecular marker” at baseline; on the other side of all 262 PET- patients, 141 were negative for the research of Bcl2/IgH rearrangement, 19 were positive, 99 were with “no molecular marker” and for 3 cases the molecular analysis is still ongoing.

According to what previously explained, the above PET and MRD results were used to divide patients in risk subgroups and to modulate the subsequent course of treatment. However, data currently available are still not enough to be able to perform a preliminary analysis of the primary and secondary trial endpoints. A first interim analysis of PFS (primary endpoint) was planned after the 40% of expected events will be occurred.

Conclusions

The retrospective analysis performed on data of the patients enrolled in the FOLL05 trial allowed us to define the PET-scan and MRD techniques as possible prognostic factors in the treatment of FL. The results

obtained were used to build the ongoing FOLL12 trial with the final goal to provide clinicians a more rational use of the available diagnostic and therapeutic resources.

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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.

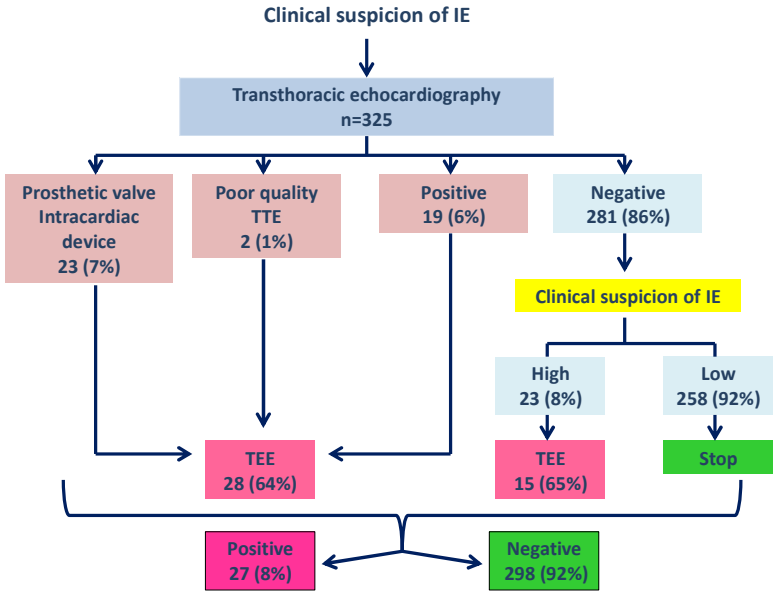
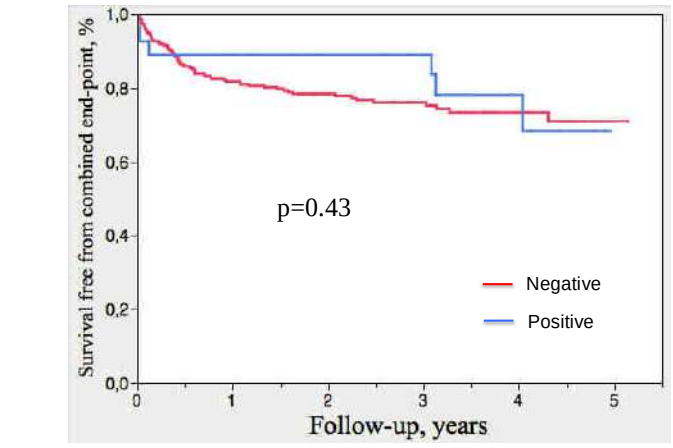


Figure 1 - Diagnostic congruity between 2012 ESC guidelines flow chart and our echo-lab activity in patients with clinical suspicion of infective endocarditis (IE) (from 2009 to June 2013).



Number 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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GHRELIN PLASMA LEVELS ARE DIFFERENTLY ALTERED BY ANTIEPILEPTIC DRUGS OR KETOGENIC DIET ONLY IN RESPONDERS

Background

Ghrelin is a peptide hormone prevalently released by the stomach (Chen et al., 2009) that has been suggested to possess anticonvulsant properties (Portelli et al., 2012). Additionally, ghrelin displays a variety of biological activities apart the main ones, which are represented by stimulation of growth hormone release and promotion of food intake (Warzecha et al., 2006). In particular, ghrelin is hypothesized to be involved in food intake dysregulation as well as in the metabolic syndrome (Horvath et al., 2001). For this reason, ghrelin levels could be related to abnormal body weight changes. However, inconsistencies in previous investigations did not allow to obtain any firm conclusion. Ghrelin was proposed to exert cardiovascular protective effects (Virdis et al.,2001) which do not appear to be explained by the stimulation of its only known receptor, the growth hormone secretagogue receptor 1a (GHS-R1a) (Gauna et al., 2007). Changes in plasma ghrelin levels have been investigated in patients affected by epilepsy in several studies (Giordano et al., 2014). A first report, based on the evaluation of 40 patients treated with valproate, revealed decreased ghrelin levels when comparing subjects affected by epilepsy and presenting obesity with patients in the normal range of body weight (Greco et al. 2005). Further investigation suggested a completely opposite alteration, as ghrelin levels were found to be increased independently of any body weight change (Berilgen et al., 2006). Furthermore others confirmed the increase in ghrelin plasma levels (Gungor et al., 2007), but this change was associated to valproic acid treatment leading to increased body weight, body mass index, and height. Notably, the majority of studies were on children, whereas in adults treated with various antiepileptic drugs (AEDs) ghrelin levels were decreased (Aydin et al., 2009) or unchanged (Varrasi et al., 2014). More recently, ghrelin levels were reported to be decreased in children treated with carbamazepine or valproic acid (Prodam et al. 2010), or to be unchanged under topiramate treatment (Ozcelik et al., 2014).

Objectives

To establish the changes in ghrelin plasma levels in children affected by epilepsy.

Materials and Methods

In the present investigation, we compared three different group of children, including: 1) control patients not affected by epilepsy; patients affected by epilepsy and fully responding to antiepileptic drug (AED) administration; 3) patient affect by refractory seizures. Additionally, part of patients belonging to the third group were subsequently treated with ketogenic diet. Ghrelin was determined by immunoassays.

Results

Notably, patients belonging to the second group presented a significant increase (more than 50%) in ghrelin plasma level when compared to the other groups. In the subgroup treated with ketogenic diet, in which all patients responded to the nutritional treatment, we observed a progressive decrease (-75%) in ghrelin plasma levels in the course of 3 month of observation.

Conclusions

These findings indicate that ghrelin levels are increased selectively in patients positively responding to AED administration, and that the reverse phenomenon is observed in refractory patients maintained on nutritional treatment. Thus, changes in ghrelin levels are in both cases possible predictors of response to therapy. In addition, the long term effects of these changes in ghrelin levels have to be carefully considered.

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CEM Curriculum: Health Sciences
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A METHOD FOR THE EVALUATION OF CUMULATIVE SOLAR RADIATION EXPOSURE IN OUTDOOR WORKERS: QUESTIONNAIRE BASED EVALUATION OF A SAMPLE OF PATIENTS TREATED FOR ACTINIC KERATOSIS AND NON MELANOMA SKIN CANCERS

Background

Long term Solar Radiation (SR) exposure is associated to various skin diseases like actinic keratosis (AK), basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and malignant melanoma (MM); SR as well as ultraviolet radiation (UVR) are carcinogens (class 1 IARC). Several individual factors are involved in the modulation of SR exposure: occupational activity is a relevant factor. In the European Union there are about 14.5 million outdoor workers (OR) and UVR is a carcinogen in 36 employment sectors.

Although the diagnosis and treatment of skin cancers have made great strides in recent years, currently the knowledge on an adequate dose-effect relationship (i.e. threshold levels for cumulative UVR exposure and increased cancer risk) is still incomplete. Possible tools to evaluate individual sun exposure are questionnaires, but to date in few studies quantitative or detailed semi-quantitative questionnaires were applied. A detailed evaluation of exposure habits and of the types of occupational and leisure activities is necessary for a more realistic estimation of the cumulative UVR received by the skin, to correlate chronic skin damage with individual SR exposure and to develop adequate preventive strategies to reduce the risk.

Objectives

To develop and test an original questionnaire for the evaluation of individual cumulative exposure to Solar Radiation (SR) in outdoor workers affected by AK, BCC and SCC.

Methods

An interviewer-administrated questionnaire, considering both working and leisure exposure, was collected to evaluate the whole-life history of solar UVR exposure in a group of patients of the Dermatologic Clinic of UNIMORE from January 2014 to August 2015.

Results

Fifty-eight questionnaires were collected in mainly male patients (81%) aged 43-91 years. With regards to occupation, the majority of the patients (57 %) reported an outdoor activity as the main profession in their life. No significant differences were observed between OW and indoor workers (IW) for the main examined demographic and pathologic characteristics.

AK was the most frequent skin disease, detected in 57.0% of the patients: 29.3% presented only AK, 13.8% AK and BCC, 10.3% AK and SCC. Comparing the two groups of workers, the frequency of AK in association with BCC or SCC was increased in OW: respectively 10.3% vs 0% for AK + BCC and 10.3% vs. 3.4% for AK + SCC. Regarding the number of skin lesions, in OW group the percentage of subjects with 11 to 15 skin lesions and with 16 to 20 lesions was higher than in IW group (7.1% VS 3.6% and 5,4% vs 0% respectively).
Considering the localization of skin lesions, OW were more likely to present lesions on the back, on the arms and on the face compared to IW. Analyzing protective habits during work activities, we found a significant association between the absence of skin lesions of the shoulders and neck and the habit to work "sometimes" or "often" in the shades. Regarding working postures, OW who adopted "sometimes" or "often" a bent-downward position were more likely to have skin lesions on the top of the head.
Regarding leisure time activities, our data showed a significant association between the frequency of exposure to UV tanning beds and the presence of skin lesions of the shoulders, neck and chest. With regard to vacation periods in summer season, the subjects who spent at least two hours outdoor between 11:00 a.m. and 3:00 p.m. revealed a lower age at the first diagnosis of skin diseases. In the same group also the total number of skin lesions was higher (8 vs. 6.5) compared to subjects who reported to spend 1 hour or less in the Sun during the central period of the day, but in this case the difference was not significant.

Conclusions

SR exposure is the main risk factor for chronic skin diseases such as AK, BCC and SCC. However, to date there are still some limits in the scientific knowledge, in particular regarding the association between these diseases and their characteristics (histopathology, localizations, age of onset, etc) and the cumulative UVR exposure, and regarding the effectiveness of protective devices in preventing skin cancers.
This study is aimed to deepen these aspects, albeit it has some limits related to the low sample size and the collection of subjective data. For a further develop of the method, allowing a semi-quantitative evaluation of UV cumulative exposure, an integration of the data collected with objective measurements is needed.
The results of this study show that OW were more likely to present AK simultaneously with NMSCs than IW, and had also a higher number of skin lesions. Skin lesions of the face were significantly more frequent in the OW group, whereas lesions of shoulders, neck and chest were less represented in OW reporting to usually work in shades. Also working posture was relevant: workers who usually adopt a downward bent position had more skin lesions on the top of the head than the other OW. With respect to leisure exposure, the study confirms the important role of recreational use of tanning beds and, considering vacation periods, the results remark the relevance of avoiding SR exposure during the central hours of the day.

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: Exosomes (30-100 nm) are membrane-surrounded extracellular vesicles (EVs) released by cells into interstitial space (both *in vivo* and *in vitro*) after the fusion of multivesicular bodies (MVBs) with the cytoplasmic membrane [1]. EVs regulate not only normal physiological processes (cell maintenance, tissue repair, immune surveillance and blood coagulation), but they are also involved in the pathology. An increased number of exosomes and alterations in their protein content has been observed in different tumors [2]. Researchers are strongly interested in the study of release of vesicles by cancer cells, because they carry a large number of tumor antigens that are secreted into the extracellular medium; thus they provide local and distant signals, playing a key role in intercellular communication. In breast cancer it was shown that some tumor markers, detected in the blood of breast cancer patients, were transported by vesicles [3]. Therefore, the characterization of proteins contained within these vesicles may be promising for the search of biomarkers in breast cancer tumors.

Objectives: Our first purpose was to develop a method for exosomes isolation, pure enough to allow a proteomic analysis of vesicles content. 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 [3]. Afterwards, the focus was shifted on exosomes purification from cell cultures: cells have been grown under usual conditions until they have reached confluence, then in a medium previously depleted of FBS-exosomes. Exosomes have been purified from this conditioned medium, through differential centrifugation [4]. For each cell line, the extraction has been performed in two set of experiments in quadruplicate to exclude experimental variation and ensure the reliability of the results obtained. To test the efficacy of the isolation method, obtained exosomes have been observed under a transmission electron microscopy (Nova NanoSem450 FEI), in order to quantify the number of vesicles and

their diameter [5]. Pellets (exosomes) have undergone lysis for protein extraction and total protein concentration has been determined by Bradford total protein assay. This has been followed by a proteomic analysis: two-dimensional electrophoresis, PD-quest analysis and Mass Spectrometry.

Results: The ultracentrifugation of plasma samples, proposed in other works, was not adequate to obtain a pure sample of exosomes: electron microscopy analysis showed the presence of vesicles in the range size compatible with the one reported in other studies, but a high dilution of the sample was necessary to obtain a reliable image, because there were a lot of protein aggregates. 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 so shifted on exosomes isolation from cell cultures. In this case, the ultracentrifugation method have allowed us to obtain a protein yield, from both cell lines, of about 1 µg/µl. Electron microscopy has revealed that the isolated particles consist of rounded vesicles with a mean diameter between 30 and 80 nm, included in the range that allows us to classify these vesicles as exosomes. A preliminary proteomic analysis has been performed [6]. Two subsequent investigations of isolation and two-dimensional electrophoresis have been carried out; in both analysis, eight total samples have been examined: four of exosomes from MDA-MB-231 and four from the silenced cell line. The resulting protein maps have been compared and 33 differentially expressed proteins, whose changes were statistically significant, have been processed and sent to the analysis in mass spectrometry (MS) for identification.

Conclusions: The exosomes purification method turned out to be efficient and pure enough to perform a proteomic analysis: the proteomic maps showed a lot of spots suitable for a qualitative and quantitative analysis. The analysis of the proteomic profiles of the vesicles pointed out differences in expression between the two cell lines: 33 differentially expressed proteins have been sent to the MS for identification.
The identified proteins will be studied in order to understand in what mechanisms they are involved. 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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CEM Curriculum: Health Sciences
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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 second 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

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Tutor: Prof. Manuela Simoni

EFFECTS OF CHRONIC ADMINISTRATION OF THE PHOSPHODIESTERASE INHIBITOR VARDENAFIL ON SERUM LEVELS OF ADRENAL AND TESTICULAR STEROIDS IN MEN WITH TYPE 2 DIABETES MELLITUS

Background:

Steroidogenesis is a complex enzymatic process in which cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) play an important role. Phosphodiesterase-5 inhibitors(PDE5i) increase cGMP, improving NO availability.

Objective:

to investigate whether long-term, chronic treatment with the PDE5i Vardenafil affects adrenal and testicular steroidogenesis in diabetic men, using liquid chromatography-mass spectrometry (LC-MS/MS).

Design:

A longitudinal, prospective, investigator-started, randomized, placebo-controlled, double-blind, clinical-trial was carried out.

Setting and Participants:

54 male patients affected by T2DM diagnosed within the last 5 years were enrolled. 26 and 28 patients were assigned to the verum and placebo-group, respectively.

Interventions:

The study consisted of an enrolment phase, a treatment phase (24weeks) (Vardenafil/placebo 10mg twice-daily), and a follow-up phase (24weeks).

Outcome measurements:

progesterone (P), 17-hydroxyprogesterone (17OHP), androstenedione (A), testosterone (T), dehydroepiandrosterone (DHEA), DHEA sulphate (DHEAS), corticosterone, 11-deoxycortisol and cortisol (C), were evaluated using LC-MS/MS.

Results:

No differences were seen in sex testicular steroids between study and control group. For the adrenal gland, steroids were considered according to the *zona* in which they are produced. Considering steroids produced in the *zona fasciculata*, no significant differences were seen in 11-deoxycortisol and C among visits, both in the study and in the control group. For the *zona reticularis*, DHEA significantly decreased during treatment

only in the study group (p=0.007). At post-hoc test DHEA showed higher levels at visit 2 and 8 than in other visits. The DHEAS/DHEAS ratio significantly increased during treatment only in the verum group. Considering the adrenal *zona glomerulosa*, corticosterone significantly changed among visits both in the study and in the control group (p<0.001). At post-hoc test, in both groups, corticosterone was significantly higher at visit 2 (p=0.028), 8 (p=0.003), and 10 (p=0.044), i.e. in coincidence with the complete clinical and instrumental examination performed only at these visits according to the study protocol.

Conclusions:

This is the first double-blind, placebo-controlled clinical-trial in which steroidogenesis is extensively investigated by LC-MS/MS in T2DM men chronically treated with Vardenafil for 6 months, and followed-up for 6 months after therapy-withdrawal. Chronically administered Vardenafil reduces DHEA levels and increases DHEAS/DHEA ratio as possible consequences of modulation of steroidogenic enzymes by tissue changes in cGMP and/or cAMP availability. A possibly stress-related increase in corticosterone is suggested for the first time.

Dr. Davide SOLOPERTO

CEM Curriculum: Translational Medicine
Tutor: Prof. Livio Presutti

STAPES MALFORMATIONS: THE CONTRIBUTE OF THE ENDOSCOPY FOR DIAGNOSIS AND SURGERY

Background

Ossicular chain abnomalies represent a rare condition, often associated with syndromic and more complex malformative forms. Clinical features of auditory ossicular malformations are really diversified, especially in children. Stapedial malformations can interest the foootplate suprastructure morphology, its articulation with the incus (monopodalic or hypoplasic stapes, crural abnomalies, absence of contact of incudo-stapedial joint), or the stapedial-oval window joint morphology (such as fixed anular ligament, hypoplasic or absent platina). With the introduction of the endoscope to middle ear surgery, knowledge of the anatomy of middle ear spaces has broadened and become clearer owing to better magnification and the possibility to look 'behind corners'.

Objectives

The aim of this study is to investigate the contribute of the endoscopic exclusive transcanalar approach for the management of stapes malformations.

Materials and methods

Between January 2008 and March 2014, 143 middle ear endoscopic surgery for conductive hearing loss were performed at the ENT Department of University Hospital of Modena and Verona. A complete audiological and neuroradiological preoperative assessment was made, to confirm indications for surgery and to check middle and inner ear status. Among 143 subjects who underwent surgery, 17 patients showed stapes malformation and underwent surgery with endoscopic exclusive transcanal approach. A complete audiological and radiological assessment before and after surgery was performed. A retrospective chart review was made at our tertiary referral centers.

Results

12/17 (70%) underwent a surgical endoscopic correction, In case of fixed platina underwent 5 endoscopic stapedotomy and 1 endoscopic stapedectomy were performed. In case of mobile platina 5 endoscopic ossiculoplasties with partial ossiculoplasty replacement prosthesis (PORP) were performed, 3 with autologous remodeling incus (IRA) and 2 with malleus head remodeling (MHR). In 1 case, only an endoscopic stapes mobilization was made. In 5/17 (30%), due to difficult anatomical findings an

endoscopic explorative tympanotomy was finally performed. The mean preoperative air conduction (AC), bone conduction (BC) and air-bone gap (ABG) were respectively 60.7 dB, 26.3 dB and 34.4 dB. The mean postoperative air conduction (AC), bone conduction (BC) and air-bone gap (ABG) were respectively 33.8 dB, 26.5 dB and 7.3 dB, with a mean improvement of the ABG of 27.1 dB. Discharge from hospital was on the first post-surgery day. No relevant post-operative complications were noted. The median follow up was 3.6 years (range 1-6).

Conclusions

The endoscopic approach results very adequate for the diagnosis and treatment of stapes malformations, checking variations of the ossicles conformation and functioning and performing safe surgery, under direct control of middle ear structures.

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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 specific brain areas, may be crucial to understand brain metabolism and how it can be manipulated.

5-Arylbenzothiadiazine type compounds acting as positive allosteric modulators of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA-PAMs) are under particular investigation in the past decade for their nootropic activity and lack of excitotoxic side effects of direct agonists. 7-chloro-5-(furan-3-yl)-3-methyl-4H-benzo[e][1,2,4]thiadiazine 1,1dioxide is one of the most active benzothiadiazine-derived AMPA-PAMs *in vitro* to date. It exists in the form of two stereolabile enantiomers, which rapidly racemize in physiological conditions. The parent compound is converted by hepatic cytochrome P450 to pharmacologically active unsaturated benzothiadiazine and to corresponding inactive benzenesulfonamide.

Objectives

The aim is to develop a method to evaluate the ability to cross brain-blood barrier and effects of saline and acute systemic administration of unsaturated benzothiadiazine derivative of 7-chloro-5-(furan-3-yl)-3-methyl-4H-benzo[e][1,2,4]thiadiazine 1,1dioxide on the release of neurotransmitters such as dopamine (DA) , 5-hydroxytryptamine (5-HT) and (ACh) in the mouse nucleus accumbens (NAc) and Hippocampus (Hip).

Methods

In the first step cerebral microdialysis was employed. Two microdialysis probes were inserted in the mouse Hip and NAc and simultaneously perfused with artificial cerebrospinal fluid (aCSF). During the experiment one group of animals was treated with saline and compared to another group with an acute systemic administration of unsaturated derivative (2 mg/kg ip).

In the second step liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) was performed to determine concentration of unsaturated derivative of AMPA-PAM and evaluate levels of DA, 5-HT and ACh in microdialysates.

Results

Results indicates that the unsaturated derivative is **able to cross blood-brain-barrier and reach the Hip and NAc** within 20 min from systemic administration without significant differences in the two cerebral areas. Its concentration increased to a maximum value of about 20 nM after 80 min and remained constant for the next 60 min. Taking into account calculated microdialysis probe recovery (about 12%), the actual concentration of the compound in Hip and NAc extracellular fluid should reach about 0,17 µM 80 min after its systemic administration of 2 mg/kg ip. Afterward its Hip and NAc dialysate concentration slightly decreased to about 10 nM in the next 6h. The presence of the parent AMPA-PAM and benzenosulfonamide derivative in Hip and NAc extracellular fluid was excluded.

The administration of unsaturated form of the AMPA-PAM modulator were followed by about 140% increase in **5-HT** concentration in Hip after 40 min and then slowly turned back to the basal levels. In NAc **5-HT** release showed a peak that reached about 200% of basal levels 20 min after administration and in the next 20 min rapidly returned to basal levels. Furthermore, the difference in **5-HT** concentration was statistically significant both in Hip and NAc.

ACh concentration was also increased in Hip and reached a peak of about 200% 80 min after administration and then rapidly returned to basal levels. Moreover, the difference in **ACh** levels both in saline and drug treated mice was statistically significant. **ACh** concentration in NAc elicited significant increase in the first 20 min after administration and was followed by rapid decrease slightly under the basal levels. No significant difference in **ACh** release in saline and drug treated mice was noted.

Drug administration has no effect on **DA** release which remained constant throughout the experiment both in Hip and in Nac. Additionally, the difference in **DA** concentration levels between saline and drug treated mice was no statistically significant.

Conclusions

Results reported that unsaturated benzothiadiazine derived AMPA-PAM is able to cross blood-brain barrier and reach the hippocampus and nucleus accumbens. It increases 5-HT and ACh release, but not DA release both in Hip and NAc. The increase in 5-HT suggests that AMPA-PAMs could affect mood and motivation. While, increase in ACh concentration levels indicates that AMPA-PAMs could exert their nootropic activity also via the potentiation of cholinergic transmission.

Results presents that combination of cerebral microdialysis technique to collect samples with brain metabolites with the following analysis by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) can be successfully applied to evaluate the levels of bioamines, brain metabolism and how it can be manipulated by pharmacologically active substances.

Dr. Roberta VALSECCHI

CEM Curriculum: Translational Medicine
Tutor: Prof. Sandra Marmiroli
CoTutor: Dr. Rosa Bernardi

ROLE AND REGULATION OF HIF-1a TRANSCRIPTION FACTOR IN CHRONIC LYMPHOCYTIC LEUKEMIA

Background: HIF-1a is an essential regulator of cell and tissue adaptation to hypoxia, and is often upregulated in tumors due to intratumoral hypoxia or activation of oncogenic pathways. HIF transcription factors consist of a and b subunits, while b subunit is constitutively expressed, HIF-1a is remarkably high during hypoxia because in hypoxic conditions, HIF-1a is stabilized, moves in the nucleus, heterodimerizes with HIF-1b and forms an active complex that it promotes the transcription of several genes mediating tissue and cellular adaptation to hypoxia. Recent evidence indicates that HIF-1a is implicated in the development of hematological malignancies including chronic lymphocytic leukemia (CLL). CLL is the most common leukemia in adults and is characterized by the accumulation of mature CD5+ B cells in peripheral blood (PB), bone marrow (BM) and secondary lymphoid organs. It has been demonstrated that in CLL B cells HIF-1a is aberrantly expressed due to normoxic miRNA-directed downregulation of pVHL. Abnormal upregulation of HIF-1a was suggested to lead to high VEGF production, thus causing the increased BM and lymphoid tissues neoangiogenesis observed in CLL patients

Objectives: CLL B cells critically depend on physical and functional interactions with stromal microenvironments in the BM and lymphoid organs. Because preliminary data of gene expression profile revealed that in CLL cells HIF-1a regulates the expression of a number of chemokine receptors and cell adhesion molecules known to promote the interaction of leukemic cells with protective microenvironment, we hypothesize that HIF-1a regulates 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-1a factor is investigated by using *in vitro* migration and adhesion assays in MEC-1 and patients' cells upon silencing by using different strategies either by shRNA-mediated interference, oligonucleotides or pharmacological inhibition and also in vivo by using CLL mouse models.

Results: We found that in chronic lymphocytic leukemia (CLL) HIF-1a is a novel regulator of the interaction of CLL cells with protective leukemia microenvironments, and is in turn regulated by this interaction in a positive feedback loop that promotes leukemia survival and propagation. Inactivation of HIF-1a impairs cell adhesion to stroma and chemotaxis, reduces bone marrow and spleen colonization in xenograft and

allograft CLL mouse models, and prolongs mice survival. Interestingly, we found that in CLL cells HIF-1a is transcriptionally regulated upon coculture with stromal cells. Furthermore, HIF-1α mRNA levels vary significantly within CLL patients, and correlate with the expression of HIF-1a-target genes including CXCR4, thus further emphasizing the relevance of HIF-1a expression to CLL pathogenesis.

Conclusions: Our results demonstrate that HIF-1a plays critical and pleiotropic roles in CLL pathogenesis and suggest that it may represent a new therapeutic target for CLL treatment to be evaluated by future preclinical experiments.

cycle XXX

Dr. Lucia BORSARI

CEM Curriculum: Health Sciences
Tutor: Prof. Marco Vinceti

RISK FACTORS ASSOCIATED TO ADVERSE PREGNANCY OUTCOME IN WOMEN WITH PRE-GESTATIONAL DIABETES

A RETROSPECTIVE COHORT STUDY IN TWO PROVINCES OF THE EMILIA-ROMAGNA REGION

Reliability of Hospital Discharge coding for pre-gestational diabetes research and disease surveillance

Background

In recent years prevalence of pre-gestational diabetes (PGDM) in pregnancy is rising, reflecting the increase in the prevalence of both type I and type II diabetes and in obesity in women of childbearing age (1,2), and there is increasing concern about possibly associated maternal and neonatal complications. Accurate population-based researches and prevalence estimates on pre-gestational diabetes are of key importance for understanding the burden of disease, time trends and maternal and neonatal outcomes. Routinely collected health data, such as Hospital Discharge (HD) records, represent a timely and cost-efficient approach for the identification of large cohorts of diabetic pregnant women, and in addition they may be particularly useful in assessing infrequent, but clinically important, outcomes.

Several studies have examined the accuracy of administrative data for identification of diabetes in the general or childhood population, while only few authors from Canada and US have evaluated the pregnant subgroup (3,4). Moreover, they have highlighted that use of administrative databases for definition of diabetes in subpopulations such as pregnant women could result challenging. During the last years, multiple electronic data sources have been integrated to create Diabetes Registers (DRs), enhancing diabetes case identification and classification. In Italy a national DR is not available yet. To date, only few patient registers exist at regional or provincial level and they have only recently been implemented (5,6). Therefore, in Italy the use of HD coding still represent one of the most important method for case ascertainment in PGDM research and diseases surveillance.

Objectives

Main objective of my 3-years research project is to assess risk factors that mainly influence adverse pregnancy outcomes (birth defects, macrosomia, miscarriage and maternal complications) in pregnant women with PGDM. The first step of the study consists in a validation analysis to investigate the reliability of HD coding to identify the population-based cohort of pregnant women with PGDM and to assess trends in PGDM prevalence.

Methods

We used HD to identified all deliveries that occurred in the period 1997 – 2010 in Modena or Reggio Emilia. Then we selected pregnant women with PGDM by the presence of ICD-9-CM codes 250.XX or 648.0 listed anywhere on the HD records. For each selected HD record, we randomly selected five controls from the non-diabetic mothers, matched according to year of birth and delivery, province of residence and referral hospital. We used DRs, implemented in Modena and Reggio-Emilia since 2010 and 2009 respectively, as gold standard for the validation analysis. Considering the retrospective design of the study, we used the date of diagnosis, when available, or the start date of disease-specific exemption as a proxy to verify if they were already diabetic in the delivery period. We further verified all doubtful cases contacting General Practitioners (GP) by e-mail or phone.

We calculated the general accordance between diagnostic codes defined in HD records and DRs, both as proportion and using Cohen kappa index. Then we calculated sensitivity, specificity, positive and negative predicted value. Finally, we estimated the row and age-specific prevalence of deliveries complicated by PGDM per 1000 total deliveries, comparing pre- and post-validation rates.

Results

Using delivery-related ICD-9-CM, we identified 3800 women, 653 diabetic and 3147 non-diabetic women, residing and delivering in Modena or Reggio E. in the period 1997-2010. The accordance between diagnosis defined by HD records and DRs was 90.7% for the total sample, with K = 0.58. In particular, we detected 350 false positive and only one false negative. Sensitivity was 99.3% (IC 95% 97.7% - 99.9%), specificity 90.0% (IC 95% 88.9% - 91.0%), PPV 46.4% (CI 95% 42.5% - 50.3%) and NPV 99.9% (CI 95% 42.5% - 50.3%). Out of the 350 false positive, 170 (48.6%) had gestational diabetes (GDM), 8 (2.3%) were affected by impaired glucose tolerance (IGT), while for 172 women (49.1%) GDM or IGT were not excluded, but we did not have sufficient information to define any diagnosis. We did not find any improvement in the use of ICD-9-CM codes for PGDM over the years. In a more detailed analysis among the 653 women with HD code for PGDM, we found a better accuracy of the codes in the province of Modena than in Reggio-Emilia (64.3% vs 34.3%, $p<0.001$) and among Italians than foreign women (51.5% vs 38.7%, $p<0.001$), while no significant differences emerged from the analysis by age groups. After the validation process the overall rate of PGDM resulted halved from 4.4 (CI 95% 4.0 – 4.7) to 2.0 (CI 95% 1.8 – 2.3) per 1000 deliveries. In the prevalence analysis by province of residence the validation process flattened the difference between Modena and Reggio E. (respectively, 3.1 and 5.9 in the pre-validation analysis vs 2.0 and 2.0 in the post-validation analysis). The age-specific rates highlighted a rising trend with age, both in the pre and post-validation analysis. Our assessment did not alter the previously evidenced higher prevalence of diabetes for immigrant women, although the difference resulted less evident (post-validation analysis: 3.3 vs 1.7).

Conclusions

Our results showed that HD coding allowed to find almost all PGDM cases (sensitivity = 99.3%), but it included also a large number of 'false positive'. Actually, more than half women classified as diabetic through HD coding were not present in the provincial diabetes registers (PPV = 46.4%), and after validation with GP and Diabetes Services we found that most of them had been affected by GDM during pregnancy. Although ICD-9-CM provides different codes to identify these two conditions (250.0 or 648.0 for PGDM and 648.8 for GDM), our study showed that GDM is often coded as 250.0 or 648.0. As a result, PGDM prevalence calculated using HD records could be largely over-estimated. In conclusion, despite HD coding accuracy results overall improved during the last years, our results show that this improvement could not involve all diseases and specifically pregnant women affected by pregestational diabetes. Our findings argue for accuracy evaluation of HDs before applying them widely to epidemiologic research, public health surveillance or population-level monitoring of care quality related to diabetes during pregnancy.

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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

It is nowadays possible to observe a remarkable increase in the use of natural products as supplements or substitutes to conventional drugs in the developed countries, triggered by many scientific results that demonstrate their effectiveness in the prevention of several diseases [1]. Herbal products are characterized by a complex mixture of different compounds (also known as plant secondary metabolites). Thus, a complete definition of all the phytochemical constituents of plant extracts by means of advanced analytical techniques, such as *metabolite profiling* and *fingerprinting*, is needed to ensure their composition, reliability and safety [2]. Moreover, medicinal plants represent potential sources of new bioactive molecules and, in this perspective, the isolation and characterization of bioactive natural compounds from plant material play a key role [3].

My research activity in this PhD was focused on one plant of nutraceutical interest (*Punica granatum* L., pomegranate) and one of pharmaceutical interest (*Cannabis sativa* L., hemp), due to the bioactivity of their components.

Pomegranate has recently gained an increasing interest, due to the health-promoting properties (antioxidant, antibacterial, anti-proliferative [4]) 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 [4].

Hemp and its preparations have been used for a long time in folk medicine [5] and, nowadays, they are gaining a renewed increasing interest, thanks to the biomedical relevance of the peculiar terpenophenolic constituents, namely the cannabinoids. In general, the main cannabinoids present in fiber type plants are cannabidiolic acid (CBDA) and/or cannabigerolic acid (CBG), followed by their neutral forms cannabidiol (CBD) and cannabigerol (CBG), while the content in the psycho-active tetrahydrocannabinol (THC) is below 0.2% [5]. From a medicinal point of view, CBD represent the most interesting cannabinoid, possessing a high anti-oxidant and anti-inflammatory activity as well as neuroprotective, anxiolytic and anticonvulsant properties [6]. Cannabinoids are usually analyzed by using GC-FID and GC-MS techniques, which lead to the decarboxylation of the native acidic compounds to their neutral forms, due to the high temperature reached; therefore a true cannabinoid profiling of the plant material is not possible with these techniques [5].

Objectives

The study on pomegranate was focused on the development of a new analytical method for the *metabolite fingerprinting* of polyphenols in pomegranate juice and peels.

As regards hemp, the aim of the work was the development and validation of a reliable method for the *metabolite profiling* of the main bio-active compounds in hemp cultivars of different origins, in order to select those that could be applied for the preparation of extracts with high pharmaceutical value. The study was also addressed to the isolation of phytocannabinoids from specific hemp varieties, with particular attention to cannabidiol (CBD).

Methods

The *metabolite fingerprinting* of pomegranate polyphenols in all the fruit constituents was achieved by means of a RP-HPLC method coupled with UV/DAD and ESI-MSn detection, based on the use of a fused-core stationary phase.

The *metabolite profiling* of hemp non-psychoactive phytocannabinoids was performed by developing a RP-HPLC method coupled with UV/DAD and ESI-MSn detection taking again advantage of the fused-core stationary phase. Ultrasound assisted extraction (UAE) was selected as the extraction technique with methanol as the extraction solvent.

The purification of non-psychoactive cannabinoids from hemp samples was carried out by means of normal phase preparative liquid chromatography (NP-LC) with silica gel as the stationary phase and chloroform and methanol as the mobile phase. The structure of the two isolated compounds was confirmed by the use of NMR spectroscopy.

Results

As regards pomegranate, the quali- and quantitative analysis of polyphenols in all the fruit constituents was performed by HPLC-UV/DAD, HPLC-ESI-MS and MS^2 analyses. The application of the fused-core column technology allowed us to obtain an improvement of the HPLC performance in comparison with that of conventional particulate stationary phases, enabling a complete separation of all constituents in a shorter time and with low solvent usage. The method validation was performed to show compliance with ICH guidelines [7]. The validated technique was successfully applied to the characterization of commercial and experimental pomegranate cultivars, which were also subjected to principal component analysis (PCA), thus demonstrating to be an efficient tool for the fingerprinting of this plant material.

As regards hemp, the phytochemical analysis of hemp non-psychoactive phytocannabinoids were performed by developing a new RP-HPLC method coupled with UV/DAD and ESI-MS^n detection, by taking again advantage of the fused-core stationary phase.

The analytical method optimized in this study was completely validated for linearity, sensitivity, accuracy and precision to show compliance with international requirements (ICH guidelines) [7], thus demonstrating

to be a valuable tool for the analysis of both acidic and neutral cannabinoids in the raw plant material. The validated method was then applied to the quantification of the main non-psychoactive cannabinoids (CBGA, CBDA, CBG and CBD) for variety distinction of nine hemp samples. Two of the analyzed samples were found to be highly rich in CBD and CBDA and, thus, they were selected for the isolation of the aforementioned compounds. The purification of non-psychoactive cannabinoids from these samples was carried out by means of normal phase preparative liquid chromatography (NP-LC) with silica gel as the stationary phase and chloroform and methanol as the mobile phase. Preparative LC allowed us to obtain the isolation of two fractions with a high content in CBD (99.6%) and CBDA (92.5%), respectively, with a mean yield of 1.2% w/w from the raw material. The isolated fractions were submitted to NMR spectroscopy in order to confirm the structure of the isolated compounds. The chemical shifts obtained were compared with those described in the literature for cannabinoids, and the two purified compounds were confirmed to be CBD and CBDA.

Conclusions

Two reliable RP-HPLC methods coupled with UV/DAD and ESI-MSn detection, both based on the use of a fused-core stationary phase, were developed and validated to perform the *metabolite fingerprinting* and *profiling* of the secondary metabolites in pomegranate and hemp. The methods were applied to several pomegranate and hemp varieties for the selection of those with a higher content in bioactive compounds, demonstrating to be valuable tools for the phytochemical analysis and the quality control of these plant materials. The purification of the main bioactive phytocannabinoids CBD and CBDA was achieved too, as the two compounds were successfully isolated by means of NP-LC and their structure was confirmed by using NMR spectroscopy. Further investigations will be performed on hemp*,* including the assessment and the application of a selective and innovative extraction technique on specific hemp varieties in order to obtain extracts with a high content in non-psychoactive cannabinoids that will be tested for their biological activities.

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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 47 HIV patients have been transplanted so far. In particular, 42 HIV infected patients underwent a liver transplant and 5 had a combined kidney-liver transplant. 16 HIV transplanted patients out of 47 have died so far.

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.

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 infections still remain the leading cause of morbidity and mortality among SOT recipients. 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 2015. 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.

The study was approved by the Ethical Committee (protocol number 163/15, 23th October, 2015). Each participant will provide written informed consent for testing and analysis of samples.

All of the immunological studies will be performed in collaboration with the Chair of Pathology and Immunology (Prof. Andrea Cossarizza).

We will collect 30 ml of peripheral blood. Plasma will be stored at -80°C until use, mononuclear cells (PBMC) will be isolated according to standardized methods and immediately used.

Part of PBMC will be used for functional analysis, by using methods based upon polychromatic flow cytometry and a novel 16 parameter flow cytometer, able to detect up to 35,000 cells/second (Attune NxT, Thermo Fisher). Cells will be stimulated with different recall antigens and phenotyped for the identification of markers of activation and differentiation (using mAbs anti- CD3, CD4, CD8, CD45RA, CCR7, CD38, CD127, CD279, HLA-DR), along with mAbs for the identification of up to 4 intracellular cytokines.

The other part of PBMC will be used for molecular analysis. In particular, monocytes will be isolated by magnetic sorting using an anti-CD14 mAb; then RNA will be extracted for the real time PCR analysis (by using a CFX96, BioRad) and quantification of genes involved in the activation and regulation of the inflammasome, such as NLRP3, AIM2, NLRC4, NLRX1.

Results

The study was approved by the local institutional review board (protocol number 163/15, 23th October, 2015).

I selected patients eligible for the project, matching HIV-positive with HIV-negative patients and controls. I obtained funds to carry on the study.

I procured the peptides for detecting the specific T cell response to different recall antigens of bacterial, viral, and fungal origin (such as CMV, *Candida* spp., smallpox, tetanus toxoid) and the monoclonal antibodies required for the detection of surface molecules associated to activation and differentiation, and for the identification of intracellular cytokines.

Next week we will start to analyze samples of control patients to definitely set up cytofluorimetric assays and the molecular method for the identification and quantification of inflammasome genes.

Dr. Lavinia GIVA

CEM Curriculum: Translational Medicine
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TRANSGENIC MOUSE MODELS TO INVESTIGATE THE ROLE OF HDL BOUND SPHINGOSINE-1-PHOSPHATE (S1P) IN VIVO

Background

S1P is a lysosphingolipid which regulates many important biological functions, such as cellular proliferation, survival and differentiation, through the interaction with five specific sphingosine 1-phosphate receptors (S1PRs) belonging to the G-protein coupled receptor superfamily. Therefore, it is not surprising that S1P is implicated in a wide range of diseases including atherosclerosis, diabetes, cancer and inflammatory disorders. In plasma, S1P is associated with the high density lipoproteins (HDL), and several studies documented an inverse relationship between HDL cholesterol levels and the extent of atherosclerotic disease. These findings suggest that the HDL atheroprotective effects could be partially attributed to S1P, in particular through the stimulation of S1PR1/3, on vascular wall cells (macrophages, endothelial cells and smooth muscle cells). Furthermore, S1P analogues, characterized by a selective agonist activity towards S1P1, exert appreciable anti-inflammatory effects in macrophages both in vitro and in vivo. Finally, S1P analogues reduce the development of murine atherosclerosis.

Objectives

In the light of that, we generated transgenic mice, able to overexpress S1PR1 or S1PR3 in specific target tissues, in order to elucidate S1P effects and to explore underlying mechanisms in vivo (S1P1-Lyz and S1P3-Lyz mouse models).

Methods

We obtained S1P1 and S1P3 knock-in mouse models by homologous recombination in embryonic stem cells, using an optimized targeting vector containing homologous genomic Rosa26 sequences. In these mice, both S1P1 and S1P3 genes were positioned under control of the CAG promoter, which is separated from S1P receptor genes by a LoxP-flanked transcriptional STOP element (CAG-Lox-STOP-Lox-S1PR). Hence, transgenes will be only expressed after the removal of Lox-STOP-Lox element by Cre-mediated recombination. That will be achieved by crossing Rosa26- S1P1 or Rosa26-S1P3 mice with ones modified with the Cre recombinase under control of the Lysozyme promoter (Lyz-Cre), a macrophage specific promoter. 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 generate athero-prone strains. For the molecular and functional characterization, nucleic acids and proteins were extracted from peritoneal macrophages and quantified for downstream applications, such as Real Time PCR, Western Blot and Immunofluorescence in order to detect receptors, cytokines or cholesterol transporters.

Results

These worldwide unique mouse models, based on the Cre-Lox technology, allow for 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.

Conclusions

The tissue-specific approach designed in this project selectively targets S1P pathways, offering insights into the underlying pathophysiological mechanisms. These worldwide unique mouse models may be effectively exploited to investigate the effects of endogenous S1P on the modulation of inflammatory responses associated to the atherogenesis as well as in a wide range of chronic inflammatory diseases.

Dr. Maurizio GRECO

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

Background

The meaning of the mechanism of interaction between lasers and tissues, is still not completely clear. A review of literature shows only data about histopathological changes after laser treatment. To obtain this information, several skin biopsies were performed, often on face or other sensitive areas. The main limitation is the inevitable scar formation after the biopsies.

Usually, lasers are considered safe treatments with a rapid healing time. Post-treatment adverse effects may occurs, such as 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 wavelengths and energies, and to discover the optimal light source to target a specific tissue or a disease.

Methods

My research interest is to analyze laser-tissue interactions and biologic effects of various laser sources by means of in vivo laser confocal microscopy. Many skin diseases (non-melanoma skin cancer, melasma, sarcoidosis, etc.) could be treated with different laser sources, in particular CO2, fraxel CO2, NdYag 532/1064 and CW 532/1064. Whereas distinct lasers interact differently with tissues, my purpose is to investigate the effects both in ex-vivo cell cultures and in in-vivo settings.

Reflectance confocal microscopy (RCM) is a non-invasive imaging technique that allow to acquire skin images with a histological resolutions.

First study - Combination of laser CO2 photodynamic therapy (PDT) and in vivo laser confocal microscopy in the treatment of basal cell carcinoma (BCC)

Two patients with histological diagnosis of nodular BCC and two with histological diagnosis of infiltrative BCC of the face, have been treated with this combined modality. We performed a long-term follow-up using clinical and dermoscopical documentation and RCM images.

Second study – Efficacy of fractional ablative CO2 laser therapy for striae distensae.

18 patients (16 women and 2 men) with stretch marks were treated with three sessions of fractional CO2 laser, once a month. Improvement has been evaluated by comparing pre- and post- clinical pictures and RCM images. This study has established the efficacy and safety of fractional CO2 laser therapy for striae distensae.

Results

The results of these studies would highlight the use of laser therapy as a choice in both inflammatory and tumoral skin diseases, in particular for the cases that can't be treated efficaciously with other treatments. Furthermore, these techniques has a high safety profile. The outcome is to improve the knowledge about this topic and to offer less invasive and safer treatments for the patients.

First study - Combination of laser CO2 photodynamic therapy (PDT) and in vivo laser confocal microscopy in the treatment of basal cell carcinoma (BCC)

Preliminary results have shown that the mean age is 48 years with a range of 32 to 80. Patients are still in follow-up (3 years). Only one patient had a recurrence (infiltrative BCC) after 6 months of follow-up, it was performed the same therapy again and he is still in complete remission. No significant complications were observed.

Second study – Efficacy of fractional ablative CO2 laser therapy for striae distensae.

Clinical improvement is visible by pictures comparing. RCM images have shown significant changes after the therapy, in particular the progressive improvement of the collagen in the dermis and the restoration of the dermal-epidermal junction architecture. In any case, worsening of striae distensae has been detected.

Conclusions

First study - Combination of laser CO2 photodynamic therapy (PDT) and in vivo laser confocal microscopy in the treatment of basal cell carcinoma (BCC)

This combination of laser and PDT led to complete recovery of the diseases (BCC nodular and infiltrative) and, moreover, a better aesthetic outcome than surgery.

Second study – Efficacy of fractional ablative CO2 laser therapy for striae distensae

These results support the use of ablative CO2 fractional laser as an effective and safe modalities to treat striae distensae.

Dr. Eleonora MARETTI

CEM Curriculum: Medicinal and Pharmaceutical Sciences
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CoTutor: Dr. Valentina Iannuccelli

LIPID MICROCARRIERS FOR ANTI-TB INHALATION THERAPY: RESPIRABILITY OPTIMIZATION BY EXPERIMENTAL DESIGN AND MANNOSE-BASED SURFACE ENGINEERING

Background

The pulmonary route appears the most reasonable and effective way to target the alveolar macrophages (AM) and eradicate surviving *Mycobacterium tuberculosis* at the primary infected site of tuberculosis (TB), especially considering that 75-80% of TB cases remain localized in the lungs. The anti-TB therapy by inhalation offers benefits compared with the current treatment in terms of patient's compliance improvement, reduction in dose amount and frequency, treatment duration and TB diffusion in other organs, thus minimizing the risk of drug-resistant mutants, toxicity and side effects. In a previous research, for a direct intramacrophagic antitubercular therapy by pulmonary administration using Dry Powder Inhaler (DPI) devices, Solid Lipid Microparticles (SLM) were developed in order to allow rifampicin, a first-line antitubercular drug, to be deposited in the lungs and be taken up effectively by AM for a rapid onset of the pharmacological action. The designed SLM were found proper for aerodynamic diameter, poor cytotoxicity and viable cell internalisation ability assayed on murine macrophages J774 (1).

Powder respirability optimization

Objectives: The efficacious aerosolization of a powder requires a good respirability to assure proper emitted dose and lung alveolar deposition.

Methods: For the achievement of the powder aerodynamic performance, rifampicin-loaded SLM were produced using the melt emulsifying technique followed by freeze-drying. However, freeze-drying technique, considered the most commonly method used to convert lipid particle dispersions into solids avoiding degradation reactions, can cause freezing or drying stresses inducing particle aggregation or irreversible fusion compromising the proper size (2) and the powder respirability. Therefore, the research intended to offer novel information regarding the effects of freeze-drying variables (type and concentration of cryoprotectants, freezing conditions, and microparticles concentration in the suspension before freezing) on SLM characteristics (size, polydispersity index, zeta-potential, circularity, bulk and tapped density) and powder respirability (aerodynamic diameter, emitted dose, and respirable fraction) in order to find which of these parameters may play a crucial role in protecting particles from stresses and maximise the respirability of the powder for inhalation. Rifampicin thermal stability during the production phases and the effect of the freeze-drying parameters on microparticle drug loading levels were also investigated.

Considering the complexity of the factors involved in a successful respirable powder, a Design of Experiments (DoE) approach was adopted as a statistical tool for optimising the freeze-drying procedure conditions.

Results: The SLM samples identified by DoE exhibited an irregular shape and features in the following value ranges: size (0.47 – 1.70 µm), polydispersity index (0.32 – 0.94), zeta-potential (-41 mV to -50 mV), circularity (0.43 – 0.66), bulk density (0.02 – 0.24 g/cm^3), tapped density (0.04 – 0.31 g/cm^3), aerodynamic diameter (0.31 - 1.02 µm), emitted dose (92.1-103.4%), fine particle fraction (8.77-70.40%), and drug loading level (11.85 – 15.88%).

Conclusion: Interestingly, the most favourable impact on the powder respirability was offered by quick-freezing combined with a certain grade of sample dilution before the freezing step without the use of cryoprotectants. In such conditions, a very high level of microparticle respirability (>50%) was achieved along with acceptable yields in the final dry powder as well as the reduction of the powder mass to be introduced into DPI capsules with benefits in terms of administered drug dose feasibility. In addition, rifampicin payloads were not distorted by thermal degradation under the adopted process conditions (3).

Mannose-based surface engineering

Objectives: Macrophages possess mannose-specific membrane receptors (MR) that can be recognized by carriers bearing mannose residues, facilitating their internalization. Therefore, as especially the infected AM overexpress MR, to further increase specificity for macrophage internalization, achieving an AM active targeting, the project considered the functionalization of SLM surface by mannose derivative used as the co-stabilizer in the SLM formulation.

Methods: The highly respirable SLM loaded with rifampicin were modified to improve drug loading level and release as well as AM internalization. Several biocompatible lipid components such as fatty acids and their derivatives, diglycerides and triglycerides were processed using mixtures of biocompatible stabilizers (sodium taurocholate and methyl mannopyranoside) in order to obtain SLM with maximum efficiency in terms of drug loading, release in simulated lung fluid, and AM phagocytosis. Lipids in the liquid physical state embedded into SLM provided Microstructured Lipid Carriers (MLC) that are known to exhibit superior advantages over SLM such as enhanced drug loading capacity and prevention of drug expulsion intended to maximize the drug concentration at the primary site of TB infection. The obtained microcarriers were examined for their intrinsic properties (size and size distribution, morphology and shape, surface charge, bulk and tap density, aerodynamic diameter, flowability, physical state of the components, drug loading and release) according to the operative parameters.

Results and conclusion: SLM mannosylation was investigated by means of Electron Spectroscopy for Chemical *Analysis (ESCA),* surface *hydrophobicity* measured by *Rose Bengal adsorption*, and Energy Dispersive X-ray Analysis (EDX) demonstrating the presence of mannose derivative on the particle surface.

Work in progress

Mannosylated derivatives of fatty acids will be synthesized as novel SLM stabilizers with improved Hydrophile Lipophile Balance and used at various concentrations. Prototypes of SLM in terms of successful functionalization, optimal breathability and chemico-physical stability, will be examined for cytotoxicity by MTT test on murine macrophages J774 and alveolar macrophages NR8383 cell lines. Further, following Red Nile labelling, SLM will be localized intracellularly by flow cytofluorimetry and confocal laser microscopy. Percoll density gradient centrifugation technique will be employed for the actual quantitative determination of rifampicin within macrophages.

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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

In the preliminary part of the study, patients undergoing treatment with SBI, were visited before initiation and ones per month during therapy. A control group of patients not undergoing SBI treatment was also enrolled. The appearance of non-melanocytic skin cancer, i.e. squamous cell carcinomas (SCC) and keratoacanthomas (KA) was also registered and correlated with the presence of atypical melanocytic lesions. Each of the lesions 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.

As an additional part of the study, we evaluated the clinical and dermoscopic features of the melanomas harboring BRAF mutation. We analyzed the lesions morphologically by means of dermoscopy and confocal microscopy. A control group of lesions wild type for BRAF mutation was also included. We analyzed the morphological differences between the two groups.

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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. 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 patients compared to healthy subjects.

Extensive literature describes biofilm production as a critical virulence factor, through which many microorganisms, including fungal cells such as *Candida spp.*, enhance their pathogenic 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.

Objectives: The aim of the project is to investigate the characteristics and peculiarities of mycobiota from healthy subjects and patients with intestinal disorders (Crohn's disease). In briefly, laboratory and clinical fungal isolates have been assessed for:

- capability to form biofilm;
- morphological and bio-molecular peculiarities of biofilm;
- susceptibility of Candida yeast cells to host cells.

Methods

Two clinical isolates of *C. albicans* (YL1 and YQ2), 43 fungal isolates from healthy subjects and the reference strain *C. albicans* SC5314 have been employed.

The immortalised murine microglial cell line BV-2 as prototype of brain macrophages has been used.

The capability of fungal isolates to produce biofilm was evaluated by quantitative colorimetric assays (crystal violet, tetrazolium salt reduction assays), while the susceptibility to host cells and morphological analysis were performed by epifluorescent microscopy.

Results

Among all fungal isolates obtained from healthy subjects, only those identified as *C. albicans* retained the ability to produce biofilm; its morphology, thickness and dispersion of new fungal cells have been quantified.

When the two *C. albicans* isolates from clinical patients were compared, significant differences were observed; biofilm production, thickness, structure density, the occurrence of hyphae and yeast-cells and resistance to intracellular killing were consistently higher in YL1 compared to YQ2 strain.

Conclusions

C. albicans is the only species capable of producing biofilm among all fungal isolates from healthy subjects. This implies its relevance as microorganism capable of switch from commensal-to-pathogen via biofilm production.

Data on the two clinical isolates from Crohn's patients indicate that *C. albicans* may exhibit different pathogenic potential possibly related to its genotypic plasticity.

Dr. Luca PINZI

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COMPUTATIONAL APPROACHES IN POLYPHARMACOLOGY

Introduction

Despite modern drug discovery continues to focus on the research of a "magic bullet" that enables the modulation and the study of a specific target, increasing evidences indicate that drugs are not highly selective, but rather show relevant interactions with more than one protein. In this context, polypharmacology is starting to have a true impact on the drug discovery process, especially in the design of drugs for multifactorial diseases as cancer. This approach allows the rational development of a unique chemical entity that hits a desired pool of selected targets, thus help overcoming problems such as drug–drug interactions, resistance and side effects.[1] Here, we present two explorations of the concept of polypharmacology: a prospective rational design of compounds with dual inhibitory activity on Hsp90 and B-Raf, and an in silico polypharmacology profiling of a publicly available database of compounds deposited in the Protein Data Bank.

DESIGN OF DUAL INHIBITOR OF HSP90 AND B-RAF AS A NOVEL PHARMACOLOGICAL APPROACH AGAINST MELANOMA

Background

Melanoma is a type of cancer that arises from the uncontrolled growth of melanocytes, cells responsible of the pigmentation in the skin. Despite it is less common than other skin cancers, melanoma is responsible for the majority of deaths related to skin cancers.[2] In the last years, several protein kinase inhibitors have been marketed for cancer treatment. Food and Drug Administration (FDA) has recently approved RAF inhibitors, such as Vemurafenib and Dabrafenib, to treat patients with the BRAF-V600E mutant melanoma. Unfortunately, responses to these pharmacological treatments are often temporary and rarely complete, with a median time to progression of 6 to 7 months.[3] Two general explanations for resistance to RAF inhibitors have emerged by recent research and clinical studies and in this context, Hsp90 may play a crucial role.[3,4]

Interestingly, the combination of Hsp90 inhibitors with B-Raf inhibitors show significant synergistic effects and the resulting drug combinations is currently being evaluated in clinical trials. Therefore, the inhibition of this cellular chaperone may be effective in patients with intrinsic or acquired resistance to RAF inhibitors.[5-8]

Objectives

In this study, we performed an integrated ligand-based and structure-based workflow with the aim of developing the first Hsp90/BRAF dual inhibitor.

Methods

Hsp90 or B-Raf inhibitors were collected from the ChEMBL database (accessed on September 7 2015) and filtered based on the enzyme assay outcome type.[7] Starting from these pre-filtered databases, different approaches were performed to discover compounds with dual Hsp90/B-Raf inhibitory activity.

- *Cross 3D ligand based virtual screening*

Hsp90 and B-Raf pre-filtered databases were merged and an all-against-all compound similarity was evaluated. From the top scaffolds ranked by B-Raf and Hsp90 similarity, common pharmacophore hypothesis queries were built.[9]

Afterwards, databases collected from different vendors were filtered to remove compounds with poor ADME properties.[9] Then, through a pharmacophore screening, the resulting databases were screened to remove molecules not likely to present a shape and chemical features close to the compounds marked as 'active' in the ChEMBL database. [9,11] The filtered databases were docked into B-Raf and Hsp90 *crystal structures* previously validated.[11] A final step of visual inspection and analysis of scores was attempted to select compounds for the bioassays.

- *Cross-docking studies of ChEMBL known active compounds on B-Raf and Hsp90 in silico models*

Starting from the previously pre-screened ChEMBL databases, Hsp90 and B-Raf known inhibitors were docked into B-Raf and Hsp90 structures, respectively.[11] In this case, no compound was selected to be tested *in vitro* due to commercial unavailability of the selected scaffolds. However, several interesting ligand-protein complexes were evaluated suggesting privileged chemical groups for both the targets.

- *Hit expansion of the active compounds resulting from our first screen*

More than 50 compounds were purchased and tested on Hsp90 and B-Raf, revealing a number of compounds with potentially interesting inhibitory activity at 100 μM concentration. A hit expansion of the compounds showing at least a 40% inhibition at 100 μM identified in the first screening was then performed. Compounds with a core scaffold similar to the previously identified active molecules were collected from different vendor databases. Using the same protocol of structure-based virtual screening applied in the *Cross 3D ligand based virtual screening* section, compounds with top scores and reliable poses on both B-Raf and Hsp90 *structures* were selected for bioassays.

Results

Around 25 compounds were selected and tested on B-Raf and Hsp90 enzymes applying the approaches showed in the previous section. Inhibitory activity in at least one target was found for several compounds and, in some cases, good percentages of inhibition were observed for both targets. Remarkably, most of the compounds showing inhibitory activity for one target have a chemical structure similar to known inhibitors of the other target. In particular, several of our newly discovered B-Raf inhibitors have a chemical structure close to known Hsp90 co-crystallized inhibitors. For these compounds, we are performing further *in silico*

studies looking for small but important modifications that provide inhibitory activity on Hsp90 while retaining the activity on B-Raf. Interestingly, some compounds characterized by different chemical scaffolds and showing inhibitory activity on both targets were identified (IC_{50} values in the micromolar range) and are now under further development.

Conclusions

Despite the simultaneous inhibition of B-Raf and Hsp90 seems to provide a clear benefit on the pharmacological treatment of melanoma, to the best of our knowledge, no B-Raf/Hsp90 dual inhibitor has been identified so far. [5,6]

Our research findings suggest that the design of the first Hsp90/B-Raf dual inhibitor is feasible and able to yield interesting structures.

POLYPHARMACOLOGICAL INSIGHTS INTO THE PROTEIN DATA BANK (PDB)

Background

The Protein Data Bank (PDB) is a well-known publicly available database of protein and nucleic acid crystal structures.[13]

More than 116,000 protein structures obtained from different organisms and through X-ray crystallography or NMR spectroscopy experiments are housed into the PDB database (http://www.rcsb.org/pdb/home/home.do, accessed on: 23/02/2016).[13] The database represents a milestone in the modern drug discovery process due to the structural data it contains. Many published studies focused on the design of compounds with pharmacological activity employing molecular modelling techniques start from the crystal structure of a target of interest (if available). Generally, the design of selective compounds remains one of the main goals in drug discovery processes. However, the information available in the PDB is not completely employed to design the coveted ´true´ selective compound. In the light of this, it is not surprising that compounds initially considered as "selective" have been recognized only *a posteriori* to be non-selective.[1]

Objectives

In this study, we devised and applied an *in silico* investigation to evaluate and predict the polypharmacological profile of ligands deposited into the PDB.

Methods

Initially, crystal structures of human proteins were collected from the PDB database (accessed on: April 6, 2015). Apo-protein structures were discarded and the resulting complexes were prepared adding hydrogen atoms, fixing tautomeric forms and the protonation state was set to the physiological pH.[10] Using an *in house* developed python script, ligands were extracted in their native poses from the binding sites. The

subsequent generated database of ligands was filtered using custom parameters, which allowed the removal of solvent molecules, fragments and peptides. [8-11] Afterwards, an all-against-all 3D ligand based virtual screening was attempted in order to build an *n x n* similarity matrix containing more than 82 million of evaluations. Thanks to the generated matrix and through the application of a hierarchical clustering algorithm, the compounds were grouped into homogenous entities. Finally, a closer inspection of the clusters was made by looking for new drug-target associations. Activity profiles of compounds showing a high similarity and reported to be active on different targets were checked in the literature.

Results

Thanks to the approach defined in the previous section, several interesting compounds with potential dual activity were discovered. Some of them were already known in the literature, providing an internal validation of our method. Others are new and will be tested experimentally to provide novel structures from which to start drug discovery projects based on polypharmacology.

Conclusions

The design of compounds that selectively bind a pool of targets of interest is starting to have a true impact on the drug discovery process.[1] One of the key strength of using ligand-protein crystal structure complexes in the drug design process is the possibility to know and exploit key protein-ligand interactions, which is a fundamental pre-requisite for drug design. These first encouraging results obtained by a close analysis of PDB suggest new molecular targets for known compounds and an interesting way to use the information available into this database for future drug design applications.

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CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD) AND HEART FAILURE (HF): THE COMPLEX RELATIONSHIP BETWEEN HEART AND LUNG DISEASES

Background

Chronic obstructive pulmonary disease (COPD) is a common, chronic and usually progressive lung disease, and a relevant source of morbidity and mortality worldwide. Persistent airflow limitation is the hallmark of the disease, and exposure to cigarette smoking is the main risk factor. Clinically, COPD patients usually complain of non-specific symptoms such as dyspnea, fatigue, chronic cough and sputum. Moreover, COPD patients often suffer from coexisting chronic diseases, likely due to shared risk factors (i.e., COPD patients tent to be old subjects, current or former smokers, and with a persistent low grade pulmonary and systemic inflammation). Cardiovascular comorbidities, in particular, are quite frequent and contribute significantly to disease severity and prognosis. Thus, current clinical research in COPD is moving beyond the lung.
The aim of the present research is to explore the relationship between cardiac comorbidities, particularly heart failure (HF), and COPD – both in the stable and in the acute setting.

Objectives

The principal aim of the present research is to comprehensively evaluate the relationship between COPD and HF, both in outpatients and in hospitalized patients. More specifically, the first aim is to evaluate the local prevalence rate of HF with reduced ejection fraction (EF) in COPD patients, and vice versa, the local prevalence of airflow limitation in HF; the second aim is to assess the differences in clinical presentations and symptoms between patient with COPD alone, with HF alone, and with both disease; the third aim is to evaluate the etiology of acute exacerbation of COPD (AECOPD), and in particular the role of heart failure in triggering such acute episodes.

Methods

The present research involves two parallel studies on the topic HF-COPD: the first study has recently concluded the clinical phase, while the second has yet to start.
The first study was a multi-center, observational trial, recruiting patients with HF or COPD. Study population included subjects aged ≥65 years and with ≥20 pack/years of cigarette smoking presenting with a diagnosis of stable COPD or stable CHF. Patients were recruited in two outpatient clinics, and all participants underwent routine echocardiographic assessment, routine spirometry, laboratory tests such as

brain natriuretic peptides and high sensitivity C- reactive protein, and symptoms evaluation with specific questionnaires.

The second study is an observational, multi-center, prospective study, which will enroll consecutive patients presenting to the hospital for AECOPD. Patients with an alternative diagnosis for dyspnea (e.g. acute coronary syndrome, pulmonary embolism, pneumothorax) will be excluded. Patients will be treated according to local medical practice, and data regarding medical history, pulmonary function tests, echocardiography, laboratory findings, and hospitalization outcome will be recorded for each subject. All medical charts will be subsequently analyzed from study investigators, to define the main putative aetiology of the index acute episode. Thus, all participants will be separated in different cohorts, according to pre-specified criteria: 1) AECOPD due to heart failure; 2) AECOPD due to respiratory causes, mainly acute bronchitis; 3) AECOPD due to coexisting respiratory and cardiac causes; 4) AECOPD due to other causes (e.g. pulmonary embolism, anxiety, depression, gastro-esophageal reflux disease). The main aim is to assess the prevalence of AECOPD due to heart failure, and to analyze if there are any differences in short and long-term outcomes between the four different cohorts.

Results

Since the clinical phase of the first study was only recently concluded, data analysis is ongoing and only preliminary results are currently available. Final study population included 241 patients. Mean age was 73 years (range 64-85). Diagnosis of HF with reduced EF in patients with a primary diagnosis of COPD was low (around 3,5%), while occurrence of airway obstruction among CHF patients was 35%.

Clinical presentation was slightly different between COPD and HF patients: not surprisingly, COPD patients complained more often of cough and phlegm, compared to HF patients. However, non-specific symptoms such as dyspnea and lack of energy were present in all patients. Interestingly, when assessing symptoms with the disease-specific COPD-Assessment-Test (CAT) questionnaire, no significant differences were found for breathlessness, limitation in daily activities, confidence in living home or energy, between COPD patients, HF patients, and patients that had both HF and COPD.

As the second study on acute exacerbations of COPD has not started yet, no data in this setting are presently available. According to previously published data, we expect that a relevant share of subjects hospitalized with a clinical diagnosis of AECOPD, will present an underling cardiac disorder as the main cause for the deterioration of respiratory symptoms.

Conclusions

COPD and HF are common, chronic and important disorders, and it is now undeniable that COPD and HF are frequent found together in the same patient. Their relationship is complex, and may present different and interesting clinical aspects, regarding both stable and hospitalized patients. We have reported that

certain symptoms such as cough and sputum may be peculiar to COPD patients, while dyspnea and lack of energy are no different between patients that have HF or COPD or even both diseases.

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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 profile and has become an important tool for the discovery of new biomarkers useful for clinical application. Mass spectrometry proteomics 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. 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 (LR peptide and LR derivatives) and do not induce TS overexpression[2]. As is known, anti-folate drugs, such as Pemetrexed, binding TS, stop both catalytic and regulation function of the protein. Up regulation of TS is then the most effect of feedback monomeric modulation. 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. Furthermore, the translational research work was conducted on a clinical trial within a randomised 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 our proteomic studies is based on the identification of novel drug candidates against trypanosomatidic infections (Human African Trypanosomiasis - HAT, Chagas Diseases

and Leishmaniasis) within the EU 7thFP project NMTrypl[3]. In particular Visceral Leishmaniasis is an infection caused by obligate intracellular protozoan parasites Leishmania transmitted by the bite of certain sandfly species. There are an estimated 12 million humans infected. It is currently endemic in Africa, Asia and 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[4]. The proteomic work has the aim of tracking the proteomic profile modulation due to Miltefosine and few its novel derivatives to study the mechanism of action. The proteomic studies on Leishmania parasites was performed in collaboration with Dr. Stefania Ferrari.

Aims

The aims of my PhD work are:

ì) to identify a pharmacodynamic biomarkers as molecular indicator of Pemetrexed effect in therapy on ovarian cancer frozen biopsies.

ii) to confirm the protein panel modulated by peptides lead treatment, with prolil-LR derivatives using statistical and biological validation assays on ovarian cancer cell line. The protein profile was evaluated also for drug as reference sample.

ìii) 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 second year is focused ì) on the identification of a protein panel that can work as a biomarkers of the pharmacodynamic activity of the Pemetrexed in a translational study within a clinical trial on ovarian cancer, using a differential proteomic approach; ii) and on the characterization of the proteomic profile modulation of Leishmania donovani parasites treated with new candidate drug-molecule 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.

Protein lysates extracted both from parasites and from frozen ovarian cancer biopsies were digested with Filter-Aided Sample Preparation (FASP) protocol, which combines the advantages of in-gel and in-solution digestion for mass spectrometry-based proteomic studies[5]. Then, differential proteomic approach was developed in order to identify the most significant differentially modulated proteins by the treatments. The analysis were performed on High-Definition (HD) ultra-high resolution (UHR) QTOF mass spectrometer (Bruker) at University of Milan Bicocca.

The quantification of differences between two or more physiological states of a biological system is very important to understand the metabolic pathway involved in drugs mechanism of action. So R and Panther

software were employed to identify the most important biological process involved in the pharmacodynamics drugs profiles.

Results and expected results

MS data collected, from samples were analyzed with Progenesis software in order to obtain the list of proteins significantly modulated by each kind of treatments.

On pre-treatment biopsies samples 1200 the statistically significantly modulated proteins were identified. At the same time the differentially expressed proteins were classified in the most biological process involved in the mechanism of action and statistical analysis are ongoing in order to identify the protein panel that could be indicator of Pemetrexed efficacy for ovarian cancer treatment.

The protein modulated by LR peptide[6] was confirmed with the study of prolil-LR derivatives. The treatment confirm the effect on 4 proteins included in the LR-effect--signature and the statistical analysis confirm the significance of differences among protein modulated by the peptides and the reference drug (Pemetrexed).

A list of 141 proteins was obtained and further analysis were done to group them into two main groups: i) proteins modulated in almost the same way by the three drug treatments; ii) proteins that were modulated differently by the three drug treatments or were modulated only by one or two of the drug treatments. Common modulated proteins are expected to characterize the mechanism of action of the compound class. Further analysis to cluster these proteins into different metabolic pathways and/or biological processes are ongoing.

The principal problem is proteins availability in database because many parasitic proteins are uncharacterized or their function is not classified.

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

Background

The standard of care for many patients with advance breast cancer is gradually evolving from empirical treatment based on clinical-pathological characteristics to the use of targeted approaches based on the molecular profile of the tumor. In the last decade, an increasing number of molecularly targeted drugs have been developed for the treatment of metastatic breast cancer. These drugs target specific molecular abnormalities, including mutated protein kinases and amplified or rearranged transcription factors. Cells harboring these 'driver mutations' have a survival advantage; therefore, targeting these alterations is a rational strategy to offer more personalized and effective treatment to metastatic patients.

Recent advances in technologies are expanding the scope of personalized medicine by providing new opportunities to develop more complete and dynamic diagnoses. Particularly, next generation sequencing (NGS) represents the most promising approaches among those currently available. The ability to perform multi-gene testing for a range of recurrent molecular alterations provides an opportunity to clarify the mechanisms of treatment resistance, to find the strategies to overcome that resistance and thus, to identify patients who may be candidates for matched targeted therapies.

Objectives

The main aim of our research is to clarify the mechanisms of resistance to treatments in metastatic breast cancer patients and, at the same time, to develop a tool for selecting the most appropriate treatment based on the molecular profile of the tumor.

Secondary aims will be:

1. Defining genomic profile of good responders and patients at increased risk for relapse or progression;
2. Identifying new targets for drug development;
3. Determining new strategies to overcome treatment resistance: combination of already available drugs (as happened with lapatinib + trastuzumab, exemestane + everolimus, etc.), development of new drugs to combine with the available ones (as happened with palbociclib + letrozole/fulvestrant, etc.), definition of a particular sequence of treatments, etc.

Materials and Methods

In this study, we will evaluate a panel of 25 genes involved in the mechanisms of treatment resistance on formalin-fixed and paraffin-embedded (FFPE) tissues of primary hormone receptor positive and/or HER2 positive breast cancers and, after progression to treatments, on FFPE tissues taken from relapsed sites. Therefore, we will evaluate tissues at the diagnosis before any treatments and at the metastasization, after the development of resistance to endocrine or 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).

Results

At the end of April 2015, the protocol has been approved from our local Ethical Committee. At the end of July 2015, thirty patients eligible for the study were identified in the archive of our Pathology Department. In November 2015, we received the multigene panel previously ordered. In December 2015 we extracted DNA from the tissues of the first patient and sequenced the 25 genes selected. In these days we are extracting DNA from the tissues of other 2 patients.

Before the end of 2016 we expect to complete the DNA sequencing of 10 patients (20 tissue samples).

Conclusions

Pending.

cycle XXXI

Dr. Laura ANSELMI

CEM Curriculum: Translational Medicine
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ANALYSIS OF METABOLIC AND PI3K/Akt/mTOR SIGNALING FEATURES IN T-ALL CELLS

Background

T-Acute Lymphoblastic Leukemia (T-ALL) is a heterogeneous malignant hematological disease, characterized by the abnormal accumulation of T-cell progenitors. It represents 10-15% and 25% of pediatric and adult ALL events, respectively. Despite many efforts in designing novel treatment protocols, combined with traditional chemotherapy, prognosis of T-ALL patients with chemoresistant or relapsed leukemia is still very poor. Moreover, a number of key signaling pathways are deregulated in T-ALL, such as the PI3K/mTOR cascade, downstream of Notch1 mutations (found in >60% patients) or PTEN gene deletion/inactivation. These alterations frequently lead to reprogramming of metabolism, thereby cancer cells display glycolytic features even in normoxic conditions to boost their rapid growth and energy demand, as simultaneously glutaminolysis fuels up TCA cycle and, consequently, oxidative phosphorylation. Such glycolytic phenotype, in turn, enables cancer cells proliferation, growth, invasion and drug resistance.

Objectives

Previous studies performed by our laboratory demonstrated the importance of network-level analysis of the phosphorylome of acute leukemia patients for a more effective therapy (Bertacchini J. et al., Leukemia, 2014), while the efficacy of the combined targeting of cellular metabolism and PI3K/Akt/ mTOR signaling was also verified in the context of primary effusion lymphoma cells, which exhibit a highly glycolytic phenotype (Mediani L. et al., Oncotarget, 2015). Thus, based on our previous experience and on the abovementioned recent findings that oncogenic signaling can drive metabolic rewiring in cancer, the broad aim of our study is to find novel therapeutic protocols for T-ALL patients, based on the new concept of precision medicine.

Specific aims: i) To describe the signaling and metabolic profile of both primary cells from T-ALL patients and T-ALL cell lines, with particular attention to wild type Notch1 or/and PTEN versus mutated/deleted Notch1 or/and PTEN.

ii) According to individual profiles from point (i), to examine whether combining signaling inhibitors (Notch1 or PI3K/mTOR inhibitors) with glycolysis/glutaminolysis inhibitors represents an alternative therapeutic approach, to improve survival of refractory or relapsed T-ALL patients.

Methods

The study will be performed using both an *ex-vivo* model, namely primary blast cells from T-ALL patients, and an *in vitro* model, namely highly characterized T-ALL cell lines which recapitulate the different features of the disease.

T-ALL primary cells (>20) and cell lines (25), displaying different mutational characteristics, which recapitulate the different T-ALL phenotypes, will be profiled by reverse phase protein array (RPPA), using antibodies recognizing key molecules of PI3K/Akt/mTOR and MAPK/ERK cascades. To verify whether chronic treatment might trigger adaptation through compensatory signaling, RPPA screening will be performed both after long-time and short-time exposure.

Mitochondrial function and levels of glutaminolysis will be evaluated by XF Extracellular Flux Analyzer using specific kits. Lactate secretion, indirect index of glycolytic rate, will be measured in the growth medium by a colorimetric assay.

Next, the cytotoxicity and the ability of the following drugs to revert the glycolytic metabolism will be monitored, according to the specific profiles. In particular, we will test the pan-class I PI3K inhibitor BAY 80-6946, as well as isoform-specific molecules, such as p110□□inhibitor CAL-101 and the p110□□□□inhibitor IPI-145, then the dual PI3K/mTOR inhibitor PF-4691502 and the mTORC1/2 inhibitor MLN0128. In addition, we will use the gamma-secretase inhibitor PF-03084014 in order to block Notch activity, and the glucose analog/hexokinase inhibitor 2-deoxyglucose (2-DG). These experiments will be performed under both normoxia (21% O_2) and hypoxia (1% O_2). Cell viability will be monitored by AnnexinV/PI staining and MTT assays, upon short-time and long-time exposure to drugs, while Western blot analysis of caspase-3 and -9, as well as presence of cleaved PARP, will allow to assess apoptosis. The software Calcusyn will be used to evaluate any synergistic, antagonistic or additive effects.

Drug associations giving the more promising results will be then validated in primary blasts from T-ALL patients, collected at diagnosis from the Policlinico of Modena. The cytotoxicity of the inhibitors will be tested in normal T-lymphocytes from healthy donors.

Conclusions

Overall, this project will correlate mutational and phosphorylome analysis with T-ALL metabolic phenotypes, allowing to define individual profiles and to predict specific treatments effectiveness.

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FALL PREDICTION IN REHABILITATION SETTINGS AND IN ELDERLY PEOPLE

BACKGROUND

Falls of inpatients may cause permanent disability or death resulting in extended lengths of stay, a decline in quality of life [Ohde, 2012] and an increased health care costs. Particularly susceptible are elderly subjects and patients at rehabilitation hospitals. It is estimated that just in the United Kingdom, patient falls in acute care hospitals cost approximately 92 million pounds per year [Da Costa, 2012]. Falls cause more than 95% of all hip fractures in the elderly and 20% of the elderly people who suffer hip fractures die within a year. The prevalence of falls in acute hospitals ranges between 2 and 6%, it is 12.5% in general rehabilitation settings (patients are frailer), moves to 20-30% at geriatric rehabilitation units, and grows up to 39% at geriatric stroke inpatient rehabilitation units [Frisina, 2010; Ross, 2012]. Patients participating in rehabilitation programs, in fact, may experience falls due to the promotion of independence and mobility, which challenges multiple systems of balance and can increase the risk for falling [Salamon, 2012].

OBJECTIVE

There is a clear need to intercept subject at risk for falling both at rehabilitation hospitals and in the community-dwelling elderly population in order to implement prevention strategies. The identification of subjects at risk is commonly performed by means of clinical or functional fall-risk assessment tools and by adjunctive instrumental assessments.

The first aim of this project is to evaluate feasibility and predictive power of the Hendrich Fall Risk Model II (HFRM) in an Italian rehabilitation hospital. In the literature, HFRM showed the best performance in terms of sensitivity and specificity in three independent studies with large samples and was validated in acute and geriatric care settings [Hendrich, 2003; Heinze, 2006; Kim, 2007]. As Myers recommended [Meyers, 2003], fall risk assessment tools have to be rigorously tested in as many clinical settings as possible.

The second aim of this project is to evaluate the contribute of instrumental evaluations of balance to the predictive power of clinical fall risk prediction tools (e.g. HFRM), as suggested in the recent literature [Panzer, 2011]. Instrumental measures of postural steadiness can be used to characterize the dynamics of the postural control system in maintaining balance during quiet or perturbed standing [Pajala, 2008]. Specific protocols of balance assessment have been developed to quantify the risk of falling, as in [Maranesi, 2015].

METHODS

First aim: Prospective cohort study: HFRM will be administered in a Rehabilitation hospital with Neurological, Orthopedic and Pulmonary Rehabilitation Wards and patient falls will be tracked. Rate of successful HFRM administrations at admission, the area under the Receiver Operating Characteristic (ROC) curve, the best cut-off score, sensitivity, specificity, positive and negative predictive values (PPV, NPV) in the prediction of inpatients falls by HFRM will be computed.

Second aim: Prospective Cohort Study: the added value of a specific protocol for balance assessment (posturography) to clinical fall risk prediction tools will be evaluated in terms of increase of predictive power.

EXPECTED RESULTS

First aim: To determine the feasibility and the predictive power of HFRM in rehabilitation settings.

Second aim: To determine the added value, if any, of the instrumental assessment of balance to the clinical prediction of future falls.

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Dr. Anna Maria COSTA

CEM Curriculum: Translational Medicine
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NEUROACTIVE STEROIDS AND EPILEPSY: A FOCUS ON NEUROSTEROIDS AS DETERMINANTS OF ANTIEPILEPTIC DRUG REFRACTORINESS IN TEMPORAL LOBE EPILEPSY

Background

Neuroactive steroids are a family of compounds synthesized after the conversion of cholesterol to pregnenolone. These molecules from peripheral glands and the nervous system itself (neurosteroids) act on targets in the nervous system, regulating brain development and physiological neuronal functions. Several lines of evidence suggest that neuroactive steroids may also affect neuronal disorders, among them epilepsy. Pharmacological manipulations of steroid levels show that they possess both anticonvulsant and proconvulsant properties. However, changes caused by seizures in tissue and/or plasma levels of these compounds have not been considered yet. This lack of information could be linked to the failure of drug treatment in temporal lobe epilepsy (TLE), the most frequent type of drug-resistant epilepsy.

Objectives

The main theme of this thesis is neuroactive steroids and epilepsy, with a particular focus on neurosteroids as determinants of antiepileptic drug refractoriness in TLE. We hypothesize that decreased levels of anticonvulsive neurosteroids, especially allopregnanolone, and/or increased levels of those proconvulsive, such as pregnenolone sulfate, could be involved in drug-resistance to antiepileptic drugs. We aim at establishing whether the levels of neurosteroids and/or all neuroactive steroids are altered in plasma, cerebrospinal fluid and/or brain tissue of animal models of TLE and patients affected by epileptic disorders. Moreover, a secondary goal is to define if a relationship exists between the changes in neurosteroids and/or all neuroactive steroid levels and the severity of epilepsy, so that pharmacological treatments aimed at restoring these molecules may result in beneficial effects on seizure recurrence.

Methods

We will analyze our molecular targets by tandem mass spectrometry and enzymes involved in their synthesis by immunohistochemistry and/or molecular biology techniques.

Results

Our results will clarify the relationship between circulating and tissue steroid levels in epilepsy in order to find out whether 1) anticonvulsive or proconvulsive steroids produced in epileptic tissue affect the therapeutic response 2) peripheral steroids are able to maintain neuroactive steroid levels in the brain. In addition, the balance between anticonvulsant and proconvulsant steroids will be also determined by directly investigating them in cerebrospinal fluid as well as in the brain regions affected by epileptic foci. These results will be finally validated through pharmacological experiments that will provide a rational basis for the therapeutic use of allopregnanolone analogues.

Conclusions

In conclusion, we hope to refine our understanding of the role of anticonvulsant and/or proconvulsant neurosteroids and/or all neuroactive steroids in difficult-to-treat epilepsy.

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HEPATOCELLULAR CARCINOMA IN ZEBRAFISH MODEL: EXPLORATION OF THE ROLE OF NEO-ANGIOGENESIS AND BIOLOGICAL AGGRESSIVENESS

Introduction

Hepatocellular carcinoma (HCC) is the most common primary cancer of the liver and it has risen to become the 5th commonest malignancy and the third deadly cancers worldwide [1]. To influence this trend it would be necessary to act on the etiologic causes. HCCs are aggressive, phenotypically and genetically heterogeneous tumors that commonly emerge on a background of chronic liver disease (CLD) and cirrhosis. Evolving information suggests that the metabolic syndrome with non-alcoholic liver disease may be an important cause of HCC in addition to viral infections, toxic exposures and hormonal. Indeed, a relevant proportion of individuals with NAFLD (non-alcoholic fatty liver disease) is at risk of progression to simple steatosis or to a more severe condition called non-alcoholic steatohepatitis (NASH), that can eventually develop into hepatic cirrhosis and hepatocellular carcinoma (HCC) [2]. Also obesity has been established as a significant risk factor involved in determining the severity of liver damage and its progression to cancer [3]. However the biology of HCC remains poorly understood mainly because of the complex clinical heterogeneity and genomic landscape of this tumor type.

Our research group selected Zebrafish (*Danio rerio*), as experimental model to test the relationship between steatosis, fibrosis and cancer development [4]. Being vertebrates, this small fish are structurally much more similar to humans than are other model organisms, such as worms (e.g *C. elegans*) and flies (e.g *D. melanogaster*), and so have been exploited successfully to model various human diseases. Furthermore, Zebrafish offer unique advantages over other vertebrates including in vivo imaging at cellular resolution and the capacity for large-scale chemical and genetic screens. Finally, the zebrafish genome has been sequenced and the annotation has revealed that 70% of human genes have a zebrafish orthologous [5]. Consequently, the genes and developmental pathways involved in liver development and disease are highly conserved among vertebrates [6].

On the other hand, the last year, Villa et al, published an important result for liver cancer landscape, in which they identify a molecular signature of five genes in liver of HCC patients, that can distinguish tumor subtypes, assist clinical staging and predict patient outcomes. This hepatic transcriptomic profile, including Angiopoietin-2 (*ANGPT2)*, delta-like ligand (*DLL4*), neuropilin (NRP)/tolloid (TLL)-like 2 (*NETO2*), endothelial cell-specific molecule-1 (*ESM1*), and nuclear receptor subfamily 4, group A, member 1 (*NR4A1*), is able to discriminate, with high sensitivity and specificity, patients with extremely rapid tumor growth and ominous

prognosis. All five genes are neo angiogenesis-related, they have roles in endothelial cell migration, angiogenesis and blood vessel morphogenesis and are also related with survival. *ANGPT2* was the most significantly upregulated gene [7]. Since HCC is a hypervascular tumor, the angiogenesis plays an important role in the progression of the disease; it is probably a driving force in HCC development. The identification of the less understood mechanisms may open new possibilities for the early diagnosis and for the prevention and treatment of HCC through the development of targeted therapies. As noted above, evolutionary conservation of genome between zebrafish and human exist; but, although the zebrafish has many attributes of a promising cancer model, one outstanding question is how similar zebrafish and human tumors are at the molecular level. Preliminary data indicate that zebrafish liver tumors possess the general molecular hallmarks of human liver cancer [8].

Objectives and Methods

In my PhD project I want to address two different aspects of liver carcinogenesis, one associated with exploration of the role of neo-angiogenesis and biological aggressiveness, the other related with the further characterization of the chronic overfeeding model (Turola et al. DMM 2015), with the addition of an inflammatory hit.

1. Zebrafish possesses a complex circulatory system similar to mammals; its development was studied and described in detail. In particular, the Zebrafish embryo has already developed a functional cardiovascular system (beating heart, aorta, cardinal vein and blood) by 24h after fertilization. In addition, multiple features of this model, such as optical clarity of the embryo, the availability of copious transgenic line and the easy genetic manipulation, make Zebrafish an exceptional model system to study not only development angiogenesis but also the *de novo* angiogenesis associated with pathological condition such as tumor vascularization [9-10]. Extensive information on *Danio rerio*, including genomics databases, developmental stages, publications and molecular tools are today available, and this has allowed us to confirm the presence in Zebrafish genome of all five homologous genes (angpt2b, neto2b, dll4, esm1 and nr4a1) of human HCC molecular signature. We want to examine cancer-related angiogenesis, by means analysis of expression profile and proteomic landscape. In particular, whether the over expression or down regulation of all 5 genes, or one of them, can modify the progression of Zebrafish tumor. The identification of genes essential for blood vessel formation will be of pivotal importance for understanding of the angiogenesis process HCC-related, for the discovery of novel therapeutic targets and for screening new drugs, taking advantages of permeability to small molecules of Zebrafish and of its easy manipulation [11]

2. In the Zebrafish model of dietary-induced-obesity that have developed we want to investigate whether the addition of an inflammatory stimulus, by means peritoneal injection of Thioacetamide (TAA) leads to

more severe chronic liver disease and eventually HCC [12]; preliminary results show that male fish (male sex in human is a recognized risk factor for HCC) develop a liver tumor similar to the human one (data not published). This model will be explored for similarity with human HCCs as the timing and the pathogenesis of HC occurrence are very close to humans. Therefore it could constitute a very useful experimental model.

Expected results

1. For Task 1, we expect to identify different behavior in term of neo-angiogenesis between aggressive and slow-growing HCCs. I expect to be also able to evidence different metastatic potential between the two, by observing the developed fish after embryo injection.
2. For Task 2, I expect to fully characterize the dietary-induced liver disease with the addition of the inflammatory hit in term of severity of disease and HCC development.

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PRECLINICAL VALIDATION OF A WEARABLE PERITONEAL DIALYSIS DEVICE

Background

End Stage Renal Disease epidemic is steadily expanding, with maintenance dialysis prevalence rates peaking at almost 2000 individuals per million population in industrialized countries. The most important disadvantage of hemodialysis (HD) is its intermittent character, resulting in extensive variations in the internal environment as well as large swings in fluid status. Peritoneal dialysis (PD) can provide a more gentle treatment, but clearance of uremic toxins is relatively low and technique failure rate are high. Amelioration of waste products clearances and advantages in survival have been advocated for intensive (i.e. nocturnal or short-daily) HD and, in PD, for automated peritoneal dialysis (consisting in multiple short overnight depurative exchanges). Currently available continuous treatments present the drawback of being performed through large and heavy medical devices, limiting patient's freedom. A miniaturized dialysis device, which combines efficient toxin clearances with gradual fluid removal allowing flexibility for the patient, would be a great asset. Principal bottlenecks towards the creation of a Wearable Artificial Kidney (WAK) for clinical use resulted the necessity of a stable vascular access and of continuous anticoagulation (for HD) as well as the need for large sorbent volumes. A WAK could be used to enhance the efficacy of PD, circumventing the problem of a direct access to the blood system. A reduction in the number of exchanges would also be allowed; moreover, continuous glucose infusion may avoid high toxic concentrations. MiniKid® (MK), developed by Nanodialysis (Oirschot, The Netherlands), redefines peritoneal dialysis by using a continuous flow of dialysate in the peritoneum and by employing a new technology for toxins removal based on nanomaterials and electrocatalytic urea oxidation. Dialysate is constantly refreshed and recycled; no large amount of sorbents is needed since they can be easily regenerated. MK is small and wearable and offers patients the opportunity to dialyze continuously, outside the hospital setting. A stable internal environment is provided and glucose infused at a slow rate keeping its concentration at the minimum necessary level.

Objectives

Nanodialysis asked Unimore to cooperate in MK development by participation in the pre-clinical validation; a joint project was submitted to *Eurostars* (European Commission programme) for financial support, and ranked 14 out of 293 eligible applications. Provided funding, we will perform preclinical testing for MK, assessing its biocompatibility and validation of its efficacy in vitro and in vivo, paving the way for future

clinical trials.

Methods

Biocompatibility testing will follow ISO 10993 certification ("Biological evaluation of medical devices"). Cells, bacteria and animals will be exposed to the following solutions: 1) spent peritoneal dialysate treated by the MK device in vitro and 2) untreated spent peritoneal dialysate from the same batches. *Genotoxicity and carcinogenicity* will be tested exposing bacteria and human peritoneal mesothelial cells (MCs) to the above-mentioned PD solutions. *In vitro cytotoxicity* tests will be performed with peripheral blood mononuclear cells and MCs. *Identification and quantification of potential leachables and degradation products* will be performed analyzing spent dialysate treated by MK at different time points for released particulate, soluble compounds or ions. *Systemic toxicity* will be assessed with a rodent model: subtotally (5/6) nephrectomized rats will be instilled daily through a peritoneal catheter with the two above-mentioned PD solutions for 12 weeks. Body weight, blood hematology and chemistry will be monitored weekly. After sacrifice, organs will be weighted and analyzed for inflammatory, proliferative and fibrotic changes. A peritoneal equilibration test will be performed, and peritoneal fibrosis, neo-angiogenesis and EMT will be assessed. As for *immunotoxicology testing*, weight and histology of lymphoid organs of treated rats will be examined; peripheral leukocyte counts will be monitored. Effects of incubation of human leukocytes with MK treated dialysate will be studied in vitro. *In vitro performance and efficacy* will be verified recirculating spent peritoneal dialysate from PD patients in the MK circuit, testing the removal of uremic toxins. *In vivo validation of efficacy* will be performed pursuing a rat model of chronic uremia (bilateral nephrectomy). Rats will be randomly assigned to receive conventional PD or continuous MK treatment for two weeks with a crossover design; primary endpoints will be total toxins clearance and ultrafiltration. Vital parameters will be assessed, and blood tests performed weekly. Peritoneal status will be evaluated as for task regarding systemic toxicity.

Results

We expect to demonstrate the absence of in vitro and in vivo toxicity of MK, as well as its efficacy compared to traditional PD.

Conclusion

We expect to demonstrate the biocompatibility and efficacy of MK, a wearable device for continuous PD.

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GLOBAL HIP FRACTURE MANAGEMENT: FALL PREVENTION, ORTHOGERIATRIC CARE, AND PALLIATIVE CARE FOR FRAIL ELDERLY PATIENTS

Study 1a. THE PRE.C.I.S.A. STUDY: AN RCT AIMED TO ASSESS THE EFFICACY OF A NEW APPROACH FOR FALL PREVENTION IN THE ELDERLY

Background and objectives

Fall prevention programs are increasingly developing, given the high social and economic impact of fall consequences in the aging population. Such programs, that may be single or multidisciplinary, often exclude patients affected by Parkinson disease or stroke, although being at even higher risk of falling. Aims of the PRE.C.I.S.A. study are: 1) to compare, with a randomized controlled trial, the efficacy of a multidisciplinary intervention for fall prevention in comparison to the usual care, on a sample of community dwelling elderly including persons with Parkinson disease or stroke; 2) to deliver a reduced set of clinical and instrumental indicators allowing the quick detection of community dwelling elderly at risk of falling.

Methods

We are carrying out a multicenter randomized controlled trial with blinded assessments on pre-test, post-test (12 weeks) and at 1-year follow-up. Patients in the treatment group receive a multidisciplinary (geriatrician, neurologist, physiatrist, physiotherapist) personalized intervention to reduce fall risk factors, in association to a multicomponent intervention based on: education on strategies for fall risk factors reduction, assessment and correction of home hazards, personalized home-based exercise program in synergy to a group-based exercise program lasting 11 weeks. Patients randomized in the control group receive structured information on fall risk factors and general suggestion on prevention strategies. The estimated minimum sample size is 365 subjects .

Expected results and conclusions

Expected outcomes for the treatment group in comparison to the control group are: at 3 months from randomization, a significant improvement of general functioning (functions, activities, social participation) and quality of life; at 1 year, a significant reduction of falls and of accesses to acute hospital services for falls and their consequences. If such expected results will be reported we could conclude that this innovative approach effectively prevent falls and ameliorate the quality of life for community dwelling elderly.

Study 1b. THE ROLE OF HAND GRIP STRENGTH TEST IN FALL PREVENTION

Background and objectives

Hand grip strength test, which has been frequently applied in geriatric research in recent years, has been associated to negative outcomes in elderly population. A low strength has been correlated to falls, hip fracture, high rate of hospitalization and mortality, and cognitive decline. However, as concerns falls it has not been yet investigated which risk factors for fall are linked to hand grip strength in the elderly. Aim of our study is to detect the factors influencing hand grip strength relation with falls in a geriatric population.

Methods

In the setting of the PRE.C.IS.A. Study, hang grip strength test is performed as part of the geriatric evaluation during the pre-test phase. Such test is performed using a validated dynamometer and according to the American Society of Hand Therapists protocol.

Expected results and conclusions

The expected finding of this study is the evidence of which risk factors for fall are correlated to hand grip strength (e.g. drugs, orthostatic hypotension, comorbidities, depression, cognitive decline, gate disorders, nutritional parameters, functional status, etc.). This study should contribute to explain why grip strength may predict falls in the elderly.

Study 2. NEW ORTHOGERIATRIC CARE MODELS IN N.O.C.S.A.E. HOSPITAL IN MODENA

Background and objectives

One of the worst consequences of falls in the elderly is hip fracture, which is correlated to subsequent disability and higher mortality. In the last years different orthogeriatric models have been developed for a multidisciplinary geriatrician-centered care of hip fracture elderly patients. The two main models are the presence of a geriatrician as a consultant in an orthopedic ward or an orthogeriatric ward coordinated by a geriatrician where the orthopedist is a consultant. Orthogeriatric care has been correlated to lower rates of inhospital complications, inhospital mortality and long-term mortality. Aim of our study is to compare the outcomes of different orthogeriatric models recently developed in N.O.C.S.A.E. Hospital in Modena.

Methods

We compared a new orthogeriatric model of care coordinated by a geriatrician working in a rehabilitation ward (47 patients) to a traditional model based on a geriatrician working as a consultant in an orthopedic ward (158 patients).

Results

The length of hospital stay resulted longer for patients in the new model group, but at the same time >50% of them were discharged at home vs 16% in the traditional model group.

Conclusions

These findings suggest that an innovative orthogeriatric model in a rehabilitation setting, thanks to more specialized and more intense rehabilitation resources, allows a better motor and functional recovery for hip fracture eldelry patients with a consequent higher rate of return at home. Our future research will be focused on another orthogeriatric model recently implemented in NOCSAE hospital: an orthogeriatric ward coordinated by a geriatrician in the setting of a long-stay geriatric unit.

<u>Study 3.</u> EXPERIENCES OF INHOSPITAL PALLIATIVE CARE FOR END-STAGE GERIATRIC PATIENTS

Background and objectives

Palliative care is recently spreading in geriatric settings because of the increasing number of elderly patients affected by end-stage chronic disorders. Given the high number of deaths among geriatric population till occurring inside hospitals, inhospital sanitary professionals should be sensitized and trained to offer high-quality palliative care to end-stage hospitalized patients. Aim of the study is to investigate which clinical factors are correlated to end-stage condition, for a more appropriate selection of hospitalized patients who should benefit from a palliative approach.

Methods

We carried out a pilot study in a geriatric ward enrolling 190 consecutively hospitalized elderly patients. 8% of them were considered “end-stage” and received palliative care.

Results

The diagnosis of end-stage condition was significantly correlated to higher comorbidity scores and lower functional scores; on the contrary, traditionally accepted palliative scores did not result useful for the choice of a palliative approach in a hospitalized geriatric population. Patients treated with palliative care received lower diagnostic exams, suspension of useless drugs, more careful symptoms monitoring and control, humanization of the hospital setting, better psychological support for them and their families.

Conclusions

This study reveals the relevant prevalence of end-stage hospitalized elderly patients and the benefits that palliative care can provide for them. The interesting results of this pilot study have recently encouraged the creation of a new model where a motivated, trained, multidisciplinary team offers palliative care to end-stage hospitalized elderly patients in a dedicated setting. Our future research will focus on this new-born inhospital palliative care model for end-stage patients inside a long-stay geriatric ward in N.O.C.S.A.E. hospital in Modena.

Dr. Stefania PADUANO

CEM Curriculum: Health Sciences
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ADVANCED METHODS FOR ASSESSING WATER SAFETY AND QUALITY: APPLICATION ON THERMAL SPRINGS AND WATERS TREATED WITH BIOCIDES

Background

Legionella and other bacteria, such as *Pseudomonas*, are emerging as opportunistic bacteria associated with exposure to water, able to cause diseases in susceptible individuals. They are natural inhabitants of a broad variety of environmental reservoirs, including natural and treated water. Water treatments are an effective strategy to prevent waterborne infectious diseases, but these procedures can select resistant bacteria and cannot be applied on hot spring waters. Moreover, the widespread presence of biofilm in man-made water systems facilitates the persistence of opportunistic pathogenic bacteria, thus limiting the effectiveness of biocides[1,2].

The assessment of water quality has been traditionally performed by culturing microorganisms from water samples. The recent diffusion of Next Generation Sequencing (NGS) and bioinformatics tools offers the opportunity for a more extensive approach for examining the microbial diversity[3]. By analyzing the microflora DNA it is possible to detect not only single bacteria, but, simultaneously, all the different species interacting in an ecological niche[4,5].

Objectives

The aim of this project is to evaluate the advantages of NGS-based methodology in order to establish the biological risk linked to exposure to different types of waters.

For this purpose, we will investigate the following aspects with both traditional methods and NGS technologies:

- the impact of different water treatments and pipe materials on biofilm and microbial water community of a hospital's hot water distribution system;
- the changes in the microbial community of an untreated thermal water, moving from spring to points of use for inhalation, bathing or mud therapy.

Methods

This study will be organized in three parts:

1) Bench-scale experiment

Coupons of commonly used plumbing materials (stainless steel, hot dip galvanized steel, brass and copper) will be inserted into four separate racks made from plastic materials. The racks will be connected to the return loops of four separate hospital water networks, three treated with chemical disinfectants (chlorine dioxide, monochloramine, or hydrogen peroxide) and one untreated (control). After 6, 12 and 24 months of water exposure, one coupon for each material will be removed and analyzed in order to evaluate the development of biofilm and its composition. Biofilm will be analyzed as follows:

- heterotrophic plate counts at 22 and 37°C, using the pour plate method (UNI EN ISO 6222:2001);
- detection, quantification and biochemical typing of *Pseudomonas* spp using culture method on Cephalothin-Sodium Fusidate-Cetrimide agar;
- detection, quantification and serotyping of *Legionella* spp using culture method (ISO 11731:1998);
- detection and quantification of *Legionella* spp by quantitative PCR with and without the Ethidium Mono Azide pre-treatment in collaboration with the Department of Occupational and Environmental Medicine, Epidemiology and Hygiene, INAIL Research, Rome, Italy;
- characterization of the microbial community by NGS technologies in collaboration with the Department of Health Sciences, University "Foro Italico", Rome, Italy.

2) On field study 1

This part of study will be conducted in the University hospital of Modena, the same used for the bench-scale experiment. The microbial community will be evaluated by comparing samples of biofilm and water, collected from distal outlets of three hot water distribution networks treated with various disinfection procedures (chlorine dioxide, monochloramine, hydrogen peroxide) and from an untreated water network as control. These samples will be analyzed for the same parameters described above.

3) On field study 2

This study will be conducted in a thermal facility of Northern Italy. We will examine differences in the microbiota inside thermal water networks, collecting samples of biofilm and water from spring to point of use. Both types of samples will be analyzed for the same parameters described above.

Expected Results

We will consider the possible application of NGS method in water safety management. Characterization of the selective pressure of different water treatments on the microbial community, including populations of waterborne pathogens, will yield new information to assess the risks and/or advantages of different disinfecting procedures. New knowledge acquired on biofilm formation and microbial diversity in thermal water distribution systems will lead to select the effective control measures in order to prevent diseases associated with pathogenic and opportunistic bacteria in persons attending the thermal facilities.

Both results will be useful for the public health purpose to guarantee the best water quality and safety for consumers.

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ANALYSIS OF PROVIRAL DNA AND SJTREC+ CELLS IN CD4+ T CELLS SUBSETS FROM HIV+ PATIENTS WITH SUPPRESSED VIREMIA

Background

The progression of HIV infection can be halted by potent drugs that block the production of the virus and inhibit its integration into the host DNA. Response to therapy is typically monitored by counting CD4+ T cells and measuring plasma viral load, which becomes undetectable in most patients. Since at present HIV cannot be eradicated and persists in the host, robust data are needed on the importance of monitoring residual viral activity and intracellular HIV reservoirs in patients with undetectable viremia. Taking into account that the virus establishes latent infection at different degrees within central (CM) and effector memory (EM) or naïve (TN) CD4+ T cells, a possible and novel approach would be to measure HIV DNA in different lymphocyte subsets. Similarly, the residual capacity to reconstitute the immune system needs to be accurately studied and monitored, possibly by measuring the amount of cells containing the signal-joint T cell receptor rearrangement excision circle (sjTREC), a marker of thymic functionality.

Objectives

- To quantify HIV proviral DNA and the presence of sjTREC in different subsets of CD4+ T cells such as TN, CM and EM cells in HIV patients and in healthy donors (HD);
- To combine information on the amount of virus present in different CD4+ T cell subsets (by quantifying HIV DNA) and on the regenerative capability of the immune system (by quantifying TREC);
- To provide crucial guidance on optimal monitoring of HIV+ patients using an innovative combined approach with flow cytometry and cell sorting along with a molecular biology approach based on droplet digital PCR (ddPCR).

Methods

A minimum of 30 HIV-infected patients successfully treated for at least two years will be enrolled with a CD4+ T cell count >500 cells/uL and with plasma viremia undetectable, from at least 15 months. 10 HD age and sex-matched will be also enrolled. TN, CM and EM CD4+ T cells will be sorted with a S3e sorter (Bio-Rad, CA, USA) in a specifically designed biosafety containment hood (Biobubble, UK). HIV proviral DNA and sjTREC DNA will be quantified in each subset using QX200 droplet digital PCR (Bio-Rad).

Expected results

Correct and functional workflow and protocols will be set up. We will measure HIV proviral DNA levels, expressed as LTR copies/1,000 cells, and sjTREC amount in CD4+ T cells subsets in HD and HIV patients. For what concerns HIV proviral DNA, we expect its absence in healthy donors T cells, but its presence at different levels in the three subsets from HIV patients. sjTREC values are expected to decrease together with the differentiation process, with highest levels in N cells and lowest levels in EM cells. Moreover, in HIV patients we will analyze if correlations exist between sjTREC, LTR levels and clinical parameters, such as actual CD4+ T cell count, CD4+ T cell nadir or time to reach undetectable plasma viremia. This combined approach not only will lead to a better comprehension of the features of HIV reservoir and its monitoring, but could also be useful for the development of eradication strategies.

Dr. Cinzia PUZZOLANTE

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BIOFILM-ASSOCIATED PROSTHETIC JOINT INFECTIONS: A STEP TOWARD A BETTER IDENTIFICATION IN ROUTINE CLINICAL PRACTICE AND COMPARISON OF DIFFERENT SUSCEPTIBILITY TESTS

Background

Nowadays is estimated that over 65% of all human infections are biofilm-related [1]. However this number is expected to grow due to the increasing number of medical interventions and positioning of medical devices. Chronic biofilm-based infections have significant morbidity, mortality and financial costs and are often recalcitrant to conventional antibiotic therapy. Moreover biofilms are unperturbed by host immune responses such as phagocytosis, despite a sustained presence of host inflammation [2].

The biofilm formation on the surface of the prosthetic device is the fundamental pathogenic characteristic of prosthetic joint infections (PJIs) and makes the etiological identification of the bacteria extremely challenging with the conventional methods as tissues cultures on agar or enrichment broths [3]. Furthermore laboratory methods usually used for the determination of bacterial susceptibility to antibiotics (e.g. MICs) are not appropriate in biofilm-related infections including PJIs and no standard methods are currently approved by CLSI or EUCAST for the evaluation of the efficacy of antibacterials in this setting [4,5].

Objectives

Our primary aim is to evaluate the use of beadmill processing combined with automated blood culture bottle methods for the etiological diagnosis of PJIs compared with direct observations of microbial biofilms.

Our secondary aim is evaluate the Minimum inhibitory concentration (MIC) versus the minimum biofilm eliminating concentration (MBEC) or the minimal bactericidal concentration (MBC) of a combination of antibiotics commonly used in clinical practice (rifampicine, daptomycin, linezolid, tigecycline, vancomycin, colistin, meropenem) in biofilms obtained from patients with implant-associated infections.

If possible, a sub-study to characterize the immunological response during a biofilm-driven infection will be performed.

Methods

Beadmill process will be performed through Glass “Ballotini” beads transferred and vortexed with the samples and then inoculating the suspension in aerobic and anaerobic blood bottles. Direct observations and structural analysis of microbial biofilms will be performed with the use of confocal scanning laser microscopy (CSLM) and appropriate staining methods.

Susceptibility tests will be performed on microtitre plates and/or on Calgary biofilm device or on a flow cell model.

Results

The protocol to enroll patients will be submitted at the local Ethical Committee. We expect to include in the study about 5-7 patients with PJIs and to enroll in the next months at least a patient with PJI to due to a high-virulent organism (eg. MRSA, ESBL+ Enterobacteriaceae, Acinetobacter spp.).

Conclusions

Pending

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EARLY PREDICTORS OF GRAFT SURVIVAL AND PATTERNS OF DELAYED GRAFT FUNCTION AFTER SIMULTANEOUS KIDNEY-PANCREAS TRANSPLANT

Background

Pancreas transplantation (PT) has become the treatment of choice for many patients with severe complications of diabetes, particularly those with end-stage renal failure. Since 1966, when the first successful pancreas transplant in humans was performed, the results of pancreatic transplantation have dramatically improved. However, graft failure rates are almost 10% at 1 year and 30% at 5 years, and complications such as pancreatitis can be life threatening. Effective early markers of graft dysfunction are lacking, the definition of the pancreatic delayed graft function (DGF) is controversial and its patterns are poorly characterised.

Objectives

This study aims to identify early predictors of pancreatic graft survival and to examine DGF patterns and how do they relate to pancreatic graft survival.

Methods

This is a retrospective analysis. Data are retrieved from the intensive therapy unit (ITU) and transplant surgery databases for all whole organ pancreas transplants performed between January 2009 and December 2011 (data collection is still ongoing).

We analysed recipients demographics and donor/graft characteristics and monitored every 6 hours for the first 2 days after surgery the values of: blood glucose (BG), base excess (BE), lactate, pH, blood pressure (BP) and intravenous infusions and fluid balances.

Data were checked for missing entries; cases were excluded if pancreas graft outcomes were unknown. Graft failure was defined by a return to exogenous insulin therapy or explant of organ.

Pancreas transplantation was performed according to a standardized clinical protocol with systemic venous drainage and enteric exocrine drainage. Donors and recipients were matched according to national organ allocation guidelines. All recipients followed a standard immunosuppression protocol comprising basiliximab induction and tacrolimus, mycophenolate and steroids maintenance.

Results

Data for 56 simultaneous kidney-pancreas transplants (SPK) were retrieved and included in the analysis so far. Ten transplanted grafts (17.9%) were from donors after cardiac death (DCD), with an average cold ischemia time of 13±3.25 hours, an average donor age of 35±13.2 years and an average pancreas donor risk index (PDRI) of 1.57±0.58. The recipients were 42±9 years old at the time of the transplant and with a BMI of 24.8±3.8. We observed 7 graft failures (12.5%) after a median time of 9.7 months (range 0-41). Fourteen patients (25%) had average BG levels >8 mmol/l in the first 24 hours after surgery but ended up with a functioning graft. The 7 graft failure patients had similar BG to the patients with a functioning graft but a significantly lower BE in the first and second day after surgery (p=0.024 and p=0.0015 respectively), a lower pH in the first 24 hours (p=0.046), had a superior volume of fluids infusion (p=0.033) and a fluid balance on day 1 and 2 of 2593 and 4293 ml versus 1454 and 1628 ml (p=0.026 and p=0.003 respectively).

Conclusions

The management of pancreas transplantation is complicated by the absence of a marker that enables graft dysfunction to be detected at an early enough stage to allow more intensive investigation or effective intervention. Although DGF after PT has been reported, relatively little is written about this entity in the literature. There is no agreed-upon definition of what constitutes DGF after PT and little is known about its etiology or its impact on short- and long-term outcomes in recipients. These preliminary results showed that BE, pH and fluid balance might predict the graft outcome better than BG.

Patients' recruitment and data collection and analysis are still ongoing. The complete dataset and the final univariate and multivariate analysis is needed in order to confirm the preliminary results and identify, if possible, the DGF patterns.

Dr. Amelia SPINELLA

CEM Curriculum: Translational Medicine
Tutor: Prof. Clodoveo Ferri

CARDIO-PULMONARY EVALUATION IN A LARGE COHORT OF PATIENTS WITH SYSTEMIC SCLEROSIS

Background

Systemic Sclerosis (SSc) is a connective tissue disease, characterized by progressive thickening and excessive fibrosis of the skin and internal organs, as well as by widespread microvascular damage. Cardiopulmonary involvement is common in SSc: pulmonary fibrosis, pulmonary arterial hypertension (PAH), electrical disorders are the most serious complications and frequent cause of death.

Objectives

To assess the prevalence and incidence of cardiopulmonary involvement in patients with SSc, as well as the type and severity of cardiac and lung involvement and their correlations with SSc clinical features, quality of life, and survival.

Methods

We analysed 241 consecutive SSc patients referred to our Rheumatology Unit from January 1999 to January 2014 (F/M 205/36; mean age 50.8 ± 14.7 SD years). All patients underwent general and cardio-pulmonary evaluation, including demographic and clinic-serological features, standard electrocardiogram (ECG), Doppler echocardiography, right heart catheterization (when requested), high resolution scan of the lungs (HRCT), and pulmonary function tests, according to current methodologies.

Results

During follow-up 38/241 patients (15.8%) died because of pulmonary complications (6 pts; 15.8%), severe PAH (15 pts; 39.5%), in 5 cases complicated by lung cancer (13.2%), cardiac involvement (10 pts; 26.3%), and other causes (7 pts; 18.4%). Statistically significant findings were found in the deceased group if compared to remaining patients. In particular, forced vital capacity (FVC) and carbon monoxide diffusing capacity (DLCO) were significantly reduced in the deceased individuals ($p<0.001$) which also showed higher rates of severe fibrosis at HRCT ($p<0.001$). Furthermore, these patients were characterized by higher incidence of ECG frequency and rhythm alterations ($p<0.001$), ischemic ECG changes ($p=0.003$), increased systolic PA-pressure ($p<0.001$), decreased values of tricuspid annular plane systolic excursion (TAPSE) ($p<0.001$), reduced left ventricle ejection fraction ($p=0.001$), right-sided dilatation ($p<0.001$), higher

prevalence of pericardial effusion (p=0.012) at echocardiography, and reduced cardiac index (p=0.007) and cardiac output (0.036) at right heart catheterization.

Multivariate analysis using Cox's regression emphasized the increased risk of death due to pulmonary (hazard ratio, 3.907; 95% confidence interval, 1.829-8.346; p <0.001) and heart involvement (hazard ratio, 2.840; 95% confidence interval, 1.288-6.259; p=0.010). In particular, ECG frequency alterations, reduced DLCO values and male gender were identified as the strongest independent predictive factors related to mortality.

Conclusions

Our findings provide further evidence of the prognostic value of cardiopulmonary involvement that is the leading cause of SSc-related morbidity and mortality. The detection of these manifestations in the early stage of the disease as well as their careful monitoring and follow-up are mandatory in order to counteract their impact on the overall disease outcome.

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STUDY ON HYDROGEN SULFIDE PROTECTIVE ACTION IN ALZHEIMER'S DISEASE RAT AND MOUSE MODELS

Background

Alzheimer's disease (AD) is a chronic disorder characterized by progressive neurodegeneration associated with cognitive decline and several behavioral deficits. Sporadic AD is generally diagnosed in people over 65 years of age, whereas familial or genetic AD (the less prevalent form of Alzheimer) is an early-onset autosomal dominant disease [1]. In developed countries, AD is one of the most economically costly disease to society: indeed, an estimated 26.6 million people worldwide had AD in 2006, and this number may quadruple by 2050.

In some regions of AD brains, such as cortex and hippocampus, two members of the γ-secretase complex, presenilin-1 (PS1) and presenilin-2 (PS2), and β-secretases process the amyloid precursor protein (APP) to generate extra-cellular β-amyloid (Aβ) fibrillar deposits (Aβ plaques); also intraneuronal tau neurofibrillary tangles composed of hyperphosphorylated tau protein develop. Both these hallmark lesions trigger pathophysiological pathways that lead to synaptic dysfunction, neurodegeneration and marked neuronal loss with consequent impairment in cognitive functions. Furthermore, free radicals, nitric oxide, glutamate, several cytokines, mitogen-activated protein kinases, Bcl-2 family members and caspases play an important role in the mentioned pathophysiological pathways. A combination of impaired cholinergic transmission and high glutamate activity underlies the main symptomatology of AD, which is characterized by memory loss and severe cognitive decline [2].

Hydrogen sulfide (H2S) is a colorless, flammable, water-soluble gas and Tabiano's spa-waters are particularly rich in H2S (strong sulfydrometric degree, that is, more than 100 mg/l) [3]. H2S is increasingly being considered as an important signaling molecule in various body systems, and accumulating evidence demonstrates that H2S donor compounds exert significant beneficial effects in several animal models of inflammation and ischemia/reperfusion injury [4]. H2S is endogenously produced also in the brain, probably with a neuromodulatory role. It has been previously reported that brain H2S synthesis is severely decreased in AD patients, and plasma H2S levels are negatively correlated with the severity of AD [5]. Recent data showed that the H2S donor sodium hydrosulfide reduces Aβ generation in cultured cells, and Aβ-induced cognitive impairment in rats, as detected in a short-term study [6]. Further, inhaled H2S resulted able to prevent neurodegeneration in a mouse model of Parkinson's disease [7].

Preliminary recent results of our research group showed that H2S and Tabiano's spa water have neuroprotective effects in slight experimental AD [8].

Objectives

The aim of my research project will be to extensively evaluate the possible neuroprotective and neurogenic effects of a short- and long-term treatment with a H2S donor and Tabiano's spa-water to counteract the progression of AD.

Methods

For my study I will use two different animal models of AD:

- rat model of AD induced by brain injection of β-amiloid1-40 (Aβ);
- AD mouse model harboring human transgenes APPswe, PS1M146V, tauP301L (3xTg-AD mice).

In the rat model I will study an early phase of AD, in transgenic mice I will evaluate the middle and severe AD conditions.

In each model I will investigate the neuroprotective effects of a H2S donor and Tabiano's spa-water through analysis of cognitive tests (learning and memory), brain morphological alterations, amyloid/tau cascade, excitotoxicity, inflammatory and apoptotic responses; and I will investigate the neurogenesis process through immunohistochemical analysis.

Expected results

Favorable results would suggest that appropriate treatments with H2S donors or Tabiano's spa-water might represent an innovative approach to slow down AD progression in humans.

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