University of Modena and Reggio Emilia

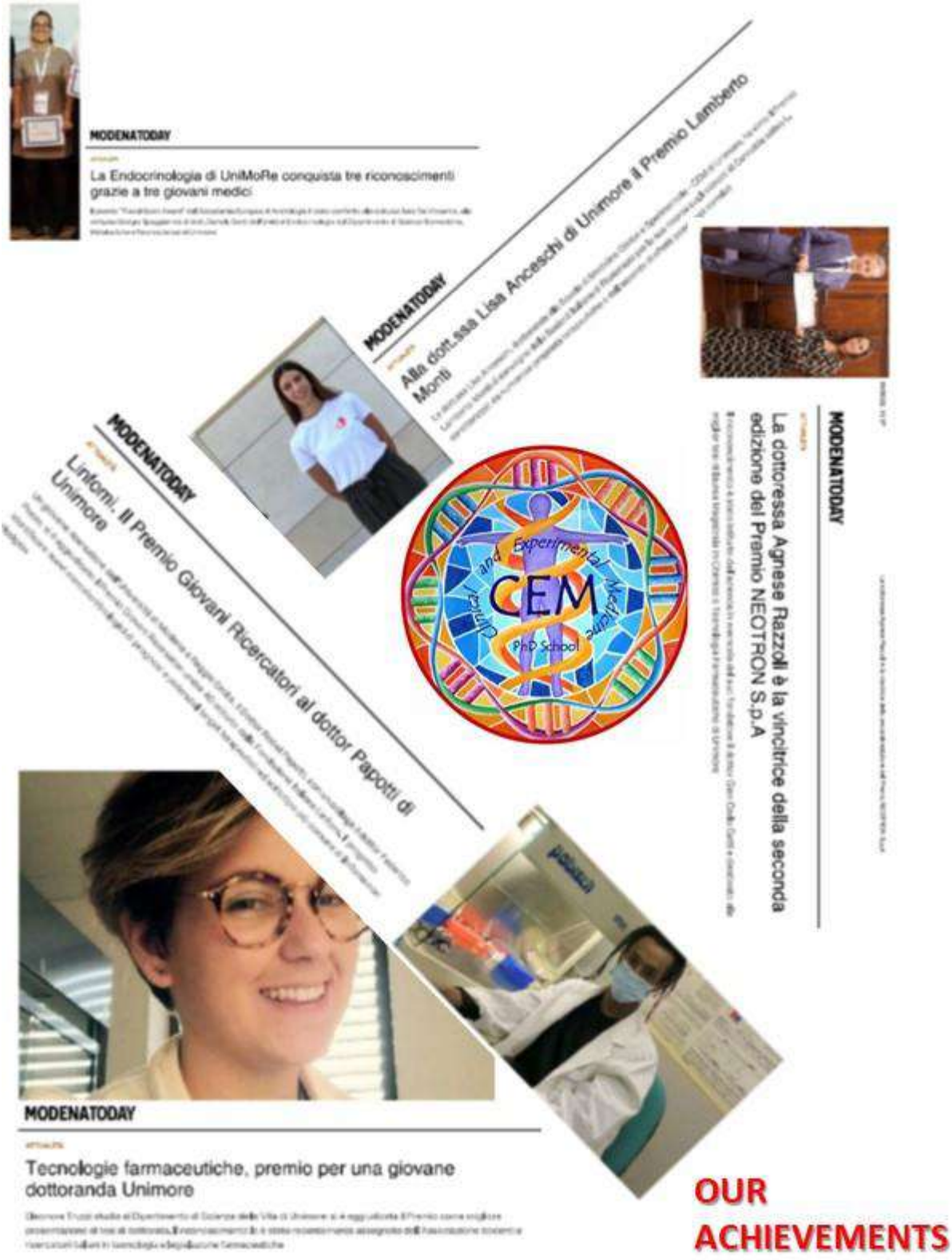
PhD COURSE OF CLINICAL AND EXPERIMENTAL MEDICINE

PhD DAY 2022

Abstracts

June 10 (9:00 a.m.), lecture room H1.1 of Physiology – Dept. Biomedical, Metabolic and Neural Sciences, (287 Campi street, Sect. Physiology and Neural Sciences - Modena)

June 17-24 (9:00 a.m.), room cs0.1 "Centro Servizi Policlinico" (71 del Pozzo street - Modena)



Many students of our PhD programme have been awarded in the last year and the previous ones. Some of them made the award public by releasing press news. Just to mention some of the awards of the last year, our PhD students received the: Eli-Lilly Scholarship (Alessia Paganelli and Cristel Ruini), ICAR 2021 Scientific Committee award (Lorenza Di Marco), Alberto Biggi award (Rexhep Durmo), American Society of Hematology award (Annalisa Tameni), Best Paper "Accademia LIMPE-DISMOV" 2021 (Francesco Cavallieri).

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

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

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

Nanomedicine, Medicinal and Pharmaceutical Sciences
Translational Medicine
Health Sciences

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

Medicinal and Pharmaceutical Sciences
Translational Medicine
Health Sciences

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

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

XXXIV cycle

Federica Violi

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

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

Background

Among the interventions aimed at changing health providers' behaviors, "Audit & feedback" (A&F) has been emerging as one of the most promising and its effectiveness has been shown in different settings. In an audit and feedback process, an individual's professional practice or performance is measured and then compared to professional standards or targets. (Ivers, Jamtvedt et al. 2012, Ivers, Grimshaw et al. 2014) Indeed, providing health professionals with structured reports on their performance can stimulate quality improvement when process or outcomes indicators highlight potential problems concerning the delivery of specific health care interventions or the clinical outcomes achieved. However, many factors influence the actual yield of A&F interventions, including the context in which they are applied, the type of targeted behaviors, and how they are structured and delivered

Type 2 diabetes mellitus (T2DM) and Chronic Heart Failure (CHF) are serious and growing common chronic conditions that are increasingly managed by health professionals in outpatient and community settings.

Over the last few years, the Emilia-Romagna Regional Health Care System promoted a reorganisation of primary care, largely based upon the principles of the Chronic Care Model and the adoption of formally structured clinical pathways. The development and implementation of these pathways require the involvement of different health professionals and demand strong clinical as well as managerial responsibility in constantly monitoring processes and outcomes of care.

Because A&F include a wide range of interventions (which differ in terms of type of quality indicators used, structure of the report feedback to health professionals, frequency of the feedback), providing those responsible for the organisation and management of clinical pathways for chronic conditions with timely and exhaustive information on relevant dimensions of the quality of care delivered (safety, effectiveness, appropriateness and equity) is of the utmost importance.

Objectives

- Aim 1: To assess the impact on the quality of care of an A&F intervention based upon information drawn from the administrative databases available.

- Aim 2: To explore the opportunities offered by additional sources of information and to fully address relevant dimensions of quality of care and health services performance through the use of qualitative research methods (*focus group*).
- Aim 3: To compare different approaches towards the implementation of A&F interventions.

Methodologies and statistical analyses

The Emilia Romagna Region is part of a project founded by the Ministry of Health to define the characteristics of an optimal A&F intervention applied to T2DM and CHF clinical pathways. In order to design an optimal A&F system, an analysis of the existing clinical pathways has been undertaken among the Local Health Authorities of Reggio Emilia, Bologna, Imola and Piacenza, relying on the available formal documentation integrated with structured interviews with local managers and clinicians. Process and outcome indicators measurable on administrative data available has been selected, also relying on a review of the literature in the field. Moreover, a qualitative analysis, through the construction of focus groups and interviews, has been conducted among health professionals and patients, to identify the determinants and the dynamics that regulate the hospital-territory continuity of care and to allow the identification of additional information and indicators required in an optimal A&F intervention for the management of effective clinical pathways. Furthermore, a structured questionnaire was administered to different Italian regions implementing A&F interventions to improve clinical practice with the aim of analyzing and comparing different regional initiatives in terms of type of competences and responsibilities involved, characteristics of target clinical behaviors of the interventions and type of indicators and data sources used.

Results:

Within the context of the questionnaires collected among the 7 Italian regions implementing A&F interventions to improve clinical practice (Aim 3), a direct comparison between the 15 suggestions made by the article of Breahut and colleagues and the main results obtained through the analysis of the projects collected is represented in the following table. We investigated the characteristics of the working group composition, the targeted clinical behaviors, the selected indicators and their informative sources, the feedback procedures to be adopted (i.e. timing and frequency of the reports, as well as their structure), the actions expected from the targeted health professionals (i.e. what health professionals were supposed to do or act on after feedback delivery) and other interventions to be carried out along with the A&F procedure to sustain/reinforce its impact. Some suggestions were respected by almost all the A&F designers, others were less represented and in some cases no information was available to decide the level of adherence to the suggestion. The results are presented in the following table.

Table. Comparison between literature recommendations and approaches proposed in the regional projects.

Items to be explored (Breahut)	Results
Nature of the desired action	
1. Recommend actions that are consistent with established goals and priorities	The intervention targets defined refer mainly to the improvement of the continuity of care, the coordination among professionals, the adherence to guidelines recommendations and clinical pathways rules, patient engagement and its relationship with doctors, the reliability of administrative data and indicators.
2. Recommend actions that can improve and are under the recipient's control	The professionals involved are mainly epidemiologists, statisticians, clinical researchers, physicians, nurses and other professional figures involved in healthcare assistance. Less represented are ICT, manager and other professional figures with coordination roles. The structure of the intervention is shared with professionals in all the projects. The identified recipients of the feedback are individual professionals (GPs or specialists), as well as multidisciplinary teams and manager or other professional figures with coordination roles.
3. Recommend specific actions	Comparison and dialogue with colleagues, implementation of healthcare practice recommended by guidelines and clinical pathways.
Nature of the data available for feedback	
4. Provide multiple instances of feedback	Reference time: mainly 6 or 12 months
5. Provide feedback as soon as possible and at a frequency informed by the number of new patient cases	Mainly half yearly, but also other frequency are reported
6. Provide individual rather than general data	Mainly aggregate data
7. Choose comparators that reinforce desired behavior change	Reference standard are identified in all the projects at different levels (i.e. international standards, average regional data, comparison with professionals of the same Primary Care Department, standard derived from national lawa, International Standard for Equity in Healthcare from HPH)
Feedback display	
8. Closely link the visual display and summary message	The report structure involves the use of tables and graphs in most of the projects

XXXV cycle

Roberto Tonelli

CEM Curriculum: Translational Medicine
Tutor: Prof. Enrico Clini

INSPIRATORY EFFORT AND RESPIRATORY MECHANICS IN SPONTANEOUSLY BREATHING PATIENTS WITH ACUTE EXACERBATION OF IDIOPATHIC PULMONARY FIBROSIS: A RETROSPECTIVE MATCHED CONTROL STUDY

Background

Patients with acute exacerbation of idiopathic pulmonary fibrosis (AE-IPF) may experience severe acute respiratory failure, even requiring non-invasive ventilatory assistance. In this scenario, the mechanical behavior of the fibrotic lung when subjected to unphysiological pressures may enhance the parenchymal damage. However, physiological data on inspiratory effort and lung mechanics in AE-IPF patients during unassisted or assisted spontaneous breathing is lacking.

Objectives

This study aimed at exploring the inspiratory effort and the respiratory mechanics, at baseline and 2 hours following non-invasive mechanical ventilation (NIV) application, in a population of patients with AE-IPF and to compare the mechanical data with acute respiratory distress syndrome (ARDS) patients matched for clinical severity.

Methods

Patients with AE-IPF admitted to the Respiratory Intensive Care Unit to receive a NIV trial from August 1st, 2016, to January 1st, 2022, were retrospectively analyzed. Esophageal pressure swing (ΔP_{es}) and respiratory mechanics before and 2 hours after NIV start were collected and described serving as the primary outcome. The paired Student's *t*-test assessed the difference between variables before and after NIV application, when distributed normally; otherwise, the Wilcoxon test was used. Correlations between positive end-expiratory pressure (PEEP) levels and changes in dynamic compliance ($dynC_{RS}$) and PaO_2/FiO_2 ratio were also assessed using Pearson's correlation coefficient and linear regression analysis. An exploratory comparison with a historic cohort of ARDS patients -propensity score-matched 1:1 by age, sequential organ failure assessment (SOFA) score, body mass index (BMI), and PaO_2/FiO_2 level- was performed using Student's t-test or Wilcoxon test and $\chi 2$ test or Fisher's exact test for continuous and dichotomous variables respectively, as appropriate. ANOVA and Kruskal-Wallis were further used to test as an interaction for whether the change in physiological variables 2 hours after NIV were different between groups.

Results

At baseline, AE-IPF presented high respiratory drive activation with ΔP_{es} = 27 (21 – 34) cmH_2O, respiratory rate (RR) = 34 (30 – 39) bpm and minute ventilation (VE) = 21 (20 -26) L/min. Two hours after NIV application, ΔP_{es}, RR and VE showed a significant reduction (16 [14 – 24] cmH_2O, p<0.0001, 27 [25 – 30] bpm, p=0.001, and 18 [17 – 20] L/min, p=0.003, respectively) while no significant change was found for dynamic transpulmonary pressure (27 [21 – 34] VS 27 [25 – 36] cmH_2O, p=0.2) expiratory tidal volume (Vte) (9.1 [8.7 – 10.1] VS 9.3 [8.7 – 9.9] mL/kg of predicted boy weight, p=0.2), $dynC_{RS}$ (28 [19 – 31] VS 26 [18 – 28] mL/cmH_2O, p=0.1) and dynamic mechanical power (71 [49 – 94] VS 60 [51 – 74] J/min, p=0.1). PEEP levels negatively correlated with PaO_2/FiO_2 ratio and $dynC_{RS}$ (r=–0.67, p=0.03 and r=–0.27, p=0.4, respectively). When compared to AE-IPF, ARDS patients presented lower baseline value of ΔP_{es} (24 [22 – 28] cmH_2O, p=0.004), RR (27 [26 – 30] bpm, p=0.001), VE (18 [17 – 21] L/min, p=0.04) and dynamic mechanical power (48 [52 – 62] J/min, p=0.01). At difference with AE-IPF, Vte and $dynC_{RS}$ increased significantly following NIV application (p=0.01 and p=0.004 respectively) with PEEP levels directly associated with PaO_2/FiO2 ratio and $dynC_{RS}$ (r=0.24, p=0.5 and r=0.65, p=0.04 respectively).

Conclusions

In this study, patients with AE-IPF showed a high inspiratory effort, whose intensity was reduced by NIV application without significant improvement in respiratory mechanics. In an exploratory analysis, AE-IPF patients showed a different mechanical behavior under spontaneous unassisted and assisted breathing compared with ARDS patients of similar severity.

Cecilia Botti

CEM Curriculum: Translational Medicine
Tutor: Dr. Angelo Ghidini
CoTutor: Prof. Livio Presutti

THE EXTENT OF VESTIBULAR INJURY IN SUDDEN SENSORINEURAL HEARING LOSS

Background

Sudden sensorineural hearing loss (SSHL) is the loss ≥30 dB in three consecutive frequencies. The most widely acknowledged theories for explaining the pathogenesis of SSHL implicate vascular ischemia, autoimmune response and viral infection. However, the exact mechanism underlying SSHL remains elusive.

Several prognostic indicators of favourable or unfavourable outcome in SSHL have been proposed: patient's age, the interval between the onset of symptoms and treatment, audiometric patterns, the severity of hearing loss, findings at vestibular evoked myogenic potentials (VEMPs). The importance of the assessment of vestibular function was evidenced by previous studies. While cervical and ocular vestibular evoked myogenic potentials (cVEMPs and oVEMPs, respectively) test otolith organs and their afferents, semicircular canal function can be reliably evaluated by the video head impulse test (vHIT) in all canal planes. A recent study showed that vHIT results were different in vestibular neuritis and SSHL with vertigo, suggesting different causes of vestibular neuritis and SSHL. To our knowledge, the function of all three semicircular canals in SSHL without vertigo has never been studied by the means of the vHIT.

Objectives

The primary aim of the study is to describe the extension of macular and canal injury in patients affected by SSHL with or without vertigo. Secondary aims are to study the association between canal and macular function with hearing prognosis and to describe ischemic patterns of inner ear damage.

Methods

All consecutive patients with SSHL with or without vertigo who referred to Otolaryngology Unit and Audiology Unit of *Azienda USL – IRCCS di Reggio Emilia* were consecutively recruited for one year, starting from the approval of the local Ethic Committee *Area Vasta Emilia Nord* in September 2020. Patients gave informed consent for inclusion in the study. Inclusion criteria were: age ≥18 years, every sex, new diagnosis of SSHL. Exclusion criteria were: incomplete follow-up, previous ear disease, contralateral hearing loss, known etiology of hearing loss, except for hydrops. The usual therapeutic and diagnostic protocol was followed. Follow-up consisted in audiometric examinations. Demographic, clinical and instrumental data were collected: initial and final audiogram, c-/o-VEMPs, vHIT, age, gender, comorbidity, symptoms, time from clinical onset to treatment, ischemic alterations at head-MRI. o-/c-VEMPs and vHIT results were compared

with hearing recovery and the presence of ischemic alterations at head MRI. Statistical analysis was performed using SPSS 20.0 (IBB SPSS, IBM Corp., Armonk, NY). Quantitative variables were checked for normal distribution using Kolmogorov-Smirnov and Shapiro-Wilk tests. Continuous variables were described by mean ± standard deviation or median and range. Comparisons between groups were performed by Pearson's chi-squared test or Fischer's exact test for categorical variables, one-way analysis of variance, Mann-Whitney U test or Kruskal-Wallis test for continuous variables. Differences between groups were considered significant at $p<0.05$.

Results

147 patients had SSHL during the study period, 61 patients were excluded, 86 patients met the inclusion criteria and were included in the study. Median age was 55.7 years (22-84). The male to female ratio was 1.32. Vertigo occurred in 73.3% (63/86) of cases. The severity of hearing loss was: mild (12.8%), moderate (47.7%), severe (18.6%), profound/complete (20.9%). Hearing loss configuration was flat (50%), down-sloping (25.6%), or low-frequency (24.4%). Hearing recovery was: complete (28.0%), partial (32.0%), or absent (36.0%). cVEMPs or oVEMPs abnormalities were found in 45.3% and 41.9%, respectively. Canal function was altered at the vHIT in lateral 16.3% for lateral, 20.9% for posterior and 10.6% for anterior canal.

Three groups of patients were identified: patients without vertigo, patients with vertigo, patients with Meniere's disease. The groups didn't differ significantly in terms of age ($p=0.10$), cardiovascular risk factors ($p=0.39$), or time from diagnosis to treatment ($p=0.98$). Hearing loss configuration and severity differed significantly among the groups: mild/moderate and low-frequency hearing loss occurred more frequently in Meniere's disease patients ($p<0.01$). Saccular dysfunction was more frequent in patients with vertigo ($p<0.05$). Utricular function didn't differ significantly among the groups ($p=0.48$). Lateral or anterior canal dysfunction was more frequent in patients with vertigo and in patients with Meniere's disease ($p<0.05$). Posterior canal dysfunction was more frequent in patients with vertigo ($p<0.05$). Complete canals deficit was present only in patients with vertigo. Isolated superior canal dysfunction was present only in Meniere's disease. Deficit of three or more receptors was more frequent in Meniere's disease or in patients with vertigo ($p<0.05$). Impaired saccular function and high number of impaired receptors were associated with poor hearing outcome ($p<0.05$). Patients with vertigo had more severe Fazekas scale scores at head MRI ($p<0.05$). Patients with ischemic patterns had also vertigo ($p<0.05$), severe hearing loss and poor outcomes ($p<0.05$).

Conclusions

A complete functional assessment of vestibular receptors and afferents in SSHL with or without vertigo could help to better define the extension of the injury and give information about the prognosis. The extent of canal and macular injury could suggest the ischemic nature of SSHL. Vertigo, high number of injured receptors and certain patterns of canal or macular injuries are associated with poor outcome.

Sara De Vincentis

CEM Curriculum: Translational Medicine
Tutor: Prof. Vincenzo Rochira

BIOCHEMICAL HYPOGONADISM IN PEOPLE LIVING WITH HIV: RELEVANCE OF SEX HORMONE-BINDING GLOBULIN (SHBG) MEASUREMENT AND COMPARISON BETWEEN LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY (LC-MS/MS) AND CHEMILUMINESCENT IMMUNOASSAY FOR SEX STEROIDS ASSAY

Background

Although the prevalence of male hypogonadism among people living with HIV (PLWH) has significantly lowered after the introduction of antiretroviral therapy, it remains high if compared with age-matched HIV-uninfected men, ranging from 13% to 40% in the age group of 20-60 years. Immunoassay-based techniques are generally used in clinical practice for the assay of serum total T (TT) and estradiol (E2), but they lack adequate accuracy and sensitivity in particular for values in the lowest normal range for men or below it. For this reason, liquid chromatography tandem mass spectrometry (LC-MS/MS) represents the recommended methodology for the assessment of TT and E2. Furthermore, increased levels of Sex Hormone-Binding Globulin (SHBG) that are typical of the HIV clinical setting may lead to normal levels of TT despite actually low free testosterone levels, resulting in biochemical hypogonadism. The importance of SHBG measurement in male PLWH has been largely emphasized by the guidelines on male hypogonadism. At present, however, data on the prevalence of hypogonadism in PLWH based on the combination of serum sex steroids measured by LC-MS/MS, SHBG and gonadotropins are lacking.

Objectives

The aim of this study is to investigate the prevalence of biochemical hypogonadism and relative estrogen deficiency in male PLWH aged<50 comparing LC-MS/MS with chemiluminescent immunoassay (CI), and to characterize the whole functionality of hypothalamic-pituitary-gonadal axis combining TT, gonadotropins, SHBG and E2 measurements.

Methods

A prospective, cross-sectional, observational study was carried out involving male HIV-infected outpatients attending the Modena HIV Metabolic Clinic from May 2013 to December 2017. Inclusion criteria were men aged 18-50 years, with documented HIV-infection and ongoing antiretroviral therapy (ART).

Exclusion criteria were prior treatment (referred or documented) with androgens, sex steroids, antiandrogens, anabolic agents, GnRH agonists, psychotropic agents; documented hypothyroidism, known pituitary, testicular or adrenal diseases, a previous conventional pituitary surgery or radiotherapy;

documented Acquired Immunodeficiency Syndrome (AIDS), active cancer, severe liver insufficiency or severe chronic renal failure. Serum TT, E2, gonadotropins, SHBG were measured by CI. TT and E2 were also assessed by LC-MS/MS. Free testosterone (cFT) was calculated by Vermeulen equation. ***Statistical analysis:*** The non-parametric Mann-Whitney U test followed by the Dunn's multiple comparison *post-hoc* test was used for comparisons of continuous variables since they resulted not normally distributed at the Kolmogorov-Smirnov test. Categorical variables were compared with Chi-square test. Linear regression was used to examine the association between continuous variables; results were expressed through β and R2 coefficients. Stepwise, linear, multiple regression analysis using a backward elimination method was applied to the data with $p<0.1$ as the criterion for a variable to enter the model.

Results

A total of 316 PLWH (45.3±5.3 years) were enrolled. Both serum TT and cFT assessed by LC-MS/MS were significantly lower compared to serum TT (639 ng/ml vs 720 ng/ml, $p<0.0001$) and cFT (105.7 pg/ml vs 118.6 pg/ml, $p<0.0001$) assessed by CI. The prevalence of biochemical hypogonadism was higher with LC-MS/MS than CI, both for TT (5.1% vs 3.2%, $p<0.0001$) or cFT (9.5% vs 7%, $p<0.0001$). The prevalence of hypogonadism (overt+compensated) was 17.1% for cFT using LC-MS/MS. Secondary form of hypogonadism was more prevalent than primary. Hypogonadal patients were significantly older ($p=0.002$) than eugonadal patients; moreover, the duration of HIV infection and of ART was significantly longer in the hypogonadal subgroup (both $p<0.0001$). Serum SHBG did not differ between eugonadal and hypogonadal PLWH; the stepwise multivariate regression analysis showed that SHBG was associated with serum TT, viral liver co-infection and duration of HIV. The prevalence of relative estrogen deficiency was of 30.0% among hypogonadal patients and 15.5% among eugonadal.

Conclusions

The measurement of serum TT by LC-MS/MS coupled with SHBG improves biochemical hypogonadism diagnosis in male PLWH. Besides, our findings confirm that SHBG is a key diagnostic tool useful to identify T deficiency in the context of HIV since prevalence of hypogonadism is about 1.9-fold increased using serum cFT rather than TT, regardless of the method used for TT measurement (CI or LC-MS/MS). On the other hand, the prevalence of male hypogonadism results underestimated by CI compared to LC-MS/MS in PLWH, both for TT and cFT. Finally, gonadotropins are essential for detecting a condition of compensated hypogonadism.

Ilaria Ottonelli

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

NANOMEDICINES ACROSS BARRIERS

Background

Nanomedicines (NMeds) offer innovative advantages over traditional drug formulations, such as: protection of sensitive drugs, selective targeting, and controlled release. One prime example of NMed applications is for Inherited Retinal Degeneration (IRD), a neurodegenerative genetic disorder that leads to the death of photoreceptors and eventually blindness. While current treatments consist of surgery or administration of antioxidant molecules, a peptide derived from the Pigment Epithelium Derived Factor has recently been demonstrated to effectively prevent cell death in IRD after intravitreal injection; however, this peptide is degraded in the vitreous and needs several injections to reach therapeutic levels. Thus, its encapsulation into NMeds could represent an advantageous approach to avoid the loss of active peptide and reduce the amount of drug needed to achieve curative levels of activity. A further step towards prolonged release could be represented by the formulation of an injectable hydrogel: such a matrix could act as a reservoir, slowly releasing NMeds over time to further reduce the number of intravitreal injections needed.

Objectives

The first aim of this project is to investigate a NMed formulation to efficiently delivery a neuroprotective peptide after intravitreal injection. A second aim is to formulate an injectable, thermo-sensitive biocompatible hydrogel to achieve a prolonged release of the neuroprotective peptide to the retina. To investigate these aims, biodegradable and biocompatible materials were chosen to formulate NMeds, such as the FDA approved polymer poly (L-lactic-co-glycolic) acid (PLGA), biocompatible lipids i.e., Cholesterol, DOTAP, and DOPE, hyaluronic acid (HA). To formulate hydrogels, biocompatible materials such as high molecular weight HA and poloxamer P407 were used.

Methods

Starting from well-established protocols for polymeric and hybrid NMeds, several formulations were designed and characterized in their chemico-physical properties. Fluorescently labeled NMeds were used to test for their biodistribution in the mouse eye after intravitreal injection to select a lead-NMed. The selected system was further characterized in terms of stability, morphology, composition, and peptide encapsulation efficiency. Peptide-loaded NMeds were then tested using *in vitro* retinal cultures to assess toxicity, uptake, and peptide activity.

For the second aim, in collaboration with the University of Angers (France), the formulation of an injectable thermosensitive hydrogel was optimized using HA and poloxamer P407. Concentrations of both polymers were varied to reach a gelation temperature around 33°C and good injectability. At the same time, rheology analyses were performed on hydrogels composed of high molecular weight HA and agarose to have a model for synthetic vitreous humor.

Results

Among polymeric and hybrid NMeds tested, the one most abundantly found in the retina consisted of a hybrid core of PLGA and DOTAP, surface coated of HA. The system was demonstrated to have a peptide encapsulation efficiency of around 20%, and residual surfactant in the matrix of less than 10%. Regarding the amount of HA on the surface, technical issues linked to poor reproducibility of the colorimetric quantification protocol still need to be specifically addressed. *In vitro* these NMeds showed low toxicity at a concentration of 8 µg/mL, but massive uptake by cultured photoreceptors 24 h after administration. Unfortunately, the peptide receptor is found on the cell surface, and this large uptake would lead to decreased biological effects. With the aim of reducing their uptake, NMeds were embedded into a hydrogel of HA and poloxamer P407. The optimized hydrogel displayed a liquid behavior < 10°C, with a gelation temperature of 34°C. This gel was loaded with fluorescently labelled NMeds and injected in an in vitro synthetic vitreous humor model to test the diffusion and release profile of NMeds throughout the model system. Fluorescent microscopy analysis is now ongoing to assess the diffusion rate of NMeds out of the hydrogel across the synthetic vitreous, compared to NMeds suspension.

Conclusions

Nanomedicines offer a variety of possibilities to increase the therapeutic efficacy of treatments. Retinal diseases are often hard to treat due to inaccessibility of the tissue and the poor patient tolerability of the high number of intravitreal injections currently needed. This makes NMed delivery a prime candidate for improved applications. In particular, the optimization of NMeds loaded with a novel neuroprotective peptide is a promising approach for IRD, and the formulation of these structures into an injectable hydrogel could further improve the advantageous features of prolonged release, reduction of the number of injections needed, protection of the peptide, and lower toxicity. Future experiments will evaluate the ratio of NMeds loaded in the hydrogel to optimize the peptide release profile. These formulations will eventually be tested on *ex vivo* and *in vivo* IRD models.

Lara Senn

CEM Curriculum: Translational Medicine
Tutor: Prof. Giuseppe Biagini
CoTutor: Dr. Anna-Maria Costa

ANTISEIZURE EFFECTS OF CANNABIDIOL LEADING TO INCREASED PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA LEVELS IN THE HIPPOCAMPAL CA3 SUBFIELD OF EPILEPTIC RATS

Background

Epilepsy is a chronic disorder displaying a high impact on psychological, social, and economic conditions of patients and their caregivers, besides the problems of the disease itself. Its prevalence of 0.5% to 1.0% within the world's population ranks epilepsy on the fourth place of neurological diseases after migraine, stroke, and dementia. Among others, temporal lobe epilepsy (TLE) is the most common form of epilepsy in adult patients. The administration of antiseizure medications (ASMs) results in seizure freedom in about 65% of patients. However, the remaining patients continue to present spontaneous recurrent seizures (SRSs). For this reason, the use of alternative drugs as new treatments is in the focus of the latest research. *Cannabis sativa L.* has been cultivated for over 6000 years to treat pain and insomnia and used since the 19th century to suppress epileptic seizures. Cannabinoids have been shown to regulate the excitability of neuronal circuits involving the endocannabinoid system and associated ligands and receptors. Therefore, cannabidiol (CBD) has gained considerable interest as an add-on medication for drug-resistant epilepsy, but its exact mechanism needs to be clarified.

Objectives

The aim of this project is to investigate the anticonvulsant properties of CBD and peroxisome proliferator-activated receptor gamma (PPARγ) levels in an animal model of temporal lobe epilepsy (TLE). A beneficial interaction was found between PPARγ and CBD, which was associated with neuroprotective properties in ischemic stroke and with stimulated neurogenesis in a model of Alzheimer's disease. Moreover, we recently found that PPARγ is involved in the antiseizure properties of EP-80317, a ghrelin analogue tested in a model of repeated seizure induction. Thus, it could be possible that the antiepileptic effects of CBD depend, at least in part, on unconventional targets such as PPARγ. To evaluate this hypothesis, we designed an experiment in which two different doses of CBD (12 or 120 mg/kg) were tested in epileptic rats previously treated with kainic acid (KA). In these animals, we evaluated the changes in levels of PPARγ immunoreactivity in response to the antiseizure effects of CBD.

Methods

Thirty-eight adult male Sprague-Dawley rats (Charles River, Calco, Italy) of 175-200 g initial body weight were continuously monitored by video-electrocorticography up to 10 weeks after an intraperitoneal kainic acid (15 mg/kg) injection. Sixty-seven days after the induction of status epilepticus and the appearance of SRSs in all rats, CBD was dissolved in medium-chain triglyceride (MCT) oil and administered subcutaneously at 120 mg/kg (n = 10) or 12 mg/kg (n = 10), twice a day for three days. Similarly, the vehicle was administered to ten epileptic rats. Brain levels of PPARγ immunoreactivity were compared to those of six healthy controls via immunohistochemical staining. The duration of the convulsive seizures was determined by ECoG recordings, while the severity of convulsive seizures was detected by behavioural observing using a modified Racine scale.

Results

CBD at 120 mg/kg abolished the seizures in 50% of rats ($p = 0.033$ vs. pre-treatment, Fisher's exact test) and reduced total seizure duration ($p < 0.05$, Tukey Test) and occurrence ($p < 0.05$). PPARγ levels increased with CBD in the hippocampal CA1 subfield and subiculum ($p < 0.05$ vs. controls, Holm–Šidák test), but only the highest dose increased the immunoreactivity in the hippocampal CA3 subfield ($p < 0.001$), perirhinal cortex, and amygdala ($p < 0.05$). Overall, these results suggest that the antiseizure effects of CBD are associated with upregulation of PPARγ in the hippocampal CA3 region.

Conclusions

The results obtained on the systemic KA rat model suggested that the antiseizure effects of CBD might lead, at least in part, to increased PPARγ levels in hippocampal and extrahippocampal regions, and especially in the hippocampal CA3 subfield. A possible limitation suggested in this study is the lack of definitive proof of the involvement of PPARγ in the antiseizure effects of CBD. In the future, we will assess whether PPARγ antagonists block the antiseizure effects of CBD.

Cristel Ruini

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

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

Background

Visual diagnosis plays a central role in the field of Dermatology. In the era of digitalization and artificial intelligence, novel imaging tools supporting physicians in diagnosis and follow-up need gained increased popularity not only in academia but also in daily clinical practice. This is particularly true if the above mentioned tools can be used to lead dermatologists to the correct diagnosis with no need for scarring, expensive and time-consuming surgical biopsies. Optical coherence tomography (OCT) and reflectance confocal microscopy (RCM) are useful in dermato-oncology, but not yet standardized and less explored in other fields. The novel device line-field confocal optical coherence tomography (LC-OCT) has not been systematically investigated yet.

Objectives

Aim of this project was the in-vivo investigation of the morphological patterns of LC-OCT-acquired 2D- and 3D-acquisitions of healthy skin, melanoma and non-melanoma skin cancers and main inflammatory skin diseases compared to their histological counterparts. First, we aimed to define main digital parameters for identifying above mentioned conditions at bedside together with their sensitivity and specificity compared to histology. Second, we intended to create algorithms for LC-OCT guided diagnosis and grading of basal cell carcinoma (BCC) and field cancerization. Third, we aimed to compare our findings with already established diagnostic techniques such as OCT and RCM, discussing the global pitfalls. In the end, we integrated artificial intelligence trained software to support operator dependent diagnostic decisions.

Methods

Six-hundred virtual skin samples including healthy skin, melanocytic and non-melanocytic benign and malignant tumors, inflammatory and bullous diseases were included in the study so far. Clinical and dermoscopic pictures were collected using Fotofinder, RCM with Vivascope©, OCT with Vivosight© and LC-OCT with the prototype of DAMAE medical When excision was necessary, the histological examination was performed. Morphological patterns, diagnosis and diagnostic confidence were blindly assessed by four expert observers. The sensitivity and specificity of LC-OCT features compared to dermoscopy and the gold standard histology were evaluated. Diagnostic confidence and interobserver agreement were rated.

Results

Most common LC-OCT findings of keratinocyte skin cancer in descriptive statistics were hyperkeratosis/parakeratosis, disruption of stratum corneum, broadened epidermis, basal and suprabasal keratinocyte atypia, dilated vessels/neoangiogenesis and elastosis/collagen alterations. Univariate multinomial logistic regression identified a preserved DEJ as less common feature in squamous cell carcinoma compared with actinic keratosis (AK) and Bowen disease, LC-OCT increased the diagnostic confidence by 24.7% compared with dermoscopy alone. We were able to in-vivo reproduce the criteria of the histological PRO classification of AK with an agreement of 75% for all lesions and 85.4% when comparing the subgroups PRO I vs. PRO II/III. The interobserver agreement was 90%. Main LC-OCT features of BCC and its histological subtypes were: atypical keratinocytes, altered DEJ, tumour nests in the dermis, dark clefting, prominent vascularization and white hyperreflective stroma for nodular BCCs, string of pearls pattern for superficial BCCs, shoal of fish pattern for infiltrative BCCs. We reported an overall BCC subtype agreement between LC-OCT and histology of 90.4 %. The multinomial regression with stepwise selection of variables identified following features as most useful in distinguishing BCC subtypes: epidermal thinning, atypical honeycombed pattern, prominent vessels/neoangiogenesis, shoal of fish pattern, string of pearls pattern and white hyperreflective stroma. In melanocytic lesions, no difference in performance between RCM and LC-OCT was observed. Good image quality correlated with better diagnostic performance for diagnosing a melanoma vs. all types of nevi with both devices. The most significant criteria for diagnosing a melanoma with LC-OCT were an irregular honeycombed pattern, pagetoid spread and the absence of dermal nests.

Conclusions

LC-OCT can enrich the actual knowledge on the in-vivo analysis of healthy skin and skin cancer and gain a central role in the world of non-invasive diagnostics in dermatology. Further studies focused on melanocytic lesions and inflammatory skin diseases are needed to gain more experience in this yet unexplored field of dermatology.

Tommaso Lo Barco

CEM Curriculum: Translational Medicine
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PROSPECTIVE EVALUATION OF GHRELIN AND DES-ACYL GHRELIN PLASMA LEVELS IN CHILDREN WITH NEWLY DIAGNOSED EPILEPSY: EVIDENCE FOR REDUCED GHRELIN-TO-DESACYL GHRELIN RATIO IN GENERALIZED EPILEPSIES

Background

Despite the continuous development of antiepileptic drugs (AEDs) in the past few decades, about 30% of patients with epilepsy still present seizures resistant to drugs. In the clinical setting, testing the response to a specific AED is time-consuming. Uncontrolled seizures and exposure to high doses of multiple medications result in possible developmental delay in children, cognitive decline in adults, and significant increase in morbidity and mortality. Ghrelin and des-acyl ghrelin, neuroactive peptide hormones, were recently found in higher concentrations in plasma of individuals with epilepsy who responded positively to drug treatment, compared with non-responders and healthy controls (*Marchiò et al, 2018*). Due to a possible anticonvulsive role seen in animal models, different plasmatic concentrations of ghrelin and des-acyl ghrelin may actually explain different responses to AEDs; however, mechanism underlying higher levels in drug responders is unknown.

Objectives

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

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

Methods

This is a 24-months-lasting prospective study, conducted in three neuropediatric Italian centers (Modena, Verona and Florence). Inclusion criteria for the group of interest were: i) subject with a suspicion of epilepsy; ii) subject between 0 and 16 years; iii) the obtaining of written informed consent by parents or caregivers.

Exclusion criteria were: acute or chronic metabolic disorders with rare/sporadic seizures without epilepsy. At variance, the control population was constituted of patients without any suspicion of epilepsy and/or acute or chronic metabolic disorders with rare/sporadic seizures. In recruited patients, we measured ghrelin and des-acyl ghrelin by immunoassays in plasma samples obtained after overnight fast at three different times: before (T0), beyond (T1) 2 months and a year (T2) after initiation of drug treatment. Demographic data, clinical features, epilepsy diagnosis, glycemia and blood level of anti-epileptic drugs were obtained and included in a multivariate statistical analysis, comparing values of ghrelin and des-acyl ghrelin in the following groups: i) patients with positive response to drug treatment ("responders"); ii) patients with drug-resistant epilepsy ("non-responders"); iii) control population.

Definition of drug resistant epilepsy was considered on the basis of Berg criteria (*Berg et al., 2006*) after a minimum of 18 months after treatment initiation.

Results

Among 50 recruited patients, 7 did not started any treatment because of the development of a benign form of epilepsy in which no treatment was required- Forty-three patients were treated with ASMs: 32 were treated with monotherapy and 11 were treated with 2 or more ASMs as mono- or polytherapy. Seventy-seven percent of children were seizure-free at T1, while 16% of children continued to present seizures at T2, but only 11% seemed to display possible drug resistance. The median concentration of total ghrelin in plasma did not change (p=0.9 Kruskal-Wallis test) in all patients before the administration of ASM (T0: 427.452 pg/mL; 282.814-730.335), after 2 months (T1: 377.760 pg/mL; 263.865-717.535) and 12 months (T2: 451,048 pg/mL; 282.255-757.250) of ASM administration. Similarly, the median concentration of DAG in plasma was unchanged (p = 0.873) in all patients before treatment with ASMs (T0: 42.858 pg/mL; 25.656–71.322) and after 2 months (T1: 43.736 pg/mL; 31.918–63.391) or 12 months (T2: 40.222 pg/mL; 27.221–58.269) of drug therapy.

The examined cohort of patients was composed of children with focal (n = 19), generalized (n = 16), or combined generalized and focal (n = 8) epilepsies. In comparison to children with combined generalized and focal epilepsy (total ghrelin: 648.159 ± 90.400 pg/mL), those affected by a generalized form of epilepsy had significantly lower levels of mean total ghrelin (397.806 ± 39.812 pg/mL; $p < 0.050$; Duncan's method). Furthermore, the total ghrelin plasma levels were analyzed according to the adopted ASMs. This analysis showed no statistically significant effect of both ASM ($F(3,8) = 0.273$, $p = 0.843$; two-way repeated measures ANOVA) and time ($F(2,8) = 3.948$, $p = 0.118$). Similarly, there was no statistically significant interaction between the type of ASM and time in all children ($F(6,8) = 1.713$, $p = 0.235$), indicating that all ASMs did not modify the total ghrelin plasma levels. The mean ghrelin-to-DAG ration was also significantly lower in patients with generalized epilepsy ($p<0,05$; Duncan's method), in comparison to those affected by focal or combined generalized and focal epilepsies. Also in this case, no significant effects of both ASM ($F(3,30) =$

0.450, p = 0.719; two-way repeated measures ANOVA) and time (F(2,30) = 0.171, p = 0.843) were reported. Similarly, there was no statistically significant interaction between the type of ASM and time in all children (F(6,30) = 1.708, p = 0.153), indicating that ASMs did not modify the ghrelin-to-DAG ratio in plasma.

Conclusion

These preliminary data refute the hypothesis of an augmentation of ghrelin levels beyond 2 months (T1) and a year (T2) after initiation of drug treatment. Moreover, we found ghrelin-to-DAG ratio significantly reduced in generalized epilepsies in comparison to focal and combined generalized and focal epilepsies.

Current data needs to be implemented by confirming the differences seen among plasmatic concentrations of ghrelin and des-acyl ghrelin of children with epilepsy showing a positive response to AEDs treatment comparing with non-responders and with healthy controls, and further characterize the mechanism underlying differences in lights of these new results.

Silvia Faccioli

CEM Curriculum: Translational Medicine – Cycle XXXV
Tutor: Dr. Francesco Lombardi

PREVENTING HIP DISPLACEMENT IN BILATERAL NON-AMBULATORY CEREBRAL PALSY CHILDREN

Background

Cerebral palsy (CP) is the most common motor disability in childhood (prevalence 2-2.5/1000). CP children have increased risk to incur in progressive hip displacement. More severe non-ambulatory quadriplegic patients, classified as Gross Motor Function Classification System (GMFCS) IV and V, are the most affected. The issue is particularly relevant because it may cause hip pain, which is the most frequent site of musculoskeletal pain in non-ambulatory CP subjects. Hip displacement is measured by means of the migration percentage (MP). The MP is the percentage of the femoral head area that is not covered by the acetabulum, calculated on an anterior-posterior pelvic radiography, acquired in a supine position. Population-based register studies reported trends of hip displacement among Scandinavian, Scottish and Australian CP children. Italy does not yet have either a national CP register and poor data have been published, relative to hip displacement in the Italian CP children.

Literature enquired postural management and botulinum toxin injections as preventive approaches, but the results were inconclusive, given the small sample sizes and low level of evidence. Conversely, surgical approaches appeared to significantly reduce the MP. Nevertheless, recent studies have demonstrated that surgery itself has a risk of recurrence. Postural management may not significantly reduce the MP, but might have a role in preventing its increase, thus overcoming the trend to relapse after surgery. Still, there is a lack of evidence and poor indications about type and timing of it.

Objectives

1. *Retrospective study* involving non-ambulatory CP children who attended the Children Rehabilitation Unit at the S. Maria Nuova Hospital in Reggio Emilia, before March 2020:
   1.1 To describe the trend of hip subluxation in our sample and its determinants.
   1.2 To investigate the prevalence of hip pain in our population and to enquire its determinants.
   1.3 To identify the point of no-return: i.e., the cutoff MP value, beyond which no reduction of the hip displacement might be expected, unless addressing surgery.
2. *Prospective multicenter RCT*: to verify if keeping a sitting position centering femoral heads is more effective than usual postural management, in preventing hip luxation in quadriplegic CP children.

Methods

1. *Retrospective study.* The study was approved by the Area Vasta Emilia Nord Ethics Board on 21 April 2020 (200/2020/OSS*/AUSLRE). This single-center retrospective cohort study included patients with spastic or dyskinetic CP, GMFCS level IV or V, age 0-18, having been referred to our Unit before March 2020. The following data were collected: MP, GMFCS level, age, sex, CP subtype, drug-resistant epilepsy, use of walkers or standing devices with weight relief, previous botulinum or hip surgery, oral or intrathecal baclofen, hip pain. Descriptive statistics are provided. Multiple linear stepwise regression was performed to analyze MP trends (1.1). Multivariate stepwise logistic regression was performed to enquire hip pain prevalence and its determinants (1.2). Receiver operating characteristic (ROC) curve analysis was conducted to investigate which value of the MP could be adopted as the "point of no return" (1.3).
2. *Prospective multicenter randomized controlled trial*. The study has been approved by the Area Vasta Emilia Nord Ethics Board (July 2020) and the protocol has been registered on ClinicalTrials.gov Register (ClinicalTrials.gov Identifier: NCT04603625). It is a multicenter RCT, involving 13 Italian sites. Inclusion criteria are: quadriplegic CP, age 1-6 years; GMFCS IV-V; MP <41%. After recruitment, patients are randomized to usual or experimental sitting, that is required to be used at least 5 hours a day, for 2 years. The primary outcome is the degree of luxation, measured by means of the MP, on pelvic radiography, at 12 and 24 months. Secondary outcomes include compliance and Health Related Quality of Life (HRQoL), hip pain, device cost, MRI lesions, concurrent spasticity treatments and physiotherapy.

Results

1. A total of 504 subjects were included: 302 GMFCS V, 209 females, 432 spastic CP
   1.1 Hip subluxation in spastic CP of the examined sample confirmed the trends previously described. Dyskinetic subtype showed overall lower MP values and a more variable behavior relative to age and GMFCS level. Age, CP severity and spastic subtype are the main determinants. The stepwise multiple regression analysis demonstrated that weight-relief walking and standing assistive devices, combined with botulinum contributed to reduce the MP progression.
   1.2 The overall prevalence of hip pain was 9.7% (6.7% were GMFCS V). Age, sex, MP and lumbar scoliosis were significant independent determinants of hip pain.
   1.3 The optimal cut-off value was identified as MP≥ 50%, with a sensitivity of 84.5% and a specificity of 100% (p-value <0.001, performing the chi-squared test).
2. Covid-19 emergency induced a relevant delay. An amendment was obtained to extend the recruitment phase to December 2023. At present 48 patients have been recruited. Next June preliminary results, relative to the patients that reached the 12-months evaluation, are going to be analysed.

Conclusions

Based on our data a divergent MP trend is observed in dyskinetic compared to spastic subjects. And weight-relief walking and standing devices, combined with botulinum contribute to reduce the MP progression in quadriplegic CP children (1.1). A lower prevalence of hip pain was found, compared to previous studies (1.2). The point of no-return under which conservative approaches may have a role is MP<50% (1.3). The RCT is still ongoing (2): it will help to define type and timing of postural management. The RCT protocol has been submitted for publication.

Alessandra Odorici

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NOVEL TOOLS TO COUNTERACT BACTERIAL AND FUNGAL BIOFILM PRODUCTION: *IN VITRO* STUDIES FOCUSED ON ORAL CAVITY

Background

Biofilm production onto biotic and abiotic surfaces is a multistep phenomenon, allowing and/or enhancing microbial capability to persist, survive and express its virulence traits. Accordingly, given their ability to produce biofilm, *Pseudomonas aeruginosa* (*P. aeruginosa*), *Staphylococcus aureus* (*S. aureus*) and *Candida albicans* (*C. albicans*) are opportunistic pathogens responsible for a wide range of clinical manifestations on different anatomical sites, including the oral cavity. The latter is described as a heterogeneous and highly complex habitat, involving host elements and resident microbial communities, that together play a key role in maintaining local homeostasis and health conditions. Nevertheless, in numerous clinical settings, oral infections commonly occur and rapidly become significant, given the reduced susceptibility of the established biofilms to everyday oral hygiene and antimicrobial therapy as well. MicroRepair (MicroR) is a recently described biomimetic compound, made of carbonate-hydroxyapatite-zinc crystals. Thanks to its biomimetic property, MicroR is able to interact with tooth hydroxyapatite, favoring enamel remineralization. Also, MicroR is expected to exert some antimicrobial activity, via release of zinc ions; thus, it should efficiently counteract the persistence of pathogenic bacteria on enamel surface, thus limiting their deleterious effects. Among various natural products, pomegranate (PomeGr), the fruit of *Punica granatum*, is receiving great attention. Because of its high content in phenolic compounds, including tannins and flavonoids, PomeGr is known to have beneficial effects against microbial infections, inflammatory diseases, diabetes, atherosclerosis and oxidative stress**.**

Objectives

Our aim was to investigate, *in vitro*, the antimicrobial properties of the biomimetic hydroxyapatite MicroR and of the natural compound PomeGr extract. These two products, used either alone or in combination, were tested for their ability to affect biofilm formation by bacterial and fungal pathogens, i.e., *P. aeruginosa*, *S. aureus* and *C. albicans*. Also, PomeGr extract was assessed for its polyphenol content and for the potential consumption of specific compounds upon incubation with each of the three microbial agents.

Methods

By previously established luminescence/fluorescence-based assays, *P. aeruginosa*, *S. aureus* and *C. albicans* were tested for biofilm production in the presence of MicroR and/or PomeGr. By standard broth dilution assay, the MIC of the PomeGr and of its solvent (citric acid) were determined. Furthermore, by high-performance liquid chromatography-mass spectrometry (HPLC-MS) analysis, the phytochemicals composition of the PomeG was assessed, particularly before and after exposure to *P. aeruginosa*, *S. aureus* or *C. albicans*.

Results

We found that both MicroR and PomeGr affected biofilm production. Yet, the efficacy of the two, given alone or in combination, varied from 37% to 98%, depending upon the bacterial or fungal agent considered; some additive effects were also evident. Next, by HPLC-ESI-MS analysis, the phytochemical profile of the PomeGr was described. In line with the literature, in our PomeGr extract, we found high quantities of the following polyphenols: Galloyl-hexoside and its isomers, HHDP-hexoside and its isomer, Pedunculagin and its isomers, Citric acid, Gallic acid, Ellagic acid, Ellagic acid–hexoside, Ellagic acid-deoxyhexoside, Digalloyl-hexoside and its isomers, Punicalin, Pedunculagin and its isomers, Granatin and its isomers and Brevifolin carboxylic acid. Furthermore, with the aim of assessing the potential antimicrobial involvement of certain components, we compared the phytochemical content of PomeGr, before and after exposure to *P. aeruginosa*, *S. aureus* or *C. albicans* (by comparing the peak areas corresponding to specific compounds in each chromatogram). Our data showed a reduction (ranging between 50 and 99%) in Pedunculagin, Punicalagin, Granatin, Di-(HHDP-galloyl-hexoside)-pentoside and their isomers upon PomeGr exposure to *P. aeruginosa;* in contrast, all the other polyphenols showed a low-to-undetectable decrease. Differently, the peak areas obtained upon exposure *to S. aureus* showed values very similar to those observed with PomeGr alone; the only exception was citric acid that showed a 9.7-fold decrease. Finally, upon PomeGr exposure to *C. albicans*, the decrease in some peak areas showed intermediate values compared to those obtained with the other two microbial agents.

Conclusions

These data provide initial *in vitro* evidence that MicroR and PomeGr are capable to impair microbial biofilm production, a key virulence trait for many oral pathogens; such antimicrobial effects are mainly evident against the Gram-negative *P. aeruginosa* and, to a lesser extent, against the Gram-positive *S. aureus* or the fungal pathogen *C. albicans.* Interestingly, certain polyphenols happen to be consumed and therefore are likely mediators involved in the PomeGr-mediated antimicrobial activity.

Taken together, these *in vitro* findings open to clinical studies aimed at defining novel protocols to counteract, as efficacious as possible, oral biofilm-associated infections.

Alessia Paganelli

CEM Curriculum: Translational Medicine
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IMPAIRED WOUND HEALING AND SKIN CANCER: SURGICAL MANAGEMENT AND ONCOLOGICAL FOLLOW-UP OF CUTANEOUS SQUAMOUS-CELL CARCINOMAS ARISING IN PATIENTS AFFECTED BY CONGENITAL EPIDERMOLYSIS BULLOSA

Background

Hereditary epidermolysis bullosa (EB) is a group of rare genodermatoses characterized by skin fragility and blistering of the skin and mucous membranes in reaction to minimal traumas. EB classification is based on the presence of specific genetic mutations and associated clinical manifestations. The development of cutaneous squamous cell carcinomas (cSCCs) is one of the most common medical complications in junctional and dystrophic forms of the disease. Complete surgical excision of cutaneous tumors represents the gold standard of treatment. However, not only recognition of cSCCs can be challenging in affected skin, but also wound closure after surgical excision poses a great therapeutic challenge in EB patients.

Objectives

The main goal of our study was to assess the postoperative outcomes in EB patients undergoing surgery for the presence of cSCC. In particular, we aimed at better understanding the main criticalities encountered both in the surgical management and oncological follow-up.

Methods

We retrospectively selected EB patients diagnosed of cSCC and surgically treated at the Oncological and Regenerative Dermatologic Surgery Unit at Modena University Hospital. Collected data included patient age and sex, date of cSCC diagnosis, relapses/recurrences, site of the neoplasm, number of surgical interventions, use of dermal substitutes, postoperative infections.

Results

We retrospectively identified a cohort of 5 EB patients with surgically resected cSCCs at our center. Of them, three patients were affected by junctional EB (JEB) and two by dystrophic EB (DEB). Mean age at the first cSCC diagnosis was 35 years. A total of 26 cSCCs were detected in the study population. All the cSCCs were located on limbs and extremities. DEB patients developed cSCCs mostly on lower limbs and feet, while forearms and hands were the most common sites of onset for cutaneous malignancies in the JEB cohort. Forty-one surgical interventions were necessary to achieve complete radical excision of cSCCs with clear

margins, varying from1 to of 4 surgical sessions per cSCC. Three patients experienced at least one recurrence, despite previous complete excision of the neoplasm (mean relapse rate: 40%). Dermal substitutes were used in most cases (n=31) but carried a higher infectious risk.

Conclusions

EB patients tend to develop numerous aggressive cSCCs that often relapse even after complete excision with clear margins. The best approach for cSCCs in EB patients is based on early diagnosis and prompt tumor removal. Regular dermato-oncological follow-up is needed after surgery. In our experience, JEB and DEB patients should undergo annual skin check-up from the age of 20 and more intensive schedules should be adopted in case of previous cSCCs.

Fulvio Massaro

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

Background

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

Objectives

The main aim of this study is to identify molecular and functional alterations of bone marrow (BM)-MSC derived from 120 samples from healthy young and elderly donors. We will evaluate:

-MSC morphology, phenotype and expansion;

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

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

-MSC response to inflammatory priming;

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

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

Methods

Mononuclear cells were isolated by Ficoll gradient centrifugation and then seeded in DMEM supplemented with FBS, L-glutamine and antibiotic/antimycotic solution. MSC were identified by analysis of cell-surface markers, according to the ISCT criteria. To evaluate response to inflammatory status, MSC were incubated for 24h in the presence of IL-1β (25 ng/ml), IFN-A (3000U/ml), IFN-ɣ (50 ng/ml) and TNF-α (15 ng/ml). Macrophages were obtained by differentiation of THP-1 cells. For polarization experiments, culture in RPMI,

100 ng/ml LPS and 20 ng/ml IFN-ɣ (for M1 polarization, additional 24h exposure) or 20 ng/ml IL-4 and IL-13 (for M2 polarization, additional 72h exposure) was performed. MSCs were plated at the bottom of the co-culture transwell system for all the time of cytokine exposure. EVs were isolated from supernatant obtained from MSC culture and ultra-centrifugation at 150.000 g-force for 1h. Total RNA from each cell culture was extracted in a single step using TriPure Isolation Reagent. Real-time PCR was performed on an ABI Prism 7900HT Sequence Detection System, using 25 ng of cDNA and SYBR Green PCR Master Mix. For miRNA expression we used the SYBR Green PCR Master Mix microRNA quantitative PCR, using as an endogenous control RNU48 gene. Cell surface markers were analyzed using specific fluorescence conjugated and non-conjugated antibodies.

Results

MSC derived from elderly donors are large, flat and more granular and display increased population doubling time and p16 and p21 expression. We reported an increased expression of pro-inflammatory genes (TGF-β, GAL1, IL-6, IL-8) in older patients in response to inflammatory priming. MSC show a significant impact on macrophage polarization towards M1 and M2 status: particularly, MSC from young donors induced a reduction of typical M1 markers expression such as CCL2 and TNF-α, and enhance the switch to anti-inflammatory M2 status, as shown by increased levels of IL-10, TGM2, and CD206 on both THP-1 cells and monocyte-derived macrophages (MDMs). Moreover, this effect is less marked when using replicative senescent MSC from the same donors. The majority of EVs derived from MSC showed a size of 100-250 nm and were actively phagocytosed by macrophages. The analysis of miRNA expression in EVs revealed a significant difference for miRNA known to be involved in macrophage polarization and particularly the expression of miR-193b-3p is strongly increased after co-culture of macrophages with MSC. This miRNA regulates a biological pathway leading to CCL2 down-expression through STAT1 regulation and preliminary data suggest that this might be one of the pathways explaining MSC immunomodulating activity.

Conclusions

MSC show differences in size, morphology, colony-forming ability. MSC gene expression profile seems to vary according to subjects' age and particularly in old donors seem to be characterized by an impaired immunomodulating activity, with a reduced inhibition of macrophage M1 status. Furthermore, macrophage polarization could be strongly influenced by EVs activity through miRNA production which seems to vary according to donors' age.

Robel Papotti

CEM Curriculum: Translational Medicine
Tutor: Prof. Samantha Pozzi

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

Background

Diffuse Large B-cell Lymphoma (DLBCL) is the most frequent aggressive lymphoma, originating either from the germinal-center (GCB group) or post-germinal cells (ABC group). Response rate to first-line therapy (R-CHOP) is approximately 60%. Currently, risk stratification includes the International Prognostic Score, PET-CT scans, Cell-Of-Origin (COO) and expression/translocation of BCL2/BCL6/MYC. Nevertheless, no molecular marker has been widely correlated with the risk of treatment failure. The Activation-induced cytidine deaminase (AID) enzyme and the *TP53* gene could play a relevant role in DLBCL aggressiveness. The first can be responsible for an ongoing somatic hypermutation phenomenon which might lead to immunoglobulins intraconal heterogeneity, whereas *TP53*, possible target of AID "misfiring", is one of the most frequently and clonally persistent mutated genes in relapsed/refractory DLBCL.

Objectives

We aim to provide a deep study of the existing prognostic tools in our cohorts and to evaluate AID both regarding its on-target activity, for example as a driver of B-cell receptor intraclonal diversification, and off-target by promoting genomic instability and mutations of *TP53*.

Methods

The project comprises two DLBCL well characterized cohorts, treated with R-CHOP or R-CHOP-like regimens and with clinical data. Cohort 1: 204 FFPE samples, provided by the Polyclinic of Modena; Cohort 2: 62 FFPE samples, provided by the National Cancer Institute of Aviano. Evaluation of prognostic significance of COO classification has been conducted through immunohistochemistry (IHC) and Hans' algorithm, *Lymph2Cx* assay on Nanostring platform and Agilent Microarray gene expression profiling. BCL2 and MYC expression through IHC and AID expression both using real-time PCR and Microarray. RNA sequencing for comprehensive mutational analysis, gene expression and *IGHV* rearrangements evaluation has been performed using a total RNA with riboerase approach specifically optimized with in-house pipelines, on a NovaSeq 6000 platform (ILLUMINA) and S4 Flow Cell. Probability of progression/death will be estimated by the Kaplan–Meier method, while survival data between defined subgroups will be compared with the log-rank test.

Results

Regarding Cohort 1: Hans algorithm identified 83 (40%) GCB and 124 (60%) ABC cases, whereas *Lymph2Cx* revealed 76 (49%) GCB, 58 (37%) ABC, and 21 (14%) Unclassified cases. The comparison between the two methods showed good concordance according to the Landis and Koch scale (0.719 k-statistic value). GCB patients (Hans algorithm and *Lymph2Cx*) had a significantly longer PFS compared to ABC subset [Hans algorithm p=0.011, HR=1.98 (1.17-3.35); *Lymph2cx* p=0.027, HR=1.93 (1.08-3.47)]. BCL2- cases were 67 (33%), BCL2+ were 134 (67%), while c-MYC- cases were 170 (85%) and c-MYC+ 31 (15%). Double Expressor cases were 12%. Cox regression analysis showed a 5-year PFS of 90% (78-96) for BCL2-/c-MYC-, 50% (39-60) for BCL2+/c-MYC- and of 52% (29-70) for BCL2+/c-MYC+. RNA-seq of 96 samples over 204 has been performed, libraries were globally well balanced, showing an average of 1.11E+08 number of reads; samples had a consistent percentage of uniquely mapper reads with a median percentage of 82.27%. We were able to optimize a predictor that confirms the COO classification previously obtained through the *Lymph2Cx* panel and to display 478 differentially expressed genes (p<0.01) among the COO subgroups. AID resulted in overexpression in the ABC subtype (p= 0.070) compared to the GCB. The *IGHV* sequencing through amplicon based NGS approach starting from the Leader region or from FR1/FR2/FR3 regions was poorly successful due to the high degradation of starting material or to mutations occurring in the binding sites of the primers, resulting in low amplification. We decided to study the IG heterogeneity by the *IGHV* gene sequence reconstruction through specific pipelines working on RNA-seq data; analysis is currently ongoing. Preliminary data on CLL fresh cases showed high concordance regarding clone detection both using commercial kit as Invivoscribe Lymphotrack assay and IG reconstruction from RNA-seq data.

Regarding Cohort 2: COO prediction based on Microarray GEP detected 30 cases ABC and 32 cases GCB. 6 cases resulted differently classified with *Lymph2Cx*. GEP analysis indicated 1295 differentially expressed probes (p <0.01, FC> 1.0), specifically 673 up-regulated and 622 down-regulated within the ABC cases. GEP data showed an AID overexpression in ABC (p=0.065, FC>2). On the contrary, *TP53* mRNA levels showed no significant differences between COO groups.

Conclusions

Here we show how in the real-life context COO classification and BCL2 expression truly prove to have prognostic significance, suggesting a worse outcome for ABC and BCL2+ subgroups. Overexpression of BCL2, rather than c-MYC, may be responsible for Double Expressor cases' poor prognosis. AID suggested a major expression in the ABC subgroup in both cohorts, with a possible link to a significant activity related to an increased genomic instability. RNA-seq data showed to be importantly informative both for the AID-related signatures and for IGHV rearrangements heterogeneity study. Our method, specifically designed to reconstruct the IG sequence starting from RNA-seq data, might enlighten diverse aspects of IG behavior in the context of DLBCL.

Luca Bedetti

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

Background

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

Objectives

This area-based prospective cohort study involves all Neonatal Intensive Care Units (NICU) of Emilia Romagna, an Italian region with a population of around 4 million inhabitants.
The primary aim is to evaluate the neurodevelopmental outcome at 2 years of life in infants undergoing TH. The secondary aim is to describe differences in terms of TH and variations in the neonatal management between Neonatal Units.

Methods

Eight NICUs joined the Neuronat Network (a network on neurodevelopmental outcome of infants with HIE undergoing TH).
We enrolled prospectively all surviving infants with any grade of HIE (mild, moderate or severe) born in Emilia Romagna at ≥35 weeks' gestation, from January 2016 to December 2019, who underwent TH according to Italian Guidelines. A common data collection form on a web platform (RedCAP) was created. The form included perinatal data (such as diseases during pregnancy, type of delivery, need of resuscitation, Apgar score and metabolic acidosis at birth), information about neurological assessment during hospital stay (such as grade of HIE according to Sarnat & Sarnat classification and electroencephalography evaluations), use of TH and complications associated with the treatment, and neurodevelopmental follow-up at 24 months of age. Neurodevelopmental assessment was performed through a neurological examination (according to the Amiel-Tison neurological assessment) and either through the Griffiths Mental Developmental Scales or the Bayley Scales of Infant and Toddler Development, depending on the local protocols. The primary outcome measure was a severe functional disability at 2 years of age, defined as the presence of cerebral palsy, cognitive score <2 SD, bilateral blindness (visual acuity < 6/60 in better eye), or bilateral deafness (requiring

bilateral hearing aids or unilateral/bilateral cochlear implants). In relation to the secondary aim, data were collected regarding methods of cooling, electroencephalographic monitoring, adverse effects of hypothermia and timing of neuroradiological investigations.

Results

During study period, 224 infants with HIE were admitted to NICUs. Given a number of 116000 term infants born in the Region in the same years, we found an incidence of HIE of 1.9/1000 live births. Among the total infants admitted for HIE, 43 were not treated with TH. Among 181 infants who received the treatment, 7 (3.1%) died and 125 (69%) completed the neurological follow-up at 24 months of age. Cerebral palsy and severe functional disability were diagnosed in 12 (9.6%) and 15 (12%) infants respectively. Regarding the use of TH, we found that almost 20% of infants who received the treatment had grade 1 HIE.

Conclusions

This is the first Italian study about HIE, TH and neurodevelopmental outcome. The results provide information about neurologic outcome at 2 years of age in infants who underwent therapeutic hypothermia in Italian Neonatal Intensive Care Units. Comparison to previous studies from other countries, the current study found low rates of severe neurological impairment in infants treated with TH. In addition, it underlines how the use of TH is changing in routine clinical practice compared to what was described in the first randomized controlled trials.

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NEUROIMAGING AND FLUIDS BIOMARKERS IN ADULTS PATIENTS WITH STATUS EPILEPTICUS

Background

Status Epilepticus (SE) is a common neurological emergency characterized by high short term morbidity and mortality. Identifying diagnostic and prognostic biomarkers could help in the evaluation and management of SE patients. From a prognostic point of view, as reported in the last SE definition, depending on the type and duration of seizures, SE can lead at the brain site to a variety of cellular and molecular alterations, which can induce subsequent irreversible damage and long-term consequences. The measurement of different CSF and serum biomarkers of neuro-glial injury and neuroinflammation and the identification of acute neuroimaging alterations related to SE could help predicting and rapidly identifying those patients who will eventually develop short and long term consequences of SE thus letting the appropriate therapeutic and clinical management to be applied. From a diagnostic point of view, especially in cases of suspected Non-Convulsive Status Epilepticus (NCSE), a subtype of SE characterized by a qualitative and/or quantitative alteration of consciousness without associated overt major motor phenomena, the diagnosis could be very challenging. In these cases, the gold standard for the diagnosis is actually the EEG through the application of the latest Salzburg Criteria for the diagnosis of Non-Convulsive Status Epilepticus (SCC). Nevertheless, there is still no consensus on them, especially among the more doubtful possible SE cases (P-NCSE). In this context the identification of neuroimaging and fluids' diagnostic biomarkers could assist the diagnosis.
Thus, the diagnosis and prognostication of SE is rapidly moving toward a multimodal and comprehensive approach based either on the evaluation of neuroimaging correlates or the determination of fluids (serum and cerebrospinal fluid, CSF) potential biomarkers of SE beside the EEG analysis.

Objectives

This study aims to:

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

Methods

This is a prospective monocentric collection of adult patients (≥14 years) with SE. Enrolled patients undergo serum and whenever acquired for clinical need CSF too, samplings during SE within 48 hours from its diagnosis. These samplings are elaborated for biomarkers measurement: neuro-glial injury biomarkers (neuron specific enolase, NSE, neurofilament light chain, NfL, and S100B), neuroinflammation biomarkers (IL-6, IL-1ß, HMGB1) and neurosteroids. When available, CSF-serum albumin ratio and CSF TAU (t-TAU and p-TAU) as markers of BBB breakdown and neuronal injury respectively are measured too. The same fluids' biomarkers are measured in healthy individuals (control group) age and sex-matched. In patients with SE, clinical information (demographic, etiology, clinical semiology, comorbidities, and therapeutic management) are collected too. Follow-up information (mortality and functional outcome measured by the modified Rankin Scale, mRs) are collected at 30 days. Patients with a clinical suspicion of NCSE undergo CTP/CTA (Cerebral Tomography Angiography) study and an analysis of the characteristics of CTP and EEG patterns and their relationship is then made. The results derived from neuroimaging and fluids' biomarkers will be combined to define if this multimodal approach could provide improvement in SE management in clinical practice.

Results

Aim 1: at present we included 21 adult focal NCSE patients studied with CTP and EEG in the acute phase. Eighteen patients (86%) had focal hyperperfusion patterns (10 cortical only, 1 thalamic only and 7 cortical + thalamus) and 3 (14%) a normoperfusion patterns. In patients with hyperperfusion patterns there was a perfect (100%) concordance in spatial localization of focal multilobar cortical hyperperfusion and focal ictal activity. Among the hyperperfused patients, all the 12 patients with continuous pattern (CP) showed cortical hyperperfusion while only 3 out of 6 (50%) with waxing and waning pattern (WWP) had cortical hyperperfusion (χ^2, $p = 0.03$).

Aim 2: at present we included 65 SE patients in which we measured serum levels of NfL and S100B. We compared them with serum levels of NfL and S100B in 27 healthy subjects (Controls). Overall, the values of NfL (SE: median 57 pg/ml, interquartile range (IQR) 141; Controls: median 6.57 pg/ml, IQR 9, $p < .001$, Mann-Whitney test) and S100B (SE: median: 0.12 ug/L, IQR 0.17; Controls: median 0.02 ug/L, IQR 0.008, $p < .001$) in SE were higher than in controls. The two groups differ for gender and age. Since NfL levels correlate with age ($\rho = 0.454$, $p < .001$) and S100B correlate both with age ($\rho = 0.411$ $p = .001$) and gender ($\tau = 0.250$, $p = .015$) we repeated the analysis after a case-control matching analysis ($n = 23$ for both groups) for age (Student's t-test, $p = 0.867$) and gender (χ^2, $p = 1$) that confirmed the results. Moreover, serum levels of NfL and those of S100B showed a positive correlation ($\tau = 0.419$, $p < 0.001$). NfL and S100B showed both a positive correlation (NfL: $\tau = 0.530$, $p < .001$; S100B: $\tau = 0.413$, $p < .001$) with short term functional outcome (mRS at 30 days). Serum NfL levels were higher in patients with refractory and super-refractory SE (RSE/SRSE: Median 163 pg/ml, IQR 372, Responsive SE: Median 37.8 pg/ml IQR 90.7, $p = .001$) and in those who presented a

clinical worsening or death at 30 days' follow-up (worsened or dead: Median 130 pg/ml, IQR 246, Recovered: Median 23.45 pg/ml, IQR 87.35, p < .001). Likewise, S100B levels were higher in patients with refractory and super-refractory SE (RSE/SRSE: Median 0.174 ug/L, IQR 0.289, Responsive SE: Median 0.099 ug/L IQR 0.149, p = .048) and in those who presented a clinical worsening or death at 30 days' follow-up (worsened or dead: Median 0.174 ug/L, IQR 0.467, Recovered: Median 0.09 ug/L, IQR 0.108, p = .026). At univariate analysis serum levels of NfL were found to be predictive of 30 days worsening of clinical conditions or death (OR 1.01 CI [1.001 – 1.012] p = .032) and of refractoriness development (OR 1.003 CI [1– 1.005] p = .051) but these findings were not confirmed at the multivariate analysis where only age and treatment refractoriness remained independently predictive of 30 days clinical worsening or death.

Conclusions

From these results, it is possible to conclude that diagnostic imaging biomarkers such as hyperperfusion CTP patterns could be used to assist and support the diagnosis of NCSE, especially in those cases in which clinical and electroencephalographic features are doubtful. Moreover, even if these results are preliminary and need future confirmation, serum NfL acquired in the initial phases of SE seem to be helpful for the prognostication in terms of short term death, functional outcome and refractoriness development.

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

Background

In colon cancer, surgery remains the most efficient therapeutic approach. It aims to treat the primary tumor and metastatic disease while assessing the lymph node status. Lymph nodes (LNs) are a significant prognostic factor in predicting disease-free survival (DFS) and overall survival (OS) in patients without metastatic disease. LN metastases are a risk factor for disease recurrence and the development of metastatic disease. Furthermore, they determine whether or not the patient should undergo adjuvant therapy. A recent study has stated that the prognosis is determined by the number of positive LNs and that their topographic distribution may carry an important role. The AJCC-TNM classification is the current staging system used in western medicine, in which a correct nodal sampling is based on the retrieval of at least 12 LNs regardless of their location. On the other hand, the JSCCR (Japanese Society for Cancer of the Colon and Rectum) classification takes into consideration the topographic distribution of the positive LNs. There are no studies that determine the superiority of one system over the other in terms of predicting a 3-year disease recurrence and OS. Due to the important prognostic value of the LN status, its correct staging is a largely debated argument.

Objectives

Primary aims:

Applicability of the JSCCR classification to our population. The outcome is described as the percentage of cases in which this classification probes to be completely applicable (Note: for the TNM system, is by definition 100%)

Agreement between disease stages applying both staging systems

Secondary aims:

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

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

Methods

This is a monocentric study that aimed to confront these two grading systems. We have determined the main differences and similarities between both staging systems. We enrolled 91 patients with a diagnosis of colon cancer in a 12-month period, from which 4 patients were withdrawn from the study due to intraoperative findings of metastatic disease. We included patients >18 years old with a diagnosis of colon cancer and a CT-scan negative for metastatic disease who accepted to continue the follow-up period in our Institution. We excluded patients with rectal cancer due to its different metastatic pattern and because often undergo neoadjuvant therapies, which modify the LN status. We excluded patients with synchronous solid tumors or oncological hematologic diseases and patients who had to undergo neoadjuvant therapies or had a diagnosis of recurrent/metastatic disease. After the extraction of the specimen, a surgeon dissected the lymph node stations according to the JSCCR classification. Both staging systems were applied to each patient.

The analysis concerns only the primary objective (feasibility and agreement) because the secondary objectives assess the risk of recurrence and related mortality rate which require 3 years of follow-up.

Continuous variables were characterized by median and range. Categorical data were summarized as absolute and relative frequencies. The JSCCR classification was defined as applicable whenever it was able to define the disease stage. The applicability of the JSCCR classification was calculated as a percentage with a confidence interval of 95% according to Wilson.

It was calculated the percentage of cases in which both systems appointed the same stage for each patient with a confidence interval of 95% according to Wilson. The degree of agreement was determined by Cohen's kappa coefficient with a confidence interval of 95%. Statistical analysis was performed with R 4.0.4. software.

Results

Our population presented a median age of 72.82 years IQR (65.40-79.42), 35 patients were female and 56 patients were male. Clinical presentation with anemization was observed in 35 patients, bowel obstruction in 2 patients, rectal bleeding in 14 patients, 34 patients have been diagnosed thanks to the screening program, and 6 patients didn't refer the primary symptom. The surgical procedures were distributed as follows: right hemicolectomy (60 patients), left hemicolectomy (7 patients), transverse colon resection (4 patients), segmentary colon resection (8 patients), and sigmoidectomy (12 patients). Seventy-nine procedures were performed with the laparoscopic technique whereas 14 procedures were executed with an open technique.

- The JSCCR classification was applicable in 87 out of 87 cases included in the study.
- Both classifications TNM and JSCCR presented an agreement of 100%, 95% CI: 94.7 - 100)

Conclusions

We found that the JSCCR classification can be applied in our population without any variability concerning the vastly used TNM-AJCC classification. The fact that both systems presented a degree of agreement of 100% while determining the disease stage could be due to the small sample size. To better determine if the differences between both staging systems could carry a change in staging a bigger sample size is needed.

The study is now entering the follow-up phase, which will be completed in December 2024. At the end of the follow-up phase, we will be able to respond to our secondary aims.

Cecilia Catellani

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

Background

Growth hormone (GH) is pivotal for growth. Children diagnosed with GH deficiency (GHD) undergo long-term GH replacement therapy at dosages that are currently not individualized. Measured growth rate does not always coincide with the expected rate and depends both on the patient's basal conditions and on personal innate sensitivity to GH. GH plays an important role in the regulation of cell proliferation, differentiation and apoptosis, in particular, at the level of the epiphyses of long bones. This leads to consider a possible oncogenic effect of treatment. Some studies reported an increased risk of bone tumors in patients treated with GH in childhood. The data collected to date evidence the need for continuous surveillance. MiRNAs are post-transcriptional regulators of gene expression, and are involved in many biological processes such as body growth, and have been extensively studied in cancer. GH could act through changes in miRNAs.

Objectives

AIM 1: to identify circulating miRNAs varying on GH treatment and to evaluate whether they could be useful to predict the clinical outcome in terms of growth. The association between miRNA level variations (before and after treatment) and all additional recorded clinical parameters will be studied.
AIM 2: to evaluate the impact of these identified miRNAs on pathways related with cancer by using an *in silico* approach and to study the role of specific miRNAs in oncogenic processes through *in vitro* cell models.

Methods

AIM 1: 10 normal-weight, prepubertal patients with idiopathic isolated GHD (IIGHD) (5 Males, 5 Females; CA:8.80±2.60 yr; bone age:7.19±2.61 yr) were enrolled at the Pediatric Endocrine Clinics in Reggio Emilia and Modena. Serum samples at two time points before the beginning of GH treatment and at 3 months on treatment were collected. The patients were treated with GH, according to the indications of the Italian Regulatory Drug Agency (AIFA Note 39). Total RNA was extracted from serum (miRVana PARIS miRNA isolation Kit) and reverse transcribed to cDNA (TaqMan Advanced miRNA cDNA Synthesis Kit). MiRNA expression profiling was performed by using the TaqMan Advanced miRNA Human Card A, which measures expression levels of 377 different human miRNAs in order to identify those miRNAs changing in response to

treatment after 3 months by either a fold change ≥+1.5 or ≤-1.5 (either up- or down-regulated, respectively). MiRNAs showing a p-value ≤0.05 in the 2 time points before treatment, varying independently from treatment, were excluded. Selected miRNAs underwent a validation step which was performed by using Taqman Advanced miRNA assays on serum RNA samples obtained from 25 prepubertal patients with IIGHD. Clinical and biochemical parameters of patients were collected at baseline, 6 and 12 months. Simple linear regression analysis and multiple stepwise linear regression models were used to explain growth response in terms of height variation (0-6 months and 0-12 months) and growth velocity (0-6 months). Statistical analysis was performed as appropriate by using STATA.

AIM 2: The selected miRNAs from the above analysis were investigated using miRNetv.2.0 platform for gene target and pathway analyses. Single miRNA predicted target genes were evaluated using TargetScan. MiRNA mimics and inhibitors were transfected in MG-63 osteosarcoma cell line by using Lipofectamine RNAiMAX and Real-Time qRT-PCR was performed using specific TaqMan assays.

Results

The profiling analysis showed that 16 miRNAs were up-regulated and 2 miRNAs were down-regulated after 3 months on GH treatment with respect to baseline (early response). Pathway analysis showed that they modulated 100 different pathways and target genes which are involved both in longitudinal growth and cancer. Eight miRNAs were selected for the validation step based on their target genes (hsa-miR-30c-5p, hsa-miR-140-5p, hsa-miR-340-5p, hsa-miR-199a-5p, hsa-miR-335-5p, hsa-miR-494-3p, hsa-miR-22-3p, hsa-miR-106a-5p). MiR-199a-5p, miR-335-5p and miR-494-3p were confirmed to be up-regulated after GH treatment in 25 patients. These three miRNAs, together with other clinical and experimental variables, contributed to explain growth response on GH treatment in terms of height variation (0-12 months) (adj R2=0.79 R2 cv=0.43) and growth velocity variation (0-6 months) (adj R2=0.75, R2 cv=0.63). Interestingly, both miR-335-5p and miR-199a-5p were predicted to target CRIM1, a protein which interacts with Bone Morphogenetic Proteins -4 and -7 contributing to bone formation. In addition, all the three miRNAs were predicted to target CLOCK, involved in circadian clock regulation linked to bone development, and ROCK1, which is increased in osteosarcoma and when inhibited, cell proliferation and migration are reduced, and apoptosis is induced.

Conclusions

MiR-335-5p, miR-199a-5p, and miR-494-3p change in patients undergoing GH treatment. This early change contributes to explain the growth response after 12 months on GH treatment suggesting that they could be used for prediction models to customize treatment. The *in vitro* study to explore the possible pro-oncogenic effects of these miRNAs is ongoing on MG-63 osteosarcoma cells and hFOB osteoblasts. CLOCK, CRIM1 and ROCK1 will be studied in both cell models after miRNA transfection to determine the effects of these miRNAs on gene expression, protein levels, cell proliferation, apoptosis and differentiation.

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DEVELOPMENT OF NEW METHODS FOR THE CHEMICAL CHARACTERIZATION OF NON-PSYCHOACTIVE *CANNABIS SATIVA* L. (HEMP) EXTRACTS AND EVALUATION OF THEIR BIOACTIVITY

Background

Cannabis sativa L. is an herbaceous plant belonging to the *Cannabaceae* family. Cannabinoids are mainly synthesized in glandular trichomes, which are more abundant in female inflorescences. The most representative compounds are Δ^9-tetrahydrocannabinolic acid (Δ^9-THCA) and cannabidiolic acid (CBDA). These native acidic cannabinoids undergo a spontaneous decarboxylation under the action of light and heat, leading to the formation of their neutral counterparts. Fibre-type *C. sativa* (also known as hemp) is characterized by a high content of cannabidiol (CBD) and a level of psychoactive Δ^9-tetrahydrocannabinol (Δ^9-THC) lower than 0.2%. CBD displays several biological activities related to the action on different targets. In addition to cannabinoids, the plant produces other classes of secondary metabolites, such as terpenes. These compounds have been less studied, even if they can be of pharmaceutical interest, due to their synergistic interaction with cannabinoids.

Objectives

A further investigation on the role of cannabinoids in the *in vitro* antiproliferative activity of hemp extracts was carried out during the third year of the PhD program. Moreover, extracts rich in both cannabinoids and terpenes were prepared and tested in *in vivo* models of neuropathic pain, epilepsy and ulcerative colitis. To do this, it was necessary to fully characterize the bioactive compounds present in different *C. sativa* extracts by means of innovative analytical techniques. As for the analytical section of this project, the work was also addressed at the development of an innovative HPLC method for the simultaneous separation of cannabinoids using Design of Experiments (DoE). In parallel, a new method based on GC-MS was developed to study in detail the terpene composition of the extracts.

Methods

For the *in vitro* assays, ethanolic extracts were prepared starting from three fibre-type *Cannabis sativa* L. varieties, having a different phytochemical composition. Cell viability was evaluated using the CCK-8 assay, whereas the expression of the main apoptosis-related proteins as well as the cytochrome *c* release were investigated through western blot. For the *in vivo* assays, two olive oil extracts were prepared starting from a CBD-type hemp variety, including one rich in cannabinoids and one having a high content of both

cannabinoids and terpenes. The Von Frey test model of peripheral neuropathic pain was applied, and the mechanism of action was investigated using CB_1 and CB_2 selective receptor antagonists, together with the determination of the expression of microglial activation markers. The 6-Hz corneal stimulation murine model was used to evaluate the antiepileptic activity of the same oils compared to pure CBD. Finally, a murine model of induced colitis was used to evaluate the anti-inflammatory effect of hemp olive oil extracts. Concerning the chemical characterization of the above-mentioned extracts, HPLC-HRMS and HPLC-UV were used for the qualitative and quantitative analysis of cannabinoids. The volatile components of the extracts were fully characterized and quantitated by means of a new method based on GC-MS. DoE was used to develop and optimize a new HPLC-UV/DAD method for the simultaneous separation of 14 cannabinoids.

Results

The *in vitro* antiproliferative activity of the CBD-type ethanolic extract was mainly due to the induction of apoptosis, as demonstrated by the activation of caspase 3 and 7 as well as the release of cytochrome *c* from the cytosol. *C. sativa* olive oil extracts rich in both cannabinoids and terpenes showed an interesting analgesic effect *in vivo* and CB_2 receptors seemed to be involved in the mechanism of action. The presence of both cannabinoids and terpenes enhanced the antiepileptic effect of the extract *in vivo* compared to pure CBD, in the so called “entourage effect”. The assessment of the role of hemp oils against ulcerative colitis is currently on-going. Thanks to the use of DoE, a new HPLC-UV/DAD method for the separation of cannabinoids was developed, which allowed the separation of 13 out of 14 cannabinoids; the co-elution of cannabigerol (CBG) and cannabinerol (CBNe), which are two geometrical isomers, was solved using a triple-quadrupole mass analyzer, monitoring the specific transitions of each compound in multiple reaction monitoring (MRM) mode. Volatile compounds in the extracts were identified according to their MS spectra; the most representative terpenes, on the basis of the full-scan GC-MS data, were then quantified. In this way, we were able to describe the difference in volatile composition between the two extracts used for the *in vivo* assays.

Conclusions

The *in vitro* biological assays suggested that CBD can be involved in the antiproliferative activity of the CBD-type hemp extract, even if the role of other minor compounds cannot be excluded, due to possible synergistic interactions. The mechanism of action of extracts and CBD is under investigation. As for the *in vivo* experiments, the results suggested that cannabinoids and terpenes may act synergistically to enhance the bioactivity of the phytocomplex against pain and epilepsy.

The HPLC and GC methods developed for cannabinoids and terpenes separation could be useful for the analysis of complex mixtures, such as plant extracts, in order to ensure a better reproducibility of the results of biological assays by monitoring not only the main cannabinoids but also other representative compounds of the phytocomplex.

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RISK FACTORS FOR DISTAL METASTASIS AND CLINICAL PROGNOSIS OF PATIENTS WITH SOVRA-GLOTTIC AIRWAYS CANCER NON-METASTATIC AT DIAGNOSIS: A RETROSPECTIVE STUDY

Background

Patients with sovra-glottic cancer may experience the onset of distal metastasis despite effective and prompt treatment. The risk factors associated with the development of metastasis have not been elucidated yet, being the role of human papillomavirus (HPV) still controversial. Moreover, while there is evidence that a metastatic disease is associated with worse overall survival, no data are available if a specific localization (e.g. lung) might affect the prognosis of these patients.

Objectives

This study aimed at exploring the risk factors and the impact on survival of distal metastasis in a population of patients with sovra-glottic airways cancer but not metastatic at the time of diagnosis.

Methods

Patients with a diagnosis of sovra-glottic cancer admitted to the Respiratory Diseases Unit and to the Ear Head and Neck Surgery Unit of the University of Modena from 2000 to 2016 with at least a 5-years follow-up were retrospectively considered eligible for enrollment. Exclusion criteria were the presence of distal metastasis at the time of diagnosis, the presence of a synchronous cancer, incomplete core data (i.e. clinical characteristics at baseline and follow-up information) at medical record analysis.

All demographic and clinical variables including HPV infection, TNM classification, treatment received, response and disease relapse were collected.

Variables were compared between two groups: those who developed distal metastasis (DM) and those who did not (NDM); t test and Wilcoxon-Mann-Whitney test served for continuous variables, whereas categorical variables were compared by $\chi 2$ test or Fisher's exact test as appropriate. The association between demographic and clinical characteristics with the onset of distal metastasis was tested by means of univariable and multivariable logistic regression models. The overall survival (OS) analysis was performed with participants' follow-up accrued from the date of diagnosis until death. Time to death was compared using unweighted Kaplan-Meier curves and the impact of developing distal metastasis on survival was analyzed through unadjusted and adjusted COX regression analysis. In order to test the hypothesis that the difference between groups might vary according to HPV status, we formally included an interaction term in

the COX regression model. Results were then shown after categorizing the population into two strata using categorical separation. In a post-hoc sensitivity analysis, OS was assessed for the DM group according to the site of metastasis using unweighted Kaplan-Meier curves. Significance was set for p values < 0.05

Results

Fifty-seven (14%) out of 408 patients developed distal metastasis during the follow-up period. The median time to metastasis development was 24 (interquartile ranges [IQR] 12-40) months.

Former or active smoker status (odds ratio [OR]=2.5 [1.9–9], p=0.04), absence of HPV infection (OR=2 [1.3–5.4], p=0.04), extended tumor size (namely cT4) (OR=1.5 [1.2–3], p=0.04) and lymph nodal N2 classification (OR=8.7 [4.5–13.4], p<0.001) at the time of diagnosis, partial radiological response at 2 or 4 months (OR=2.8 [1.3–5.7], p=0.02 and OR=2.5 [1.3–5] p=0.03 respectively), and local relapse (OR=4.5 [1.7–9.4], p<0.001) were significantly associated with the development of distal metastasis.

The adjusted 5-year risk of dying was 3-fold higher (HR=3.4 95%CI [1.9–6], p<0.0001) in DM group. After categorization for HPV infection, the 5-year risk of dying was higher only in those subgroup of patients who had HPV infection (adjusted HR = 4.2 95%CI [2–8.6], p<0.0001). Finally, among those who developed DM, the subgroup with lung involvement showed 5-fold greater survival rate (p=0.049) compared with others.

Conclusions

A significant number of patients with local sovra-glottic cancer may develop distal metastasis despite treatment during follow-up. Smoking status, advanced malignant disease at the time of diagnosis, and poor response to treatment were independent factors associated with this late event. The metastatic dissemination significantly reduced survival but only in those patients who did not have HPV infection.

The early phenotyping of patients at major risk of late metastasis onset might improve the clinical management and the follow-up of these patients.

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COMPARISON OF BONE AND METAL-AUGMENTED REVERSE SHOULDER ARTHROPLASTY: A RETROSPECTIVE COMPARATIVE STUDY OF CLINICAL AND RADIOGRAPHIC OUTCOMES

Background

Glenoid wear is a common finding in shoulder osteoarthritis (OA) and represents a significant challenge to address in most cases of shoulder replacement. Total shoulder arthroplasty failed to address glenoid wear, for the greater stresses in the bone and implant that increase the risk of glenoid component loosening. Furthermore, the fatty atrophy of the posterior-superior rotator cuff in cuff tear arthropathy (CTA), induces loss of external rotation (ER) that is difficult to recover with a standard glenoid component. The pattern of glenoid-based bone loss is highly variable, and often, advanced shoulder OA can be associated with multiplanar glenoid deformity (posterior, superior or both). Reverse shoulder arthroplasty (RSA) addresses posterior wear and excessive glenoid retroversion, as well as superior inclination, with eccentric glenoid reaming, bone grafting or a posterior/superior augmented glenoid baseplate.

Objectives

The purpose of this study was to compare clinical and radiographic outcomes of patients who underwent glenoid- augmented RSA with bone and with metal.

Methods

This was a retrospective comparative study of 68 patients (70 shoulders) (M/F:28/42, mean age: 70 ± 9 yrs) underwent RSA with augmented glenoid component using bone (Bone Increased Offset [BIO] – RSA) (44, [63%]) (BIO-RSA group) and metal (Metal Increased Offset [MIO] – RSA) (26, [37%]) (MIO-RSA group). Surgeries were performed from February 2017 to April 2019. Inclusion criteria were a preoperative diagnosis of primary shoulder OA or CTA and a minimum 24-months follow-up. Clinical outcome measures included the Western Ontario Osteoarthritis of the Shoulder (WOOS) Index (score expressed as decimal from 0 [worse] to 1 [best]), and active shoulder mobility (active anterior elevation [AAE], active lateral elevation [ALE], ER, and internal rotation [IR]). Preoperative glenoid morphology was evaluated by computed tomography scan according to the criteria described by Walch (A1, A2, B1, B2, B3, C and D) and Sirveaux (E0, E1, E2 and E3), in primary OA and CTA, respectively.

Three views postoperative radiographs (anterior-posterior, axillary and outlet) were performed to assess the following radiographic parameters: glenosphere position, glenoid β angle, humerus and glenoid

radiolucencies, tuberosity resorption, scapular notching, heterotopic ossifications (HO), bone graft healing and viability (BIO-RSA group).

All radiographs were assessed independently by three raters and, when there was disagreement, the three met to discuss the assessment and a consensus was reached.

Descriptive statistics (absolute and percent frequency, mean, median, standard deviation [SD], and range) for each group were calculated for all variables. Delta scores were calculated for clinical and radiographic scores as the difference between postoperative and preoperative values. The preoperative scores and delta scores of the two groups were compared using the Mann-Whitney test. The level of significance was set at 0.05.

Results

Bio-RSA patients showed posterior glenoid wear (B type glenoid) in 28/44 (64%) and superior wear (E2 and E3 glenoids) in 9/44 (20%); MIO-RSA patients showed posterior and superior wear in 14/26 (54%) and 10/26 (38%), respectively. Both prostheses provided significant differences between preoperative and postoperative WOOS index ($p<.0001$), and active shoulder mobility (all $p<.0001$). Analysis of delta scores of the two groups failed to show difference in clinical scores and shoulder mobility. Preoperative glenoid retroversion was 21° ± 7 in BIO-RSA and 15 ± 3 in MIO-RSA group. BIO-RSA showed a higher rate of tuberosity resorption ($p<.004$) and cortical thinning ($p=0.027$). Glenosphere position was high in 5 shoulders of BIO-RSA group and in 1 of MIO-RSA ($p=0.022$). Glenoid β angle was 86.1 ± 7 in BIO-RSA and 88.2 ± 7 in MIO-RSA group. Viable bone graft was found in 29/15 (66%) and bone graft radiolucency in 16/44 (36%) (lines < 2 mm). The rate of glenoid and humerus radiolucent lines, scapular spur, scapular notching and HO was similar in the two groups.

Conclusions

Bone and metal are both reasonable options for glenoid-side lateralization in RSA. The good value of postoperative β angle confirm that, bone and metal are both effective to restore the right global glenoid inclination. Radiological findings in the BIO-RSA group create concerns about the survivorship and effectiveness of bone graft in the long-term.

Veronica Manicardi

CEM Curriculum: Translational Medicine
Tutor: Dr. Alessia Ciarrocchi

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

Background

Cutaneous melanoma is the most threatening form of skin cancer. Its morbidity and mortality are mostly associated with metastatic disease. Despite the large amount of molecular and clinical work, the molecular determinants leading to melanoma metastasization are still poorly defined. Recently, the ENCODE project showed that more than 80% of the non-coding genome has a biochemical function and cooperates to control gene expression in particular through the activity of distal elements as Enhancers (ENHs) and Super Enhancers (S-ENHs). It has been postulated that sequence alterations in these regions may affect their regulatory function and cause aberrant gene expression programs. Chromatin exists in multiple functional states that are defined by precise histone modifications and that correlate with gene expression. This implies that variations in gene expression can be anticipated by changes on the nature and activation status of chromatin markers in non-coding regulatory elements. All together these findings pave the way for the study of non-coding regulatory elements (REs) and chromatin functional status as possible predictors of gene expression variations.

Objectives

The aims of the project are: 1) to identify the network of non-coding REs (S-ENHs and classical ENHs) that sustain metastatic progression of melanoma, 2) to define the complex network of upstream signals that, converging on these REs, lead to the activation of pro-metastatic gene expression programs, 3) to define a prognostic gene signature that correlates with the metastatic behavior of melanoma.

Methods

ChIP-seq for H3K27ac and RNA-seq on a retrospective cohort of 20 primary tumors (PTs) and 20 distant metastases (DMs) of melanoma from the Research Biobank of the AUSL-IRCCS of Reggio Emilia (ASMN cohort) were performed. TCGA Skin Cutaneous Melanoma (TCGA-SKCM) expression and clinical datasets were downloaded through TCGABiolinks R package. Unsupervised clustering, Principal Component Analysis (PCA) and differential gene expression analyses were performed on all three datasets. Diffbind and DESeq2 R packages were used for differential analysis on ChIP-seq and RNA-seq data respectively. The TCGA-SKCM expression data were pre-processed to filter out genes with low coverage. TCGA-SKCM patients were filtered to keep only PTs and DMs. Gene Ontology (GO) analysis on differentially expressed genes (DEGs) was

performed through the enrichR R package. S-ENHs and ENHs were called based on their input-normalized H3K27ac signal intensity in 12.5 kb stitched regions performed by the ROSE algorithm, using ±3Kb as promoter region. To predict transcription factor (TF) binding sites within the DM-associated REs, the S-ENHs and ENHs corresponding sequences were used as input for the FIMO algorithm. HOCOMOCO and JASPAR were filtered on a list of upstream regulators predicted by TRRUST and TRANSFAC&JASPAR enrichment, then used as reference motif databases. FIMO motif search was performed setting q-value threshold <0.1.

Results

In order to identify the core of genes deregulated during the metastatic progression, the transcriptional profile of PTs and DMs from two different cohorts, the TCGA-SKCM and the ASMN, was analyzed. PCA and Unsupervised Hierarchical Clustering analysis could not clearly discriminate PTs from DMs in both datasets. Differential analysis was performed with an absolute log2Fold Change higher than 0.4 resulting in 442 genes significantly deregulated (FDR<0.1) in TCGA-SKCM dataset and 1753 significantly DEGs in ASMN cohort. GO enrichment highlighted pathways involved in skin development and cell adhesion. Altogether, these analyses resulted in poorly representative data likely due to the heterogeneity and low purity of both datasets samples. Thus, the profile of H3K27ac, marker of active transcription, was investigated genome-wide on the same ASMN cohort used for RNA-seq to deepen our knowledge on gene expression regulation during melanoma metastatic progression. Differential analysis on ChIP-seq data identified 1753 differentially activated regions, 30% of which were mapped as distal intergenic, whereas 24% were assigned to transcription starting sites. Unsupervised clustering analysis showed that chromatin activation status by means of H3K27ac sharply discriminates PTs and DMs. Furthermore, two separate clusters within the group of PTs were identified corresponding to in situ (Cluster 1) and dermal infiltrating lesions (Cluster 2). Aiming to identify REs and their related gene programs sustaining DMs spreading, we applied the ROSE algorithm finding 1340 S-ENHs and 26469 ENHs associated with DMs. 2406 genes and 13153 genes were predicted to be controlled by the identified DMs-associated S-ENHs and ENHs respectively. Noticeably, more than 50% of the ENHs-predicted targets were simultaneously associated with 5 or more distal REs suggesting that classical ENHs may establish functional cooperation despite their genomic proximity to sustain strong activation of cancer supportive programs. Next, we investigated whether DMs-associated ENHs or S-ENHs were governed by the same network of TFs. We used the FIMO algorithm to search for motifs of predicted TFs on these non-coding regions. We identified 19 S-ENHs-binding TFs and 6 TFs controlling ENHs thus defining a core of 21 TFs that influences the activity of DMs-associated REs.

Conclusions

Altogether our data suggest that mapping transcriptionally active elements across the genome describes clinical aggressiveness better than transcriptional profiling. Moreover, we showed that cooperation among distal elements either by local proximity (as in S-ENHs) or tridimensional interactions is crucial to coordinate the activation of gene programs that sustain cancer cells progression to metastatic spreading. Finally, we highlighted the existence of a core of TFs which finely tunes the activation of DMs-associated REs in melanoma to execute the gene program underlying metastatic progression of this disease.

Francesca Combi

CEM Curriculum: Translational Medicine
Tutor: Prof. Giovanni Tazzioli

MICROSURGICAL TREATMENT OF UPPER LIMB LYMPHEDEMA AFTER BREAST SURGERY AND RADIOTHERAPY FOR BREAST CANCER – ROLE OF AXILLARY WEB SYNDROME (AWS) AFTER SENTINEL NODE BIOPSY

Background

The research project is taking place in the Breast Surgical Oncology and Plastic and Reconstructive Surgery Units of the University Hospital of Modena. Women who are diagnosed with non-metastatic breast cancer, are treated with surgical excision. A conservative choice or a demolitive one is done depending on the local extension of the disease. Simultaneously a surgical staging of the axillary lymph nodes must be performed, to rule out loco-regional lymphatic spread. When metastatic lymphatic involvement is found, the surgical choice is complete axillary dissection (ALND); otherwise, patients undergo sentinel lymph node biopsy (SLNB). 15-20% of women develop upper limb lymphedema after ALND while 2-3% after SLNB. In most cases, it can be mitigated with rehabilitation but when conservative treatment fails, women experience functional discomfort and a dramatic worsening in quality of life. Axillary Web Syndrome (AWS) is a cause of morbidity in the early postoperative period after axillary surgery. It is characterized by fibrous cords of subcutaneous tissue, extending from the axilla into the medial arm. Both AWS and BCRL appear to be associated with lymph node removal and an inflammatory process impacting the lymphatic system. Several studies describe that patients developing AWS in the early postoperative period, are more prone to suffer from arm lymphedema afterward. At present, tailored programs are being created in the University Hospital of Modena, to follow and treat patients who develop BCRL. In this context, early detection and treatment of AWS could improve the prompt taking charge of patients affected by breast cancer who underwent axillary surgery.

Objectives

1. To create a dedicated program inside the University Hospital of Modena, to evaluate and treat women with BCRL. **2.** To define the patients that may benefit from microsurgical treatment and which technique (cooperation with Plastic Surgery Group). **3.** To investigate risk factors for AWS to early recognize it and treat it as a predictive expression of future BCRL. **4.** To adapt surgical procedures based on the recognized risk factors for lymphatic complications and to monitor the future outcomes.

Methods

We focused on studying risk factors for the development of AWS in patients that underwent SLNB for breast cancer. We focused on surgical risk factors, considering some technical aspects that could potentially be avoided or changed to prevent the onset of this complication, which lies in the spectrum of lymphatic impairment and that could preannounce consequent BCRL. We performed a case-control study. Cases were female patients aged more than 18, that underwent surgery with ipsilateral sentinel node biopsy for breast cancer in the period from Jan2018 to Jan2022 and that referred to the Breast Unit Rehabilitation program for the onset of AWS. Controls were selected among patients that underwent the same surgical procedure for the same primary cancer in the same period. The exposure to multiple technical variables of surgical intervention was investigated, such as: side of surgery (right versus left); involved quadrant (upper-outer, upper-central, upper-inner, inner-central, lower-inner, lower-central, lower-outer, outer-central, central); type of breast surgery (conservative versus demolitive); type of primary lesion mark (vegetable charcoal versus none); type of surgical incision for SLNB (same as breast incision versus separate axillary incision); identification technique for intraoperative SLNB (Indocyanine Green versus 99(m)Technetium); number of excised sentinel lymph nodes.

Results

149 cases were eligible. 450 controls were selected, with a proportion of three controls for every case (3:1). No significant difference was found among cases and controls regarding side of surgery, involved quadrant, type of breast surgery, type of primary lesion mark, type of surgical incision for SLNB, and lymph node identification technique. Conversely, the difference in the number of excised lymph nodes resulted to be significant. We separated cases and controls into two classes: removal of 0 to 2 lymph nodes and removal of ≥3 lymph nodes. The odds of being treated with ≥3 lymph nodes removal are greater in cases than in controls with an OR=2.1 (95%CI 1.10-3.99).

Conclusions

Literature shows that patients developing AWS after breast cancer surgery are more prone to develop BCRL. To efficiently dedicate the resources of the "Lymphedema Program" that is created in Modena University Hospital, we conducted a case-control study to understand if some surgical behaviors can affect the onset of AWS, in order to adapt surgical procedures to reduce the risk for patients to develop lymphatic complications. The majority of surgical factors that were analyzed resulted to be not significant, meaning that these technical aspects do not affect the outcomes. The removal of ≥3 lymph nodes appeared to be a significant risk factor. From a clinical perspective, this result should lead to avoiding excessive excisions during surgery whenever possible. At the same time, this result defines a group of patients that has a higher risk of developing AWS. This subgroup may thus benefit from early rehabilitation and a stricter follow-up to treat the condition when diagnosed.

Jacopo Demurtas

CEM Curriculum: Public Health
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CONVERSATIONAL AGENTS IN COVID-19: ACCEPTABILITY, USABILITY AND INTENTION TO COMPLY. EVOLUTION OF COVIDGUIDE AND DEVELOPMENT OF AFYAGUIDE

Background

During the first phases of the pandemic many apps were launched to tackle the surge of covid-19, among those the CovidGuide app, developed through a collaboration among German, Italian and Swiss researchers and IT specialists and used mostly in Germany, Switzerland and Italy. The app is a conversational agent (CA) with a neural network artificial intelligence (AI) supporting users in defining the right time to treat and point of care for their healthcare problem. To be safe and effective, the advice of the app should be followed by the user. This can help reduce health systems overloading.

Objectives

The aims of this phase of the project were 1) to evaluate factors leading patients to consider and eventually follow the advice of a CA, and 2) to study the development of a specific tool for African countries.

Methods

In order to evaluate aim 1 different approaches were used:

- A systematic review was carried out, CRD42021277509, following the MOOSE and PRISMA statement, with the main objective to assess the efficacy and safety of self-triage tools in primary care and secondarily to evaluate the impact on service use/diversion (including possible multiple contacts with health services), compliance with advice received, patient/career satisfaction, equity and inclusion (e.g. barriers to access, characteristics of patients using the online/digital service compared with the face-to-face service).
- CovidGuide database containing consultations results from inception to April 19 2022 was analyzed and the main descriptive statistical analyses were performed.
- A sample of 174 patients was surveyed for intention to comply and factors influencing it, through a module linked to the CovidGuide in German speaking countries. Items scores were evaluated on a 7 point Likert Scale, exploratory (EFA) and confirmatory factor analysis (CFA), were performed.

Regarding aim 2, based on the structure, experience and data from the CovidGuide, an international collaboration among researchers involved in the previous phase and Kenyan researchers and IT specialists has been set up. In presence and online meetings were planned and carried out in order to discuss and plan the development of the AfyaGuide, a CovidGuide-based self-triage tool for Kenya, taking into account the specific

needs, conditions and characteristics of this African country with lack of resources and of primary healthcare physicians.

Results

The systematic review yielded, after duplicates were removed, 5324 records. 5273 were deemed to be not eligible, leaving 51 full texts to screen. Due to great differences in methodologies applied and in results reported, a narrative synthesis has been carried out, since meta-analysis was not feasible. 10 papers were included (so far). Results suggest, in accordance with previous research, that accuracy of self-triage tools was generally low, with a certain risk of overtriage or undertriage undermining patients' safety. However, studies evaluated so far were usually on small population samples, with different comparisons and often of low quality.

The total number of consultations with CovidGuide were 374.179. 62% patients were females, 75% in the age range 14-49 years, main symptoms reported were viral syndrome not otherwise specified and, interestingly, throat symptoms complaints. The most frequent advice given was the indication to see the doctor (point of care) today (time to treat). This was the output of 143.981 consultations (38%).

Of interest, 75.692 patients, 20% of cases, received indications for self-monitoring with safety net (i.e. indications on what to do in case of symptoms' worsening).

Findings of the study on the intention to comply were obtained from a population coming mostly from Germany, where the CovidGuide is embedded in the healthcare system and represents a sort of gatekeeping to access the emergency service. 6 factors were identified and users' intention to comply with recommendation seems to be affected mostly by the perceived trust in app's advice and not by the CA's persuasiveness.

Finally, during the last months a specific tool for Kenya, AfyaGuide, has been set up. Its applicability and validity in a setting with lack of resources and of primary healthcare physicians, will be tested by a pilot study, involving 1500 Kenyan adults with Covid/influenza like symptoms, now in a methodological planning phase.

Conclusions

The intention of patients to comply with the indications of a CA may be investigated with different methods. In German speaking countries perceived social presence seems to impact on the intention to comply, even if the advice is perceived as sound and trustworthy. Matching of the data gathered through the survey with the data of the assessment/consultation included in the database is currently ongoing and will give us more information on this issue.

Finally, the results of the pilot validity study of the AfyaGuide will provide important information on the applicability and feasibility of alternatives to the need for patient-of face-to-face consultations that may result in overcrowding of the limited medical facilities and increase burden of healthcare professionals.

Rexhep Durmo

CEM Curriculum: Translational Medicine
Tutor: Dr. Annibale Versari

TOTAL METABOLIC TUMOR VOLUME AND LESION DISSEMINATION CALCULATED FROM PET/CT SCAN BEFORE FIRST LINE THERAPY ARE PREDICTORS OF OUTCOME IN PATIENTS WITH FOLLICULAR LYMPHOMA

Background

Follicular lymphoma (FL) is the most common indolent B-cell lymphoma with complex disease biology exemplified by significant clinical heterogeneity. Most patients have a long median survival, but 20-30% of patients will have aggressive disease that relapses quickly or is refractory to standard therapy. The early identification of patients who are at high risk of treatment failure currently represent an open research question. 18F-fluorodeoxyglucose positron emission tomography-computed tomography (FDG PET-CT) feature like total metabolic tumour volume (TMTV) has been proposed as an imaging biomarker in FL. However, a definitive consensus has not been achieved, with conflicting data published in the literature. Recently, we have identified a new FDG PET/CT feature, lesion dissemination (Dmax), as a promising prognostic biomarker in newly diagnosed Hodgkin Lymphoma patients.

Objectives

The aim of this study was to investigate the prognostic value of TMTV and Dmax in a large cohort of treatment-naïve FL patients from the FOLL12 trial (NCT02063685).

Methods

FOLL12 trial is a multicenter, randomized, phase III trial comparing standard (Arm A) vs response adapted maintenance therapy (Arm B) in treatment of naïve adult patients with grade 1-3a FL. In this study we included patients for whom FDG PET/CT before immunochemotherapy (ICT) was available and centrally reviewed. The TMTV was obtained from baseline scans by summing the metabolic volumes of all individual nodal and extra nodal lesions, using the 41% SUVmax threshold method. From the TMTV the furthest distance between lesions was calculated (Dmax) and normalized according to body surface area (SDmax). Kaplan–Meier analysis was used to estimate the survival curves and log-rank test to assess statistical significance. Cox proportional-hazards model was used for univariate and multivariate analysis. Optimal cutoffs for survival analysis for TMTV and Dmax were identified by maximally selected log-rank test and confirmed by 1000 bootstrap resamples. Main study endpoint was 5-year Progression Free Survival (PFS).

Results

From the FOLL12 trial, 692 patients were included; 48% were older than 60 years, 89% had stage III-IV disease and 40% had a high-risk follicular lymphoma international prognostic index (FLIPI-2) score. Overall, the 5-year PFS was 79% (95% CI, 76 to 82%): 86% (95% CI, 82 to 89%) for arm A and 72% (95% CI, 67 to 76%) for arm B. Median TMTV was 242 mL (IQR=446), median SDmax was 0.283 m^{-1} (IQR=0.248). The optimal cutoff identified were 200 ml for TMTV and 0.4 m^{-1} for SDmax. In univariate analysis, 5-year PFS was significantly lower for patients with high TMTV (60% vs 75%; HR=1.87 [95%CI: 1.41-2.49], p<0.001) and SDmax>0.4 m^{-1} (56% vs 69%; HR=1.67 [95%CI: 1.24-2.25], p=0.001). TMTV and SDmax remained significant prognosticators when adjusted by randomized arm, FLIPI-2, and ICT with HR respectively of 1.48 (95%CI: 1.09-2-01) and 1.42 (95%CI: 1.05-1.92). Combining SDmax with TMTV we were able to show a role of SDmax in the identification of patients at different risk of progression among high TMTV cases: patients with TMTV>200ml and SDmax<0.4m^{-1} had a HR of 1.59 (95% CI: 1.16-2.17) vs 2.53 (95% CI: 1.73-3.61) for TMTV>200 and SDmax>0.4m^{-1}.

Conclusions

Pre-treatment TMTV and SDmax, reflecting metabolic tumor burden and its spread, are independent predictors of PFS in patients with FL receiving frontline ICT. The combination of TMTV and SDmax may provide significantly better risk stratification in guiding tumor-tailored therapy.

Marta Perin

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

DEVELOPMENT, IMPLEMENTATION, AND FIRST EVALUATION OF A CLINICAL ETHICS SUPPORT SERVICE. A process evaluation study using Normalization Process Theory

Background

The values and preferences differences among patients, their relatives and healthcare professionals (HPs) play a pivotal role in the healthcare decision-making process. They often result in conflicts between stakeholders and cause moral distress in clinical practice.

A Clinical Ethics Committee (CEC) is a multi-professionals service that aims to support HPs in dealing with complex clinical cases characterized by conflicting ethical perspectives through ethics consultation (EC). CECs are growing entities worldwide. Several studies showed a generally positive opinion on such ethics support by users, especially physicians. In 2020, a CEC was established in an Oncology Research Hospital in the North of Italy. It is the first in the Emilia Romagna Region. The process was driven and monitored by the Bioethics Unit (BU). Working in the same Local Health Authority, the BU promotes research projects related to the ethical issues of clinical practice, ethics education, and ethics consultation for individual HPs and care teams. This research project consists of developing and implementing a multidisciplinary Clinical Ethics Committee (CEC) and a Process Evaluation (PE) of the service 16 months from its implementation.

Objectives

-To collect data about the research context where the intervention is implemented. Findings will be helpful in understanding the preliminary components of a CEC's implementation;

-To evaluate the CEC implementation process by identifying factors that might promote and inhibit the routine incorporation of the CEC in everyday clinical practice life. Findings will be useful to improve the ongoing intervention and to identify tools further to evaluate the CEC's impact on the local context.

Methods

We followed the *MRC framework* as the research framework of the overall project. It has a 4- phased approach: from a pre-clinical research phase to a final step in which the intervention is introduced into the health service. This intervention is a 0-I study, and is composed by

1) Phase 0 - a preliminary study context. It is an observational, retrospective, mix-method study of the activities provided by the BU since its implementation.

2) *Phase I – Process Evaluation (PE) study* of the CEC's implementation process after 16 months. We applied the Normalization process Theory (NPT) as the methodological research strategy. NPT helps explain empirically identifiable mechanisms that motivate and shape implementation process, while providing a means of apprising factors that might promote and inhibit the routine incorporation of complex intervention in everyday life. We combined a) a quantitative assessment of the CEC's diffusions, knowledge and utilization among HPs; and b) a qualitative evaluation from users and providers HPs in terms of barriers/facilitators, expectations and needs.

Results

Phase 0: Our results showed that BU activities have generally increased over time, with a more significant increase in research in 2020. Since its implementation, 686 hours have been spent on training (36%), ethics consultations with individual HPs (11%), and care team meetings (53%), particularly with adult palliative care teams. We interviewed 18 HPs who differently collaborated in BU's activities. Our analysis revealed the following themes: 1. the reasons for contacting the BU and the type of collaboration; 2. the role of the bioethicist; 3. the impact of BU activities on HPs, in terms of developing deeper and more mature thinking; 4. the need to extend ethics support to other settings.

Overall, our results showed a positive perception of BU's activities, especially regarding the usefulness of EC during team meetings and experiential education. These findings may contribute to understanding and explaining the contextual factors which may impact the development, implementation and use of a local CEC.

Phase I: the related study protocol was developed and approved by the local CE (n° 2022/0026554). The study has several components and data collection strategies, targeting different populations. Specifically, it is aimed at: **a)** collecting quantitative data by an Internal database to quantify the amount of activities performed by CEC and the amount of resources used; **b)** assessing the spread, use and knowledge of CEC among HPs by a quantitative survey to all HPs employed at the local Health care Authority; c) to explore the opinion on CEC functioning and role by semi-structured interviews to both Managers/Heads who formally supported and promoted the intervention and CEC's members; d) To explore the experience with EC provided by CEC by HPs who submitted an EC request; e) To explore opinions from HPs on the whole CEC service by survey to HPs who participated to the training on EC provided by the CEC.

This phase is ongoing. Preliminary results on points **a and b** will be presented at the PhD day.

Conclusions

By a rigorous methodological approach, we expect to identify the relevant components that contribute to the CEC's successful implementation and integration into everyday practice. Our findings would also identify required modifications to improve the service and develop practical strategies for enabling and sustaining the CEC delivery in clinical settings.

Domenico Penna

CEM Curriculum: Translational Medicine
Tutor: Prof. Stefano Luminari

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

Background

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

Objectives

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

Methods

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

Results

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

Conclusions

The results highlighted that in Covid-19 patients:

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

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

Rebecca Borella

CEM Curriculum: Translational Medicine
Tutor: Prof. Andrea Cossarizza

REPROGRAMMING OF NEUTROPHILS' METABOLISM IN SEVERE COVID-19

Background

Neutrophils are amongst the most abundant effector cells of the innate immune system, with a primary role in the response against extracellular pathogens as well as in acute inflammation and tissue damage. Indeed, innate immune cells play a central role in the immunopathogenesis of severe COVID-19. Accordingly, we previously described metabolically impaired mature monocytes expressing inhibitory checkpoints, together with increased amounts of immature monocytes in patients with COVID-19 pneumonia. Regarding neutrophils, formal evidence of their functional status in severe COVID-19 patients are still missing.

Objectives

The main objective of my studies is to better understand the mechanisms underlying neutrophil functions and the excessive neutrophilic response during SARSCoV-2 infection. Specific objectives are:

- to characterize granulocytes' phenotype in peripheral blood from severe COVID-19 patients.
- to investigate the role of circulating neutrophils' metabolism in their function.
- to analyze plasma levels of cytokines, chemokines, and other soluble factors involved in the regulation of neutrophils.

Methods

Blood from 88 patients with severe COVID-19 pneumonia and from 59 healthy donors (HD) was collected and immediately processed. To identify neutrophils, 100 uL of whole blood pre-incubated with Human Fc Block were treated with a mix of mAbs anti-CD45, CD15, CD16, CD11b, CD63, CD62L, CD66b, CXCR2, washed, lysed, and acquired by using Attune NxT acoustic Flow Cytometer. Neutrophils were magnetically purified from blood and oxygen consumption rate (OCR), extracellular acidification rate (ECAR), and oxidative burst were quantified by using the Seahorse XFe96 Analyzer. The ultrastructure of neutrophils from COVID-19 patients and the identification of intracytoplasmic deposits of glycogen were obtained by Transmission Electron Microscopy (TEM). Intracellular glycogen was quantified by using the Glycogen Assay Kit. *PYGL, PGM1, GYS1, UGP2, HIF1A* mRNA levels were quantified by real-time PCR. HIF-1α protein expression was determined by Western Blot. NETs released by neutrophils treated with or without glycogen phosphorylase (PYGL) and HIF-1α inhibitors were measured by using the NETosis Assay Kit. Plasma levels of myeloperoxidase (MPO), elastase (EL) and MPO-DNA complexes were obtained using ELISA kit. The quantification of cytokine plasma

levels was obtained using a Luminex platform. The reanalysis of scRNA-seq data from bronchoalveolar lavage (BAL) from COVID-19 patients and HD (GSE145926) was performed using Seurat version 3. Gene Ontology functional profile and Reactome pathway over-representation analysis were performed on at least 1.5-fold upregulated genes between COVID-19 and HD neutrophils. Flow cytometry data were analyzed by using FlowJo software version X. Statistical analysis was performed by using Prism 8.0.

Results

Circulating granulocytes are altered in severe COVID-19 patients

COVID-19 patients had a higher proportion of immature neutrophils negative for CXCR2 and more degranulated neutrophils if compared to controls. Indeed, when compared to HD, plasma from patients showed highly increased levels of MPO and EL, as well as of NET (measured as MPO-DNA complexes).

Circulating neutrophils displayed a remodeled metabolism

Hypoxemia characterizes severe COVID-19 patients and HIF-1α is a major regulator of cellular adaptations in hypoxic conditions. Indeed, HIF-1α protein expression in circulating neutrophils from patients was increased compared to controls and *HIF1A* gene expression was upregulated in neutrophils from BAL obtained from COVID-19 patients, while genes involved in oxidative phosphorylation were downregulated. The analysis of neutrophils bioenergetic profile revealed a decreased spare respiratory capacity and respiratory burst in neutrophils from COVID-19 patients versus controls. However, glycolysis and glycolytic capacity were strongly increased in neutrophils from COVID-19, which sustain NET formation.

Circulating neutrophils use glycogen for NET formation

HIF-1α can induce storage of glycogen in the cytoplasm that can be quickly mobilized to meet energy demand. By using TEM, large intracytoplasmic deposits of glycogen were detected in neutrophils from COVID-19 patients. Intracellular glycogen and mRNA levels of glycogen phosphorylase L (*PYGL*), which catalyzes glycogenolysis, were both increased in neutrophils from COVID-19 patients if compared to controls. Finally, PYGL was regulated by HIF-1α, and in fact the inhibition of PYGL and HIF-1α reduced NETosis.

Cytokines and soluble molecules involved in neutrophils' regulation are profoundly altered

BAFF, CCL2, CCL3, CCL20, CXCL10, G-CSF, GM-CSF, IFN-γ, IL-6, IL-18, VEGF, TNF were significantly higher in COVID-19 patients. The percentage of neutrophils was correlated with CCL4, CXCL1, CCL20, and IFN-γ and CCL20 was correlated with pO_2/FiO_2.

Conclusions

Neutrophils from patients with severe COVID-19 pneumonia have a remodeled metabolism that can prove an effective target for innovative therapy. Increased glycolysis and glycogenolysis are crucial for NET

formation which can be inhibited, thus, suggesting possible novel strategies against COVID-19 or other inflammatory diseases. Moreover, the presence of extremely high plasma levels of MPO and EL in patients suggests potential treatments against COVID-19.

Caterina Vacchi

CEM Curriculum: Translational Medicine
Tutor: Dr. Andreina Manfredi

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

Background

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

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

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

Objectives

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

Methods

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

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

As regard the assessment of prevalence, all subjects will be evaluated cross-sectionally while, as regards the assessment of incidence, patients without signs or symptoms suggesting pulmonary involvement will be re-screened in case of appearance of respiratory symptoms or every 6 months. All subjects with newly diagnosed pSS will screened prospectively every 6 months for a period of 5 years to evaluate predictive factors and the onset of ILD with respect to the natural history of pSS.

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

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

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

Results

During the last year, we have enrolled 45 pSS patients, increasing the population number by 20,45% and reaching a total of 265 subjects involved up to now (241 females and 24 males). Among them, 70 showed ILD (26,41%). Fourteen subjects were males and 56 females. Four patients are awaiting a CT-scan after the detection of Velcro-crackles.

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

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

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

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

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

Massimiliano Salati

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CLINICOPATHOLOGIC FEATURES, TRANSCRIPTOMIC LANDSCAPE AND TREATMENT IMPLICATIONS OF THE IMMUNOSUPPRESSIVE CD73/NT5E IN BILIARY TRACT CANCER (BTC)

Background

Despite the improved outcome with the addition of the anti-PD-L1 durvalumab to standard treatment, the vast majority of BTC do not benefit from chemo-immunotherapy. Immunosuppressive microenvironment is a dominant feature of BTC, involved in tumour progression and drug resistance.

Objectives

Here, we investigated the biological role and treatment implications of the adenosine-producing enzyme CD73 in a clinically-annotated cohort of BTC.

Methods

Immunohistochemistry for CD73, CD4/CD8 and FOXP3, whole-exome and transcriptomic sequencing were performed on resected specimens of 80 BTC (Illumina Platform). Spatial Transcriptomics was performed by using Visium Spatial Gene Expression-10x Genomics. Tumor growth was assessed in 2D and 3D culture by using MTS assay and spheroid growth analysis. Between-group comparisons were made using the chi-square test for categorical variables. The Kaplan-Meier estimators were used to calculate survival probability and the log-rank test to make comparisons between curves. The prognostic performance of each covariate on OS was first evaluated by means of Cox proportional hazard univariate model, selecting those variables with a p-value <0.05 for multivariate analysis. For all tests, a two-sided p-value <0.05 was considered to be statistically significant, with a confidence interval at 95% (95% CI) [15]. The statistical analyses were performed using the SPSS software (version 26; SPSS Inc., Chicago, IL, USA).

Results

High CD73 expression (CD73high) was associated with older age (p=0.01), gallbladder subsite (p=0.03), and nodal involvement (p=0.04). CD73high tumours were significantly enriched in FOXP3+ T lymphocytes (p<0.001). CD73high status was independent predictor of poorer prognosis at the multivariate analysis (p=0.03), with ECOG PS ≥2 (p=0.001) and the pathological stage (p=0.025) and was associated with a remarkably shorter RFS in patients treated with adjuvant chemotherapy (p=0,011). Transcriptomically, CD73high tumours were significantly enriched in upregulated EMT, TNF-alfa/NFKB, hypoxia and G2/M

checkpoint signaling pathways and p53, BMI1, MEL18, EGFR and K-RAS genes. In in vitro models, siRNA-mediated depletion and CRISPR-CAS9 gene KO of CD73 sensitized both BTC 2D and 3D culture to cisplatin/gemcitabine treatment. The pharmacological inhibition of CD73 by AMCP improved the sensitivity of BTC cell lines to cisplatin/gemcitabine treatment. Finally, spatially resolved transcriptomics of CD73high revealed a critical role of CD73 in tumor immunity and therapeutic response.

Conclusions

We showed that CD73high BTC display aggressive biological features, poorer prognosis and resistance to standard chemotherapy. The therapeutic targeting of this adenosinergic ectonucleotidase by clinically-available compounds has the potential to enhance the efficacy of conventional treatment in BTC.

Kateryna Solodka

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ELECTROLYTE-GATED ORGANIC FIELD-EFFECT TRANSISTOR-BASED BIOSENSORS FOR THE DETECTION OF BIOMARKERS OF MULTIPLE SCLEROSIS IN PLASMA

Background

Multiple sclerosis (MS) is a chronic and inflammatory disorder of the central nervous system characterized by progressive axonal demyelination and neurodegeneration. It is estimated that over 2.8 million people suffer from this disease worldwide and, because of its high prevalence, MS is considered as the major cause of non-traumatic neurologic disability in young adults. Therefore, highly sensitive methods are required for the detection and quantification of MS biomarkers in order to correctly assess the diagnosis and monitoring of the disease.

In recent years, electrolyte-gated organic field-effect transistor (EGOFET)-based immunosensors have emerged as a promising alternative strategy for the ultra-sensitive and label-free detection of biological analytes. EGOFETs are three-terminal devices in which the source and drain electrodes are connected by an organic semiconductor (OSC) layer that acts as a conducting channel, and is separated by an electrolyte from the gate electrode. The application of a potential difference between gate and source (V_{GS}) leads to the formation of two electrical double layers, at the gate/electrolyte and electrolyte/OSC interfaces, promoting the accumulation of charge carriers in the OSC, which results in a current flow between the source and drain electrodes (I_{DS}) following the application of a second potential difference (V_{DS}).

Objectives

The accurate detection and quantification of biomarkers that can provide an accurate view of the health state of patients with multiple sclerosis is an unmet need. The state-of-the-art techniques for the detection and quantification of biological analytes of interest in the pathology of MS are based on ELISA and SIMOA assays. Although these techniques are highly sensitive and specific, they also present some disadvantages, as they are expensive, time-consuming, require specific equipment and trained personnel, and fluorescent labeling.

The goal of this project is to develop a biosensor based on an EGOFET architecture, to detect and quantify, with high sensitivity and specificity, biomarkers of multiple sclerosis. In particular, the first part of the project was focused on the fabrication of an immunosensor for neurofilament light chain (NF-L), a candidate biomarker for MS.

Methods

Transistors were fabricated using test patterns (TPs) with interdigitated source and drain Au electrodes. Prior to the deposition of the organic semiconductor, the TPs were cleaned following a standard procedure, and then the organic molecule TIPS-pentacene was deposited on the substrate by spin-coating. A polycrystalline gold wire was used as gate electrode, and specific anti-NF-L antibodies were immobilized on the gate surface by means of cys-Protein G. Every step of the gate functionalization was monitored with cyclic voltammetry. Sensing experiments were performed in 50 mM PBS containing increasing concentrations of NF-L (from 100 fM to 10 nM). Current-voltage (I-V) characteristics were recorded by applying a sweeping gate-source from (V_{GS}) -0.1 to -0.6 V, while maintaining a constant drain-source voltage (V_{DS}) of -0.2 V. All measurements were performed at room temperature inside a Faraday cage. Complementary analysis of the gate surface was performed by atomic force microscopy and scanning electron microscopy.

Results

A monotonic decrease in the drain current was observed following the incubation of the gate electrode with increasing concentrations of the target protein. This concentration-dependent change in the drain current was ascribed to the binding events taking place on the gate surface, between the NF-L protein and its corresponding antibody. In addition, following the binding events occurring on the gate surface, a concomitant shift of the threshold voltage (V_{th}) towards more negative values, and a decrease in transconductance (g_m) were observed.

The biosensor exhibited the maximum response, defined as the normalized relative current change, in the subthreshold regime. The observed trend suggested the presence of two different regimes, which were interpreted as the simultaneous formation of two layers on the gate surface: a first antigen-antibody layer, and second layer corresponding to weak protein-protein interactions, observed at high NF-L concentrations. The successful fit using the Guggenheim-Anderson-de Boer (GAB) adsorption model and further morphological characterization of the gate electrode supported this hypothesis.

Conclusions

In summary, our EGOFET biosensor is demonstrated to selectively detect NF-L in a wide dynamic range of concentrations, providing a label-free, rapid, and reproducible response, indicating its potential as an alternative sensing platform for the detection of NF-L in multiple sclerosis.

XXXVI cycle

Sara D'Alessandro

CEM Curriculum: Translational Medicine
Tutor: Prof. Livio Casarini

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

Background

Thyroid is an endocrine gland responsible for the secretion of hormones triiodothyronine (T3) and thyroxine (T4) whose release is stimulated by pituitary thyroid-stimulating hormone (TSH). The increase of thyroid cancer prevalence occurs in females around the age of 20 years and peaks near menopause at 50, while around 70 years in men. Several studies demonstrated that the incidence of thyroid cancer in women is 4-fold higher than in men, suggesting that estrogens, such as the estradiol (E_2), are involved in the pathogenesis. In light of structural similarities, we hypothesize that the TSH receptor (TSHR) may form heteromers with the G protein-coupled estrogen receptor (GPER), modulating proliferative signals in thyroid cells. Similar findings were achieved by our Research Group, which demonstrated the formation of heteromers between GPER and the follicle-stimulating hormone receptor (FSHR), as well as lutenizing hormone/choriogonadotropin receptor (LHCGR).

Aims

The aim of this project is to define whether estrogens and its G protein-coupled receptor, GPER, modulate TSH-like proliferative signals in papillary thyroid cancer cells. The formation of TSHR-GPER heteromers and their interactions with TSH and E_2 will be investigated.

Methods

Experiments were performed using papillary thyroid carcinoma (K1) and follicular epithelial (Nthy-ori 3-1) thyroid cell lines. Transfected COS7 were used as control cell line to evaluate the activation of specific transduction pathways. The physical interaction between TSHR and GPER was evaluated by Bioluminescent Resonance Energy Transfer (BRET) on these cell lines, and by Proximity Ligation Assay (PLA) on either cell lines and primary healthy and cancerous thyroid tissues obtained by the Pathology Section (University Hospital of Modena, Modena, Italy) under Ethics Committee permission.
GPER and TSHR expression were evaluated by RT-PCR and immunofluorescence staining respectively on thyroid nodules needle-aspirated washing liquid fluids and histological sections of papillary thyroid cancer (PTC) and healthy primary thyroid tissues.

Cells signaling studies were performed using cells transfected with plasmids coding GPER and TSHR, and treated with their specific ligand, E_2 and/or TSH. Intracellular increase of cyclic adenosine monophosphate (cAMP) and Inositol-1-phosphate (IP1) was evaluated by BRET and Homogeneous Time Resolved Fluorescence (HTRF). cAMP and IP1 were considered as a measure of $G\alpha_S$ and α_q protein subunits activation, respectively, which transducing proliferative and survival signals. Under the same conditions, cell viability was investigated by MTT assay.

Results

Close interaction between TSHR and GPER was found in COS7, K1 and Nthy-ori 3-1 cell lines by PLA, with markedly increased number of interactions in the non-tumor condition mimicked by the Nthy-ori 3-1 cell line. Negative results obtained in the transfected COS7 cell line by BRET. Similar results were obtained by PLA in PTC and healthy primary thyroid tissues. Moreover, receptor co-expression leads to differential modulation of TSH- and E_2-induced proliferative cAMP an IP1 pathways. cAMP increased in both TSHR-expressing and GPER/TSHR-co-expressing cells, upon TSH treatment. Intracellular IP1 levels increased in TSH-treated cells, under TSHR expression, although the hormone failed in inducing IP1 production under GPER/TSHR co-expression. The presence of E_2 did not influence cAMP, nor IP1 production. GPER expression is associated with reduced cell viability in Nthy-ori 3-1, but not in K1 cell line, not depending on TSHR expression or treatment with hormones.

RT-PCR and immunofluorescence analyses revealed lower expression of TSHR and the absence of GPER expression in PTC than healthy thyroid follicles.

Further experiments will be performed to dissect the role of estrogens and GPER on thyroid cancer development, via modulation of cell viability and death signals.

Conclusions

Although signals revealing GPER and TSHR interactions are not enough to finally demonstrate the formation of heteromers, GPER may act as a switch of $G\alpha_q$-dependent intracellular pathway activated by TSHR-TSH binding. Moreover, my results suggest that GPER expression, rather than the presence of estrogens, plays a role in regulating cell proliferation differentiating tumor from healthy thyroid cells. These data suggest that GPER may play a role in increasing the incidence of PTC in women.

Beatrice Melli

CEM Curriculum: Translational Medicine
Tutor: Dr. Daria Morini

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

Background

With an increasing incidence worldwide, cutaneous melanoma is one of the most aggressive human cancers. Approximately 10% of all cutaneous melanoma occurs in families whose members have multiple primary melanomas and different susceptibility genes have been identified among these families. The pattern of heritability is consistent with an autosomal dominant inheritance that presents an incomplete penetrance where several environmental factors, such as exposure to UV rays, can contribute to the development of lesions. CDKN2A is currently the most clinically relevant melanoma-susceptibility gene, accounting for about 20–40% of hereditary melanoma. Germline susceptibility has also been associated with mutations in CDK4, BAP1, TERT, POT1, ACD, TERF2IP genes, and other with variants in intermediate-risk genes, MC1R and MITF and activating mutations in two genes, GNAQ and GNA11, both associated with uveal melanoma.
For those reasons, since 2013, AUSL-IRCCS RE proposes for familial melanoma cases the use of the genetic test “Sanger” for the sequencing of the CDKN2A (exons 1a, 1b, 2, 3) and CDK4 (exon 2) mutational hotspots.

Objectives

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

Methods

The study is conducted in collaboration with Medical Genetics, Skin Cancer Unit (SCU) and the Molecular Pathology laboratory. The libraries for the NGS are prepared from the patients’ genomic DNA using a panel for familiar melanoma with probes for the coding regions and splice junctions of the CDKN2A, CDK4, BAP1, MITF, POT1, ACD, TERF2IP, MC1R, TERT (plus promoter), GNAQ and GNA11 genes. The NGS analysis are performed on the MySeq tool (Illumina), with an average coverage of the regions of interest> 200x. The data are analyzed according to Illumina's pipelines for calling and filtering variants. The variants frequency identified in each gene of the panel are compared with the literature data, by a χ-square test or by multivariate analysis that considers covariables in the different cohorts.

Only adult individuals with a previous diagnosis of familial melanoma and consenting to participate in the study were included. The currently used Sanger test (CDKN2A/CDK4) is performed in parallel for the newly enrolled patients.

Results

NGS confirmed the data obtained by the Sanger test in CDKN2A and CDK4 genes. In a total of 44 patients' analysis through NGS the data allowed to identify other variants thanks to sensitivity and search for variants in susceptibility genes not previously examined. Since NGS allowed to analyze a lot of genes, the data obtained found several variants of unknown significance (VUS) classifiable based on bioinformatics evaluation. In detail, NGS has found 7% of pathogenic variants contrary to <3% of pathogenic variants found through Sanger sequencing. POT1 R273W variant, analyzed by NGS, is classified as a pathogenic in literature and it enabled the diagnosis of familiar melanoma in the patient. The great sensitivity of the NGS methods also allowed the analysis of a region insertion in CDKN2A gene, not previously examined in Sanger due to the limitation of primer design for the Sanger sequencing.

Conclusions

Considering these preliminary founding, it is possible assert that the genetic testing for melanoma susceptibility genes might be recommended in melanoma families after adequate genetic counseling of the patient. In a significant number of patients, it was possible to identify susceptibility variants already known in the literature, new variants classifiable as probably predisposing based on bioinformatics evaluation. Furthermore, understanding the association between gene variants and cancer risk might be of great help for clinicians involved in cancer screening and surveillance in these families.

Silvia Giovanella

CEM Curriculum: Translational Medicine
Tutor: Prof. Riccardo Magistroni
CoTutor: Prof. Gianni Cappelli

PERSONALISED MEDICINE IN GENETIC KIDNEY DISEASE: THE DIAGNOSTIC EFFICACY OF A PANEL STRATEGY

Background

Chronic kidney disease (CKD) is a worldwide public health problem, with adverse outcomes of kidney failure, cardiovascular disease and premature death. End-stage kidney disease (ESRD) represents the final stage of CKD and it has an incidence of 127 per million population in Europe [1], with a huge impact on quality of life and on healthcare costs.

The main reported causes of ESRD are diabetic nephropathy (19%), glomerulonephritis (17%) and hypertensive nephropathy (16%), but in 20% of cases, the etiology is missing [2].

Recent studies have shown that a genomic approach can identify the underlying disease of CKD in about 30% of pediatric cases and 5-30% of adults [3]. The identification of the genetic causes allows for defining a more accurate prognosis and a more suitable therapy as well as allows the evaluation of the risk of transmitting the disease to the offspring and considering alternative solutions for pregnancy planning.

Therefore, to identify a possible genetic component in suspected cases, several approaches may apply. The whole-exome technology is mainly used in undiagnosed CKD or unclear clinical presentation. In case of high suspicion of the underlying condition, the panel strategy can be preferred. In this case, the diagnostic rate depends mainly on the number of genes covering the single disease category.

At the moment, the scientific community did not express a consensus regarding the best diagnostic algorithm for the evaluation of suspected mendelian renal diseases. Thus, to evaluate the validity of a panel approach compared to other technologies, we established an international group called DECIDE (Diagnostic EffiCacy kIdney Disease European), involving five other centers that used the same diagnostic strategy. This analysis could lead to the definition of a more efficient diagnostic algorithm for kidney diseases.

Objectives

The multicenter retrospective study, DECIDE (Diagnostic EffiCacy kIdney Disease European), aims to evaluate a genomic analysis's efficacy and sensibility. The analysis was conducted through a panel that enables the assessment of multiple types of variants in 44 genes. The collection of a large amount of data, presumably 2000 tests, from the same target population and with the same diagnostic strategy will also permit to perform an epidemiological evaluation of the uncertain significant variants and assess a genotype-phenotype correlation.

Methods

Patients followed by the Nephrology Division at the University Hospital of Modena, between 2018 and 2021, with high suspicion of genetic kidney disease, were tested with the Nephropathies Solution Panel (NES, SOPHiA Genetics), containing 44 genes related to kidney diseases. Sequencing data were processed for single nucleotide variants, indels, and copy number variations via the SOPHiA DDM platform. The pathogenic and likely pathogenic variants (scored according to the American College of Medical Genetics and Genomics) were confirmed by the Sanger technique, platform Applied Biosystems® 3500xL. Clinical and genetic information, such as the family tree were collected. In case of negative results, further studies were applied, where possible, such as other specific panels and CGH (Comparative Genome Hybridization) array.

To widen the cohort of patients who underwent the NES assay, we established an international group involving six European centers, where Modena is the coordinator. The data of over 2000 patients would be collected. The clinical and genetics data will be aggregated, and the patients will be divided based on their clinical presentation. Results about other genetic assays will be considered for the algorithm optimization. The diagnostic efficacy will be expressed as the diagnostic sensitivity of the test separately for each class of clinical presentation. The enrichment of pathogenetic variants will be expressed in terms of odds ratios. To evaluate the genotype-phenotype correlation a Kaplan-Meier analysis comparing the variants classification (C3 vs C4/C5) and the type of variants will be performed.

The study was approved by the Emilia Romagna Ethics Committee (Prot.AOU 0036074/21) and at the local centers of Madrid and Granada, in the other centers the documentation is in progress.

Results

During the first step of the study, 280 patients were tested with the NES panel and enrolled in the DECIDE study. All the clinical and genetic information were collected and the patients were divided into seven classes of clinical presentations: cystic kidney (presence of renal cysts by radiologic examination), glomerulopathy (urine abnormalities and suspect for a mendelian condition), CAKUT (congenital abnormalities of the kidney and urinary tract), tubulopathy (dysfunction in specialized channels and transporters), nephrocalcinosis (renal stones, renal calcifications), negative phenotype, ND (any other kidney clinical picture with high suspicion of a genetic condition). To evaluate the diagnostic efficacy, we considered only the index cases, so the 223 patients were divided into 86 cystic diseases, 77 glomerulopathies, 9 CAKUT, 15 tubulopathies, 19 nephrocalcinoses, 6 negative phenotypes and 11 ND. The diagnostic efficacy for each clinical presentation was respectively: 35% (cystic), 30% (glomerulopathy), 11% (CAKUT), 33% (tubulopathy) and 10% (nephrocalcinosis). Considering the overall population, the efficacy was 30%. The main genes implicated in these diagnoses were PKD1, COL4A5 and COL4A3 in accordance with previous literature [4]. Concerning the other technological approaches, we have defined two diagnoses of Nephronophthisis through a specific panel and CGH array.

Conclusions

Our approach shows a high diagnostic capacity (30% efficacy), significantly reducing the share of patients with an unknown diagnosis. The approach appears to be less effective in patients with nephrocalcinosis presentation, showing room for further improvement in the diagnostic sensitivity.

The development of this project, involving 6 centers, has great potential in terms of genetic data collection, evaluation of the panel diagnostic efficacy for each clinical presentation and epidemiological evaluation of uncertain significance variants.

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Mohammad Gol

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ANTIEPILEPTOGENIC EFFECTS OF TRILOSTANE IN THE KAINIC ACID MODEL OF TEMPORAL LOBE EPILEPSY

Background

Trilostane selectively inhibits the enzyme 3β-hydroxysteroid dehydrogenase in the adrenal cortex and leads to an increase in neurosteroid levels in the brain. Previously, it was found that epileptogenesis could be anticipated by reducing the synthesis of allopregnanolone in a model of temporal lobe epilepsy.

Objectives

The aim of this project is to define whether epileptogenesis could instead be delayed by increasing the brain availability of neurosteroids with trilostane.

Methods

We designed an experiment in which trilostane (50 mg/kg) was administered once daily for six consecutive days, starting 10 minutes after the intraperitoneal administration of kainic acid (15 mg/kg) to induce status epilepticus (SE). Rats were euthanized 1 or 10 weeks after SE induction.

Results

In comparison to the control group, the total duration of convulsive seizures during SE was not significantly reduced in trilostane-treated rats. The latency to develop the first convulsive and nonconvulsive seizures of the SE was unchanged. Additionally, the treatment with trilostane did not affect the neuronal cell density and the area of lesion in the hippocampus. However, the mean duration of convulsive seizures during SE was markedly decreased by trilostane. Notably, the repeated administration of trilostane significantly increased the latency to develop both the first electrocorticographic and convulsive tonic-clonic spontaneous seizures. In subiculum, a significant change was observed in activated microglia morphology by comparing rats repeatedly treated with trilostane with those treated with the vehicle.

Conclusion

Overall, our results indicate that the repeated administration of trilostane delays the onset of epileptogenesis, an effect probably related to enhanced availability of neurosteroids in the brain.

Teresa Urbano

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ENVIRONMENTAL RISK FACTORS AND PREDICTORS OF PROGRESSION OF MILD COGNITIVE IMPAIRMENT TO DEMENTIA IN A NORTHERN ITALY POPULATION

Background

Dementia is an umbrella term that includes many types of disorders with Alzheimer's Disease (AD) being the most common diagnosis. Mild Cognitive Impairment (MCI) is regarded as a transitional state between normal aging and clinically overt dementia, where subjects experience decline in one or more cognitive domains without meeting criteria for a dementia diagnosis. MCI can be classified as amnestic, if subjects experience inability to recall stored information, and non-amnestic, if memory function remains substantially unaltered [1].

Assessment of levels of several biomarkers is fundamental for the prediction and understanding of neurodegenerative processes associated with MCI progression to dementia, as well as dementia staging and response to treatments. They include cerebrospinal fluid (CSF) and blood measures of pathological β-amyloid$_{1-42}$ / β-amyloid$_{1-40}$, tau protein, neurofilaments, and regional brain volumes [2]. In addition, several risk factors have been related to MCI and dementia vulnerability, but the strongest risk factor for their onset and progression still remains age [3]. Exposure to air pollutants, electric and magnetic fields, pesticides, metals and metalloids, as well as dietary habits and lifestyles have also been hypothesized to affect dementia risk [4-6].

Objectives

To assess potential prognostic markers, environmental and lifestyle determinants, which may be associated with dementia onset and predict MCI progression to different forms of dementia.

Methods

This is an ongoing longitudinal prospective study approved by the Ethics Committee *Area Vasta Emilia Nord* and begun in 2019. Individuals with newly-diagnosed MCI have been recruited in the Neurology Clinics of two Emilia Romagna provinces, Modena and Reggio Emilia. Participants underwent both clinical (neuropsychological assessment, lumbar puncture to assess amyloid ratio and tau protein levels) and experimental investigations (determination of several trace elements, pesticides residues, neurofilaments light concentrations in serum and CSF). At recruitment, each patient was asked to fill in a questionnaire regarding personal information, medical, residential and occupational history, lifestyles, and pesticide exposure. Follow-up visits are carried out after 18-24 months from the baseline visit to assess the progression

rate. Dietary habits were evaluated through a validated semi-quantitative food frequency questionnaire (FFQ) from the *European Prospective Investigation into Cancer and Nutrition* (EPIC), specifically developed for the Central-Northern Italy population. Through the EPIC-FFQ, the intake of nutrients and contaminants has been assessed by using the Italian version of the *EPIC-Soft* program (Progetto EPIC, Milano, Italia) [7].
All statistical analyses have been performed with crude and adjusted models and using Stata software (Stata Corp., v. 17 College Station, TX, 2021).

Results

Of the 146 MCI subjects recruited to date, one withdrew the consent to participate in the study, while 145 accepted to participate. Of these, 99 returned the questionnaires. The study population is composed of 60 men (45 recruited in Modena and 15 in Reggio Emilia) and 86 women (60 recruited in Modena and 26 in Reggio Emilia) with a median age of 61 years (interquartile range 56-66 years).
Analysis of cadmium, copper, iron, manganese, selenium, zinc and selected pesticides and dialkyl phosphate (DAP) metabolites concentrations have been performed for 104 subjects. The median serum and CSF concentrations of the trace elements were higher in men than women, except copper and iron whose levels were higher in the latter subgroup's serum and CSF, respectively. Men and women showed almost the same median levels of DAP metabolites and organochlorine concentrations, while polychlorinated biphenyls levels were not detectable in none of the samples analyzed.

Conclusions

To date, still no cure exists for dementia, independently of its clinical type. Efforts are being made to identify the role of environmental (e.g., metals and pesticides) and lifestyle-related risk factors in MCI aetiology and progression to dementia. The choice of conducting this study by enrolling newly-diagnosed MCI patients may allow us to distinguish potential causal pathogenic factors from epiphenomena that may derive from the neurodegeneration process.

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Giovanni Neri

CEM Curriculum: Translational Medicine
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DEVELOPING ANTI-GD2 CAR-T CELLS TO TREAT SMALL CELL LUNG CANCER

Background

Small-cell lung cancer (SCLC) is a high-grade neuroendocrine carcinoma accounting for 15% of all lung cancers and is predominant in current or former smokers. To date, it is distinguished by a dismal prognosis that reflects the lack of appropriate therapeutic strategies (1).

Chimeric Antigen Receptor (CAR) T cells is a novel approach that has been adopted initially to treat blood malignancies such as Acute Lymphoblastic Leukemia (ALL). It consists of patient's lymphocytes genetically engineered *ex-vivo* to recognize a specific tumor-associated antigen (TAA) via an engineered receptor (CAR) which is composed mainly by an extracellular antibody ScFv binding moiety and an intracellular co-stimulatory domain (2). The CAR binding to TAA triggers the immune response leading to CAR-T secretion of cytotoxic molecules such as granzyme B, TRAIL, INF-γ. TNFα etc. (3). The advantage of CAR-T cells is not only represented by the intrinsic plethora of cytotoxic substances they secrete, but also by their homing ability and by their possibility to expand and persist *in vivo*. Considering the treatment efficiency on blood tumors, CAR-T cells have been recently translated to solid tumors (4).

According to a study published by Furukawa and colleagues, SCLC retains high expression levels of the disialoganglioside GD2 on its surface, while it is known it has a highly restricted expression in healthy tissues (5). Hence, engineering CAR-T cells to target GD2 antigen could represent a novel and a promising approach to contrast SCLC either as a stand-alone treatment or in combinatorial regimens.

Objectives

The three-year-long research project aims to:

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

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

Methods

Cell culture techniques: isolation of PBMC, collection of lymphocytes, retroviral transduction and culture of lymphocytes. Culture of SCLC cell lines. Culture of FLYRD18 packaging cell line. 3D cell modelling using spheroids or VITVO™ platform (6). *In vitro* cytotoxicity studies via Promega Glomax Discover Multimode Microplate Reader, immunophenotyping using BD FACS ARIA III.

Molecular techniques: Cytokine release assay using Luminex technology with custom made kit by ThermoFisher. Lentiviral transduction to insert Luciferase transgene into SCLC cell line. Retroviral transduction with dsRED of SCLC cell line and retroviral transduction of T lymphocytes to express the Green Fuorescent Protein (GFP) and/or the anti-GD2 CAR.

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

Results

Anti-GD2 CAR T cells were successfully manufactured using a previously established protocol (7). CAR T cells were characterized via flow cytometry to know the CAR T lymphocytes cytotoxic, memory and exhaustion profile. SCLC cell lines were tested for GD2 expression, and we identified GD2+ and GD2- lines. Both two cell lines were transduced with dsRED or Luciferase to use them either for imaging and cytotoxicity studies. The findings derived by these assays showed a potent antitumor activity of CAR T cells against SCLC which elicit a significant tumor killing. To better study the effect of CAR T cells against SCLC we wrote an ethics committee to collect primary tumor samples from patients to isolate tumor cells to use for *in vitro* or *in vivo* experiments. We set and performed *in vitro* studies at FACS and Glomax to assess SCLC cells viability, we exploited dsRED tumor cells to collect images at EVOS fluorescence microscopy. To investigate an off-the-shelf allogeneic CAR T approach, we started developing an expansion protocol for $\gamma\delta$ T cells using zoledronate. Initial findings showed 20% of $\gamma\delta$ T cells at 15 days and 30% $\gamma\delta$ T cells at 30 days.

Conclusions

CAR T cells provided remarkable results to the immunotherapy field. SCLC is still lacking an efficient treatment that might significantly impact the diagnosis. To fill this unmet medical need, we consider to translate our anti-GD2 CAR T cells to solid malignancies against SCLC as already done with glioblastoma (8).

We developed anti GD2 CAR T cells and studied the immunophenotype to assess the population of cytotoxic T lymphocytes, the memory subset, and the exhaustion markers. Transduced cells with dsRED were used to collect *in vitro* images and cells infected with luciferase were used to precisely evaluate the tumor killing, which has been proven to be significant in the case of GD2+ cell line. In addition, an ethics committee was written to collect primary tumor cell lines. Further steps will be to collect more data on in vitro cytotoxicity to have solid data before moving on *in vivo* experiments. In addition, $\gamma\delta$ T cells were developed as a universal platform to engineer CAR T cells on.

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CEM Curriculum: Translational Medicine
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FOCUS ON CATASTROPHIC AMYOTROPHIC LATERAL SCLEROSIS: a biomarker study

Background

Amyotrophic Lateral Sclerosis (ALS) is characterized by a wide range of clinical presentation, heterogeneous progression and variable survival, with some patients who may be referred as "catastrophic", having an extremely deleterious progression leading to death or tracheostomy within one year from symptom onset. Even if a minority, estimated as less than 10% of the total ALS population, this group is extremely fragile and represents a great challenge even for the most experienced clinicians, as their conditions relentlessly worsen before support procedures are carried out. We hypothesize that systemic and neurological inflammation may aberrantly interact to precipitate ALS, especially in this specific population.

Objectives

The aim of the project is to deeply characterize with clinical, neurophysiological and immunological signatures a cohort of "catastrophic" ALS patients with respect to "classical" ALS patients.
For this purpose, we aim to describe immune profiles of "catastrophic" and "classical" ALS patients, including systemic inflammation and microglial biomarkers in order to delineate a panel of biomarkers useful for the diagnosis and prognosis linked to ALS progression.

Methods

ALS patients were recruited from a case series from Emilia-Romagna region, Northern Italy, who were visited at the ALS Center of Modena University Hospital between January 1, 2007, and January 1, 2021.
A "catastrophic" evolution was defined as either a survival time less than 12 months from onset or a decline in ALSFRS-r greater than 3 points/month. The monthly decline in ALSFRS-s was calculated by subtracting the ALSFRS-r score obtained at the first visit minus the score of the last available visit, divided by the time in months between the first and last visit. "Classical" ALS progression runs an ALSFRS-R slope of 0.3-3 points/month, excluding slow progressors. Patients have been deeply clinically and neurophysiologically phenotyped, with genetic analysis by NGS performed as part of the diagnostic process.
We measured the cerebrospinal fluid (CSF) and serum biomarkers of 12 catastrophic ALS patients in comparison to 85 patients with a classical evolution. The panel of biomarkers included systemic inflammatory cytokines and interleukins (IL-17A, IL-18, IL-18BP, MCP-1, TNF-β1, INF-γ, IL-10, IL-12, IL-1a, IL-6, TNF-α), microglial (serpinA1, CHI3L1, TREM-2), neuroaxonal (neurofilaments pNFH and NFL) and synaptic biomarkers

(neurograninA). Blood-brain-barrier damage index was also calculated. After distribution analysis, parametric or non-parametric tests (Student t-test or Mann-Whitney test, respectively) were utilized to evaluate differences between the two groups for continuous variables, whereas Chi-squared test was used for categorical variables. ROC analysis was performed to analyse the specificity and sensitivity of each analyte. Correlations between serum and CSF levels of each analyte were analysed by Spearman's correlation, as well as possible influences of other demographic factors such as age, diagnostic latency, time from onset to sampling.

Results

Demographic and clinical characteristics were comparable between the two groups, with exception for prominent fronto-temporal frailty in catastrophic group (33% vs 7% in intermediate, p=0.05) and for genetic status, with higher C9orf72 mutations in catastrophic ALS (33% vs 5,88%, p=0.006). The distinct median survival and the respiratory status at sampling reflect the different prognoses of each group.

Overall, neurofilaments and CHI3L1 concentrations in serum and CSF correlated, whereas for SerpinA2 and TREM2 were independent. Catastrophic ALS patients had higher levels of NFL in CSF [20850.68 pg/ml (13124-30140) vs 6261.5 pg/ml (3142.5- 9512.669)] and serum [201.64 pg/ml (163-358) vs 109.5 (73.67-141)] compared to classical ALS patients ($p < 0.001$ and $p = 0.001$ in CSF and serum, respectively), while pNFH were significantly elevated only in CSF [8137 pg/ml (6801.5- 10944) vs 4133 (2471-7049), p=0.005] without reaching statistical significance in serum [2131pg/ml (1085-3500) vs 1311.5 pg/ml (577-2497), p=0.077]. In CSF, serpinA1, CHI3L1 and TREM-2 were significantly higher in catastrophic ALS patients [9.59ug/ml (6.94-13.31) vs 4.49(3.20-6.19), p=0.0349; 129.36 (100.9-200.45) vs 72.72 (48.87-127.3), p=0.0390; 27.2 ng/ml (20.1-32.9) vs 18.56 (15.29-23.6), p=0.0049 respectively], while on sera we observed a higher tendency for CHI3L1 concentration without reaching statistical significance (p=0.4155) while serpinA1 and TREM-2 levels were lower in catastrophic ALS (p=0.4062 and p=0.3006, respectively). Neurogranin levels were equally distributed between the two groups and independent of the cognitive status. All systemic inflammatory biomarkers had similar concentration between the two groups, with only IL-17BP levels being higher (p=0.0778 on CSF and p=0.0506 on serum) in catastrophic patients without reaching statistical significance. Moreover, a higher blood-brain barrier damage was observable in catastrophic ALS [0.94% (0.69-1.15) vs 0.46% (0.355-0.63), p=0.009]. ROC curves for discriminating catastrophic ALS from classical forms showed an area under the curve (AUC) of 0.79 for CSF serpinA1 and 0.9 for CSF NfL, with AUC of 0.87 combining CSF NfL, serpinA1 and CHI3L1 together.

Conclusions

Despite some statistical inconsistencies of the results due to limitations in sample size, our study supports the idea that in catastrophic patients the microglial inflammatory response is highly activated compared to

patients with a slower disease, while systemic inflammatory reaction seems to be marginal. Moreover, combining markers of neurodegeneration with CSF serpinA1 and CHI3L1 levels may hold promise to discriminate against aggressive evolution at presentation.

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CEM Curriculum: Nanomedicine, Medicinal and Pharmaceutical Sciences
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IDENTIFICATION OF IMPURITIES IN CANNABIGEROL EXTRACTED FROM HEMP: CANNABIGEROVARIN (CBGV) AND CANNABIGEROBUTOL (CBGB)

Background

Cannabis sativa L. is a prolific producer of a peculiar group of isoprenylated resorcinyl polyketides well known as phytocannabinoids. Over 150 phytocannabinoids are produced in the plant of which only few have been isolated and characterized. In the present work, we focused our attention on cannabigerol (CBG) that is the precursor of all other phytocannabinoids. It is generally present as a minor component compared to either the dominant cannabidiol (CBD) in the most common fiber-type cannabis varieties, or tetrahydrocannabinol (THC) in drug-type cannabis. Nevertheless, CBG-predominant cultivars have started to be bred due to the remarkable pharmacological properties of this phytocannabinoid, especially as a antimicrobial, anti-inflammatory, cytotoxic and antidepressant agent, besides its non-psychotropic nature. CBG-rich cannabis varieties are, therefore, genetically selected for the extraction of CBG to make a pure marketable substance. Commercial CBG is generally labelled as ≥98 % pure, bearing two main impurities, one of which is cannabigerovarin (CBGV) and another compound with the hypothetical structure of a cannabigerol with a butyl side chain, commonly named CBG-C_4. The former was first reported in 1975, its chemical and pharmacological properties have been investigated, although not extensively, whereas the latter has never been characterized to date.

Objectives

The aim of the present work was to characterize the impurities of commercial CBG, provide a simple method for their qualitative and quantitative determination and make the two impurities available to perform in vitro and in vivo pharmacological studies to define their biological profile.

Methods

The high-performance liquid chromatography (HPLC) technique coupled to both diode array (DAD) and high-resolution mass spectrometry (HRMS) detection was employed for the analysis of commercial CBG powder. Chromatographic separation was performed using a Poroshell 120 EC-C18 column (3.0 × 100 mm, 2.7 µm) and eluting 0.1% aqueous formic acid (A) and 0.1% formic acid in ACN (B) as mobile phase. Method: Isocratic elution at 75% B, 6 min; Isocratic elution at 98% B, 8min; re-equilibration to the initial conditions (70% B), 13 min (total run time). Flow rate 0.5 mL/min. Injection volume 10 µL. The DAD recorded all wavelengths from 190 to 900 nm and the wavelength of integration was 210 nm. The liquid chromatography apparatus was

interfaced to a heated electrospray ionization source (HESI) of an Orbitrap Exploris 120 mass spectrometer. The identification of CBG impurities was accomplished employing the HRMS technique and subsequently confirmed by comparison with the same compounds obtained by chemical synthesis. The latter involved the conversion of the brominated resorcinols into the corresponding alkenes by performing the Wittig reaction using propionaldehyde or acetaldehyde (to get the butyl and propyl derivative respectively). Subsequently, the alkenes were reduced and deprotected. The precursors obtained were converted into the products of interest (CBGV and CBGB) by reacting with geraniol and a catalytic amount of *p*-toluene sulfonic acid. The crude was purified by silica gel chromatography. The structures of all compounds were assessed by HRMS, nuclear magnetic resonance (NMR), UV and FT-IR spectroscopy.

Results

Analysis of commercial CBG powder by HPLC-UV showed the presence of two peaks eluting before that of the main ingredient (3.30 min), specifically at 2.26 min and 2.70 min. To obtain information on the chemical structure of unknown compounds, HRMS analysis, through Orbitrap technology (Exploris 120), was employed. The HRMS trace showed the presence of the same two peaks eluting before the main ingredient (2.28 and 2.72 min respectively). Both peaks showed the same HRMS fragmentation patterns of CBG with the only exact difference of 14.0157 amu between the first and second impurity fragments and between the second impurity and CBG. Therefore, the first impurity was putatively identified as cannabigerovarin (CBGV), the propyl homolog of CBG, while the second impurity was putatively identified as cannabigerobutol (CBGB), the butyl homolog of CBG. Injection of the corresponding synthesized analytical standards confirmed such a hypothesis. The developed and validated method was applied to four commercial CBG samples. Three samples were characterized by a constant ratio between the two impurities with higher amounts of the butyl species compared to the propyl one, while the fourth sample showed an inverted ratio between CBGV and CBGB in favour of the former. At this stage there is no supporting evidence to address the concentration trend. It could be either simply reproduced from the amount of CBGV and CBGB present in the belonging hemp variety or be ascribed to the extraction process. In this regard, it would be essential to screen the concentrations of these two compounds and their ratios in different hemp varieties.

Conclusions

Commercially available “pure” CBG was analyzed by HPLC coupled to UV and HRMS, highlighting the presence of two main impurities, which were identified as cannabigerovarin (CBGV) and cannabigerobutol (CBGB), the propyl and butyl homologs of CBG, respectively. A fast and straightforward HPLC-UV method was developed and validated according to ICH guidelines to provide reliable quantification of such impurities in commercial CBG samples. Although the concentrations found were below 0.5 %, it should not be excluded that higher amounts might be present in the starting hemp material. Therefore, it could be crucial to determine such

compounds in different hemp varieties and investigate their ratio to disclose a potential correlation among the different CBG homologs. As new cannabinoids are added to the inventory, the challenge of a comprehensive phytocannabinome fingerprinting no longer seems such a distant goal.

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CEM Curriculum: Translational Medicine
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CLINICAL-INSTRUMENTAL PHENOTYPING AND BIOMARKERS ANALYSIS IN THE EVOLUTION OF PATIENTS AFFECTED BY PARKINSON'S DISEASE

Background

Parkinson's Disease (PD) represents the second most common neurodegenerative disease after Alzheimer disease. PD is a heterogeneous disease from a genetic point of view, with familial and sporadic cases. At present 23 genes or loci have been identified, in particular LRKK2 and GBA are the most common variants identified. The diagnosis of PD is mainly based on clinical data supported by bioimaging like brain MRI and single photon emission computed tomography (SPECT) with Ioflupane I123 injection (DaTscan™). These instrumental approaches are now integrated by quantitative methods to identify predictive data on disease progression, such as DatQuant software, capable of extrapolating volumetric quantitative data from the DATSCAN exam. The recent technological evolutions in terms of quantitative and heterogeneous data about PD patients have brought to new approaches of investigation based on clustering to identify subgroups and predict different disease progression pathways.

Objectives

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

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

Methods

The clinical research project has been structured in this way:

- Definition of the clinical and instrumental data to collect (like UPDRS; Hoehn and Yahr, MOCA, Schwab and England, PDQ-39)
- Definition of cohorts of patients according to genetical profile (LRKK2 and GBA) and control group
- Database creation through 1:1 match according to sex, age, age at PD onset between genetical and control subjects
- Clinical and instrumental data collection, in particular PD motor profile and scales, drug assumption (Levodopa Equivalent Daily Dose), Charlson Comorbidity Index (CCI), comorbidities and cardiovascular events (CAD), DatQuant volumetric data
- Statistical analyses, mainly based on ANOVA, paired t-test, Mann Whitney test, correlations and regression.

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

Results

The preliminary results of the analyses conducted are the following:

- We have created two matched cohorts of patients with this numerosity: 14 LRKK2, 46 GBA, 60 controls.
- In terms of cardiovascular risk factors, no significant differences have been found among any of the groups. The only statistically significant comparison has been identified between genetic PD patients and controls in terms of CAD prevalence (performing paired t-test, P = 0.05).
- Considering the DatQuant volumetric data, two parameters linked to Caudatus and Putamen nuclei (striatal binding ratio, SBR) have statistically significant differences between the two cohorts (performing Mann Whitney test, P=0.043 and P=0.028 respectively).

Conclusions

The preliminary analyses conducted so far have identified some clinical and bioimaging-based data that may potentially discriminate between genetical PD and idiopathic PD subjects. Further analyses and additional are needed to develop a phenotyping approach which may describe the two cohorts. In particular, the next step of the investigation will be based on integration and correlation with other clinical scales in order to give a clinical explanation to the volumetric data identified as potential biomarkers from DatQuant analyses.

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CEM Curriculum: Translational Medicine
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AUTOFLUORESCENCE IN NON-MELANOMA SKIN CANCER: IN VITRO EVALUATION

Background

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

Autofluorescence (AF) is the property of tissues to absorb and re-emit light with specific wavelengths. This phenomenon is due to the presence of molecules called fluorophores.

Spectrophotometric devices can read autofluorescence emission spectra of the skin.

As already demonstrated by previous studies, AF spectroscopy can be used as an optical biopsy tool for the early detection of NMSC ex vivo. Variations in intensity ratios have already been proposed as important clues for cancer tissue detection.

Objectives

- To investigate how AF differ in healthy and cancer cells in in vitro models of cutaneous cancerization.
- To investigate which are the most efficient excitation wavelengths to emphasize these differences
- To provide quantitative methods based on multivariate analysis to differentiate between healthy and cancer cells.

Methods

We evaluated 77 AF intensity spectra. Of these, twenty-four spectra were derived from healthy-cell AF, 22 from cancer AF and 32 spectra were obtained from Petri dishes only to assess how the support might affect AF final spectrum.

Normal keratinocytes were obtained from discarded tissue from healthy donors undergoing standard dermatological surgery and were compared to SCC-154 cancer cells.

The luminescence spectra, excited at different selected wavelengths with variable-power monochromatic LEDs (at 280, 310, 365, 405, 470 and 533 nm) were acquired with a portable high-sensitivity Ocean Optics spectrometer (model QE-Pro) at the Engineering department (DIEF of UNIMORE).

Different supports were tested (plastic Petri dishes, Eppendorf test tubes, multi-wells), at different incidence and detection angles. Petri transparent dishes, with a black light-absorbing paper positioned at the back, provided the better results.

Results

AF intensity spectra (at all excitation wavelengths) demonstrated a general hypo-fluorescent aspect of cancer cells compared to healthy controls.

Main skin chromophores identified in the luminescence spectra at an excitation wavelength of 310 nm included: NAD(P)H (450 nm), flavonoids and flavins (520-550 nm), lipofuscin (600 nm) and porphyrins (700 nm). All the features appear generally dumped in the cancer cells. Hypo-fluorescence of cancer cells was detected in vitro, confirming the results of our previous ex-vivo study.

Principal Component Analysis (PCA) and Partial Least-Squares Discriminant Analysis (PLS-DA) were also performed in order to classify unambiguously healthy vs cancer cells, independently on the excitation source and cell concentration, therefore eliminating such potential confounding factors.

Conclusions

The present in vitro study confirms AF alterations in the cancer cells, and not only in cancer tissue. An overall hypo-fluorescence is observable in cancer cells. These findings bring new insights in AF spectra classification and in the understanding of the fluorescence properties of non-melanoma skin cancer.

Adriana Romanzi

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

Background

Hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (iCCA) are the main primary tumors affecting the liver. Although traditionally considered very different, they share several similarities. Within the HCC group, we identified a subgroup of patients expressing a 5-gene neoangiogenic signature, Transcriptomic signature (TS). They are characterized by the scarce response to any type of therapy and extremely low survival. In TS-positive aggressive HCCs, the most expressed gene is Angiopoietin 2 (ANGPT2), which is associated with increased angiogenesis and proliferation, epithelial-mesenchymal transition (EMT), and PD1/PDL1 activation. Of note, 50% of the iCCA we tested for the neoangiogenic signature were found to be TS+, suggesting the existence of an iCCA subgroup characterizable by the TS-like HCC. Besides the severe clinical behavior, also the imaging features in TS+ HCC and iCCA are similar. However, this relationship has not been explored so far as HCCs are usually examined as a homogenous group, failing to identify similarities with other tumors.

Objectives

This project aims to clarify the main mechanisms underlying the development of aggressive HCC and to elucidate whether these features are shared by iCCA. In particular, we will evaluate the effects of specific angiogenic growth factors (Angiopoietin-2 and/or VEGF) in tumor progression, and the ability of their inhibitors to counteract their effects. We will also assess the role of hypoxia in HCC and iCCA tumoral transformation.

Methods

HCC and iCCA immortalized cell lines, respectively HepG2 and HuCCT-1, were used to build up 3D culture models to test the effect of angiogenic factor treatment. We also used EGI-1 cell lines (derived from extrahepatic cholangiocarcinoma - eCCA) as a negative control. All these tumoral cells were seeded in low attach 6 wells plates and filled with expansion medium for 3/5 days. Following the expansion of the cell culture, HCC, iCCA and eCCA-derived spheroids were stimulated with either 200 ng/mL ANGPT2 or VEGF, or with 100 ng/mL of ANGPT2/VEGF mix. After 48h, we evaluated migration in 2 different ways depending on cell lines' behavior. In particular, for HepG2 we visualized the cells and captured three images/well, to count the number of migrating cells. For HuCCT-1 and EGI-1, instead, we used the Boyden Chamber assay.

To determine the expression pattern of markers involved in EMT (i.e. E-cadherin, N-cadherin, Vimentin, Beta-catenin), HCC, iCCA and eCCA cell-derived adherent spheroids, treated as above described, were pelleted after 3h and 48h from stimulation for protein evaluation by western blotting. In parallel experiments, adherent spheroids were grown on a coverslip and fixed in formalin 4% for immunofluorescence staining with E-cadherin and N-cadherin antibodies.

Results

In both HepG2 and HuCCT-1, but not in EGI-1, treatment with ANGPT2, VEGF or their combination, stimulated the migration of the adherent spheroids. Concerning EMT biomarkers, in HepG2 E-cadherin expression dropped significantly starting from 3h and remained low even at 48h. Notably, E-cadherin expression in untreated spheroids was lower at 48h than at 3h. In HuCCT-1, E-cadherin expression levels were not modified by the treatments either at 3h or at 48h. N-cadherin expression levels increased, regardless of treatment, in HepG2 from 3h, while in HUCCT-1 from 48h. Vimentin expression strikingly increased after 48h stimulation in both HepG2 and HuCCT-1, but not in untreated controls. No differences were observed in Beta-catenin expression levels despite the stimulation in both cell lines. Regardless of treatment, the expression of EMT markers remained unchanged in EGI-1. Immunofluorescence analysis of the adherent spheroids revealed a marked decrease in E-cadherin expression at the periphery of the migrating cells in both HepG2 and HuCCT-1, paralleled by an increase of N-cadherin expression.

Conclusions

These results demonstrate a similar response to proangiogenic stimulation of both HCC and iCCA cells, but not eCCA cells, thus underlying a) the possible phenotypic differences between eCCA and iCCA, and b) the pro-invasive effects of angiogenic growth factors on either HCC or iCCA cells. Further evaluations (such as mRNA expression levels) are ongoing.

Giorgia Guaitoli

CEM Curriculum: Translational Medicine
Tutor: Dr. Federico Piacentini

ASSOCIATION BETWEEN ONCOGENIC SIGNALING AND DNA REPAIR IN NON-SMALL CELL LUNG CANCER

Background

In oncogene-addicted non-small cell lung cancer (NSCLC) alterations of oncogenic kinases (such as EGFR mutations or EML4-ALK rearrangements) determine constitutive activation of pathways involved in regulation of cell survival and proliferation. In the last decades, prognosis of oncogene addicted disease dramatically changed after the advent of tyrosine-kinase inhibitors (TKI), although resistance invariably appears. Mechanisms of resistance to TKI may be classified in main categories such as on target alterations, activation of by-pass pathways or other mechanisms including apoptosis inhibition or histological transformation.

Moreover, there is growing evidence about dysfunctions of DNA repair pathways in NSCLC, and different biomarkers (such as ERCC1, PARP, BRCA1, ATM) are under investigation to explore their prognostic/ predictive role and as possible therapeutic targets.

There is a link between tyrosine-kinase-activated and DNA repair pathways, as recent evidence suggests that TKIs may influence DNA repair system by enhancing genomic instability. On the other side, DNA repair alterations may contribute to the development of resistance to TKI treatment.

Indeed, in EGFR mutated NSCLC, treatment with first or second generation TKI leads to activation of NFkB pathway with subsequent induction of Activation Induced Cytidine Deaminase (AICD), which concurs to generate T790M mutation as resistance mechanisms. It was shown that pharmacologic inhibition of NFkB pathway decrease the frequency of T790M while AICD knockout prevent its development.

Additionally, it was demonstrated that EGFR-mutated lung cancer with acquired TKI resistance depend on PARP-1 for survival, suggesting a possible combination of TKI with PARP inhibitors.

In ALK-driven disease, less evidence is available, but mutations in DNA repair-associated genes were reported in primary resistance to crizotinib.

Objectives

The main objective of this project is to explore whether the association of TKIs with DNA repair inhibitors may enhance the activity of the two molecules and reduce (or delay) the development of resistance, resulting in prolonged duration of treatment.

Within the large field of oncogene-addicted diseases, this project will be mainly focused on EGFR-mutated and ALK-rearranged NSLCL.

Methods

In this project, we will use commercial cells lines (PC9 and H3122, harbouring EGFR exon 19 deletion and EML4-ALK rearrangement respectively) and patients-derived cell lines established in our laboratory, both before TKI treatment and at the time of progressing disease.

Cell lines will be treated by different TKI and, after treatment, we will assess the phosphorylation and expression level of DNA repair proteins by immunofluorescence, western blot and RNA sequencing.

Furthermore, we will infect sensitive cells with a lentiviral vector containing a specific CRISPR-Cas9 cleavage site and several reporter gene modules expressing different fluorescent patterns depending on the repair pathway employed by the cells. After infection, we will treat cell models with different TKI and evaluate the DNA repair capabilities using fluorescence microscopy, in order to understand how specific kinase inhibition may affect DNA repair efficiency.

On patients-derived cell lines, we will evaluate DNA repair pathways after TKIs exposure by matching pre/post TKI mutational signature analysis. If alterations in these pathways emerge after treatment, we will validate this observation *in vitro* on pre-treatment cell lines to confirm that exposure to drugs determines the decrease of these genes and to investigate which mutational processes occur during TKI exposure.

Finally, we will expose cell lines to different combinations of TKIs and DNA-repair inhibitors (such as PARP, ATM, DNA-PK and RAD51 inhibitors) and evaluate cell viability by proliferation tests in order to explore if the inhibition of DNA repair pathways may lead to cell death due to synthetic-lethal interactions.

The project will be carried out at Institut Gustave Roussy in Villejuif (France) at UMR981 INSERM-Identification of molecular predictors and new targets for cancer treatment.

Conclusions

The aim of this project is to evaluate the relationship between oncogenic signaling and DNA repair pathways and to provide useful observation about resistance mechanisms and possible therapeutic associations that may improve long-term target therapies effectiveness.

Simone Lasagni

CEM Curriculum: Nanomedicine, Medicinal and Pharmaceutical Sciences
Tutor: Prof. Erica Villa

HCC FEATURE RELATED WITH ITS RECURRENCE AFTER LT AND RESPONSE TO TYROSINE KINASE-INHIBITOR DRUGS IN HEPATOCELLULAR CARCINOMA

Background

Hepatocellular carcinoma (HCC) is the fifth most common cancer in men and the seventh in women. It is the second most common cause of death for cancer worldwide in males and the sixth in females. This type of tumor is highly malignant and characterized by rapid progression and a poor prognosis.

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

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

Objectives

The aim of this study was to analyze the biological, histopathological and clinical characteristics of patients with HCC in order to understand the development of HCC after LT, with particular attention to the expression in the explanted liver of a series of proteins linked with different mechanisms including neo-angiogenesis and lymphangiogenesis.

Methods

Formalin-fixed paraffin-embedded samples from explanted liver tissue from recruited patients were subjected to immunohistochemical analysis for Angiopoietin-2 (ANGPT2), Podoplanin, Clec-2, α-SMA and Lyve-1. Briefly, after deparaffinization and rehydration, antigen unmasking was performed with 1 mM EDTA buffer, pH 8, at 98°C for 15 minutes. The sections were then incubated in methanol 5% and H_2O_2 1% for 5 minutes for blocking endogenous peroxidases; nonspecific sites were blocked using a blocking solution reagent with bovine serum albumin 3% for 30 minutes at room temperature. Sections were then incubated with goat anti-ANGPT2 (AF623; R&D Systems) primary antibody at working dilution of 1:50, or with rabbit anti-Clec1b/Clec-2 (LS-B12627; LSBio) primary antibody at working dilution of 1:80, each at 4°C overnight in humidity chamber, or with prediluted Podoplanin D2-40 PAb (Cell Marque, Roche diagnostics) primary antibodies. Sections were then incubated with prediluted OmniMap anti-goat (for Angiopoietin-2), anti-

rabbit (for Clec1b/Clec-2) and anti-mouse (for Podoplanin D2-40 PAb) horseradish peroxidase-conjugated secondary antibody (Ventana Medical Systems, Tucson, AZ) for 20 minutes in humidity chamber and then with detection kit reagents (ultra-view universal horseradish peroxidase multimer and diaminobenzidine [DAB] chromogen, Ventana Medical Systems) following the manufacturer's instructions. The sections were then counterstained with hematoxylin, dehydrated, and permanently mounted for microscopic examination. Images of stained liver tissue were processed with ImageJ software (http://rsbweb.nih.gov/) to obtain the intensity value of DAB signal. The statistical difference between the individual groups was analyzed by applying the Student's t-test.

Results

Immunohistochemical analysis of explanted livers showed that the intensity of ANGPT2 signal is significantly higher on the endothelium of vessels in recurrent tissue (OD=0.420±0.008) than in non-recurrent tissue (OD=0.350±0.005) ($p<0.0001$). ANGPT2 expression in hepatocytes was not significantly different between recurrent and non-recurrent patients, both in tumoral and non-tumoral tissue.

The intensity of Podoplanin signal was significantly higher in the endothelium of lymphatic vessels in recurrent tissue (OD=0.486±0.012) than in non-recurrent tissue (OD=0.446±0.007) ($p=0.0021$).

The analysis regarding Clec-2, α-SMA and Lyve-1 is ongoing.

Conclusions

The rate of tumor recurrence after LT is 15%-20%, and survival is greatly reduced because treatment options are very limited in this situation. Nowadays, it is still very difficult to predict HCC recurrence after liver transplantation, which continues to represent an unresolved medical need.

Our data show that in HCC the endothelial expression of ANGPT2 and Podoplanin is higher in recurrent tissue than in non-recurrent tissue. This suggests the possible value of ANGPT2 and Podoplanin as a biomarker of tumor recurrence post-LT, which could help in the selection of the best LT candidates and the use of possible new targeted therapies.

Filomena Giulia Sileo

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CUMULATIVE COVID-19 INCIDENCE IN PREGNANCY: A POPULATION-BASED STUDY IN REGGIO EMILIA, NORTHERN ITALY

Background

The novel coronavirus disease (COVID-19) was declared pandemic on the 11th of March 2020 by the World Health Organization. The pandemic has caused more than 6.2 million deaths and has infected over 512 million people as far as May 5th 2022. As for other viral infections, pregnancy might represent a vulnerable status with an increased risk of morbidity, disease severity and fatality of COVID-19 due to pregnancy-associated physiological changes in cardiopulmonary and immune systems and increased demand for oxygen. Compared with non-pregnant women of the same age with COVID-19, they were less likely to have symptoms. However, severe COVID-19 was diagnosed in 10% of pregnant women, requiring admission to an intensive care unit in 4% of them. Despite having lower frequency of pre-existing medical comorbidities, the pregnant women were more likely to die for Covid-19 or need for: intubation, ECMO, non-invasive positive pressure ventilation, or for high-flow nasal cannula O2 supplementation. Clinical characteristics and laboratory findings were compared in pregnant vs. non-pregnant women diagnosed with SARS-CoV-2 infection but almost all studies published so far have tested only women admitted to the hospital for any reason. Only few studies have tested pregnant women receiving routine obstetric care in the first trimester, thus not reporting any comparison with the general population on women of reproductive age. To the best of our knowledge, no study has reported yet on the likelihood of being tested for SARS-CoV-2 and the cumulative incidence of COVID-19 among pregnant women compared to non-pregnant women of reproductive age and not necessarily admitted when diagnosed with SARS-CoV-2.

Objectives

The primary objective of this study was to evaluate the impact of being pregnant on COVID-19 risk and prognosis in the Italian province of Reggio Emilia by comparing the risk of undergoing a SARS-CoV-2 test and of testing positive for COVID-19 for women that were pregnant at the time of the test with the same risks for the general population of women of reproductive age.

Methods

We conducted a retrospective population-based cohort study using registry data of the Reggio Emilia Province, including all women between 15 and 49 years of age (women resident on 1st January 2020 or 1st

January 2021 or in both dates). Within this cohort, women's person time was classified as "non-pregnant", "pregnant", and "puerperium" (first six weeks after childbirth) according to deliveries and miscarriages occurred from the 1 of march 2020 up to 30th of September 2021 as registered in the Certificate of Delivery Database (CedAP) and the Hospital Discharge database (SDO).

We identified all women undergoing a SARS-CoV-2 test and we calculated the Incidence Rate Ratio (IRR) to compare the likelihood of being tested for SARS-CoV-2 in pregnant and not pregnant women. The risk of testing positive was compared between the two groups by means of Cox model adjusting for age considering all women, women with at least one pregnancy and in women with at least one childbirth. In the analysis we considered only the first infection and only the swabs performed until the first positive swab (included).

Results

During the study period, 117606 women between 16 and 49 years of age were included in the study. Among these, at least one pregnancy occurred in 6608 (5.6%) women, with 5319 and 1501 childbirths and miscarriages occurring in the study period, respectively. The total number of COVID-19 cases was 11929 (10%): among these, 11211 cases occurred among women that were never pregnant in the study period, 462 cases occurred among women that had a pregnancy during the study period (but not while they were pregnant) and 256 cases of COVID-19 occurred during the pregnancy. The total number of swabs performed in these women was 96949. The incidence rate (IR) of swabs was 1.3 among women without pregnancies, 6.2 among those with a pregnancy and 4.7 in puerperium, with an IRR of 5.0 (95%CI 4,9-5,1) e 3.6 (95%CI 3.42; 3.88). The IRR remained significantly higher among women with a pregnancy also after excluding all swabs routinely performed at admission (IRR=2.7, 95%CI 2.57-2.74). Finally, we calculated the Hazard Ratio (HR) of being positive during pregnancy, which was higher during pregnancy (1.16, 95%CI 1.03-1.32) than outside pregnancy. Results were similar considering women with at least one childbirth (1.23, 95%CI 1.04; 1.46) and with at least one pregnancy although not significant (1.16, 95%CI 0.99; 1.35). The excess risk decreased after excluding swabs performed at hospitalization (HR 1.1, 95%CI 0.96-1.24). In the puerperium, the HR was 0.6 (95%CI 0.41-0.92) comparing the puerperium period for women with at least one childbirth with women without pregnancies.

Conclusions

This is the first study comparing the likelihood of being tested and being positive for Sars-CoV-2 among pregnant and non-pregnant women in the reproductive age, considering all women, even when not admitted in the hospital for delivery or any other reasons. The probability of being tested is higher during pregnancy than during time out of pregnancy as well as the risk of being positive. The risk however decreases if screening swabs are excluded. For women in the post-partum period the probability of being tested is higher than in pregnant and non-pregnant women but the risk of positivity is significantly lower.

Marta Starnoni

CEM Curriculum: Translational Medicine
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CoTutor: Dr. Marco Pappalardo

THERAPEUTIC MICROSURGICAL SOLUTIONS FOR BREAST-CANCER RELATED UPPER LIMB LYMPHEDEMA

Background

Chronic and debilitating lymphedema is mainly caused by lymph node dissection and radiotherapy for oncologic surgery of the upper and lower extremities. The institution of a Lymphedema Unit in the Policlinic of Modena is a crucial point in order to provide an individualized treatment plan for patients affected by breast cancer-related lymphedema (BCRL). Several aspects should be taken into account including the recruitment of a multidisciplinary team, physician training, diagnosis, clinical examination, imaging techniques, patient selection and a proper treatment. Actually, only a limited number of surgical treatment options are possible. A close collaboration among different specialists is necessary in order to offer the best treatment solution according to the patient's clinical condition.

Since last year, a multidisciplinary team has been recruited in the Policlinic of Modena. The role of each specialist has been clearly defined from diagnosis to patient selection, classification, and treatment of lymphedema.

Objectives

The aim of the project is to set up a Lymphedema Unit in the Policlinic of Modena in order to offer the best surgical treatment to patients affected by lymphedema.

The project includes:

- To spread information about the new Lymphedema Unit to family doctors, urologists, gynaecologists, and breast surgeons;
- To recruit patients who underwent axillary lymph node dissection (ALND) and radiotherapy (RT);
- To establish a dedicated lymphedema clinic;
- To start a physiotherapy pathway with proper complex decongestive therapy protocol for patients undergoing lymphatic microsurgery;
- To start a collaboration with nuclear medicine doctors and radiologists to select the information needed from the imaging procedures;
- To keep going microsurgical training of plastic surgeon trainees on super-microsurgical and innovative imaging techniques.

Methods

- A list of patients who underwent ALND and RT (from January 2016 to January 2021) has been generated by the hospital server;
- Initial patient selection has been performed throughout a telephone questionnaire. Questions related to the swelling, pain, paraesthesia, heaviness of the upper arm, episodes of cellulitis and need of physiotherapy have been asked. Based on this questionnaire a score priority has been given, from higher to lower lymphedema-risk patients;
- E-mails related to Policlinic Lymphedema Pathway have been sent to GPs, various specialists and lymphedema associations;
- Senior nurse coordinator has been contacted in order to book and set-up a dedicated day for lymphedema clinic equipped of indocyanine green near-infrared fluorescence lymphography;
- A detailed Lymphedema Assessment Form has been created to easily report lymphedema severity, clinical and radiological examination, treatments, results and follow-up of the patient;
- Rehab medicine doctor has been contacted to coordinate a team of physiotherapists providing an advanced lymphedema treatment training;
- Nuclear medicine doctors and radiologists have been advised about radiological features to point out during imaging procedures of lymphedema surgical patients.

Results

- During the last year, 120 patients who underwent ALND and RT have been interviewed with a telephone questionnaire to evaluate lymphedema symptoms for further clinical and radiological examination.
- A dedicated lymphedema clinic has been established at the University Hospital of Modena on Monday morning.
- 45 patients who reached a score of 4-5 have been asked to come for clinical evaluation at our new lymphedema clinic. During the first visit, a proper history interview and clinical examination have been performed for all patients. All patients filled a validated questionnaire Lymphedema Quality of Life Tool - LYMQOL for the upper limb. A lymphedema assessment has been created to include information regarding data, diagnosis, past medical history, pain assessment, current location of swelling.
- Based on the clinical examination including subjective and objective measurements, five patients did not present lymphedema, 18 patients have been categorized as Cheng's Lymphedema Grade 1, 15 patients as grade 2, 5 patients as grade 3 and 2 patients as grade 4. 15 patients have been further evaluated in a second visit with ICG lymphography to investigate the superficial lymphatic pathway and the presence of superficial lymphatic vessels for surgical planning. Based on clinical and radiological methods, 3 patients have been selected for VLNT and 12 patients for LVA.

- A proper training has been provided to physiotherapists according to the Complex Decongestive Therapy Protocol for lymphedema patients with an initial treatment phase followed by the maintenance phase. As such, 5 patients started the initial treatment phase of Complex Decongestive Therapy protocol including manual lymphatic drainage, skin care, compression therapy.
- Training of the plastic surgeon included improvement of super-microsurgical technical skills throughout the execution of micro-anastomosis on demo vessels < 0.5mm of diameter using 11-0 and 12-0 nylon suture under the microscope for three times a week and every session lasting four hours

Conclusions

A new lymphedema multidisciplinary team for the treatment of upper limb lymphedema have been established. Patients have been evaluated clinically and radiologically for a proper diagnosis and staging of lymphedema. Individualized treatment has been proposed for all patients.

Emanuele Vitale

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RUNX2 AND RAIN: CODING AND NON-CODING INTERPLAY IN CANCER PROGRESSION

Background

Transcription Factors (TFs) involved in embryogenesis are often hijacked in tumors, where they reactivate morphogenetic pathways promoting cancer development. RUNX2, the key regulator of bone morphogenesis, was found to be aberrantly expressed in breast (BC) and thyroid (TC) carcinomas and associated with cancer aggressiveness. Although its well-established oncogenic properties, the molecular details of RUNX2 regulatory activity in BC and TC are still largely unknown. Our group recently identified RAIN, a new long-noncoding RNA (lncRNA) transcribed from two RUNX2 enhancers and implied in the RUNX2 re-expression in BC and TC. Aside from its involvement in RUNX2 expression, our preliminary data suggest that RAIN could have additional RUNX2-independent regulatory functions in BC and TC.

Objectives

- To unveil the gene expression program and the genomic dynamics governed by RUNX2 in sustaining BC and TC progression.
- To understand how RAIN impacts cancer biology in a RUNX2-independent manner.

Methods

Primary and metastatic BC (Hs578T and MDA-MB231) and TC (TPC1 and MDA-T41) cell lines were chosen as models for our analyses. RUNX2 and RAIN Knock-Down (KD) were obtained with a lentiviral CRISPRi system. The transcriptomes of edited cells were profiled by RNA-seq, and differential analysis was performed comparing RUNX2/RAIN KD and control cells. The lists of differentially expressed genes upon RUNX2 or RAIN KD were compared to identify common and exclusive targets.

ChIP-seq and ChIRP-seq approaches were used to map the RUNX2 and RAIN genome-wide distribution. ChIP-seq analyses of RNA-PolII and chromatin histone markers (H3K27ac, H3K4me1, H3K4Me3) were performed to functionally characterize the RUNX2-bound elements. The ROSE algorithm was interrogated to identify Enhancers (ENHs) and Super-Enhancers (SENHs). The FIMO algorithm was exploited for TF motif search analysis. A new bioinformatic approach was developed to integrate our genomic data and TCGA clinical data into a hierarchical network modeling the RUNX2 regulatory activity in TC.

Results

ChIP-seq analysis on TC cells showed an equal distribution of RUNX2 in proximal and distal regulatory regions. Most of the RUNX2-bound regions appeared enriched in H3K27ac and RNA-PolII, showing features of transcriptionally active genomic elements. To characterize the RUNX2-bound distal regulatory elements, H3K27ac enriched regions containing RUNX2 peaks were used to interrogate the ROSE algorithm. ENHs and SENHs were assigned to the nearest TSS to predict target genes, subsequently validated using RNA-seq data. We focused on SENHs as key regulators of oncogenic gene expression programs. 525 and 492 RUNX2-associated SENHs were found in TPC1 and MDA-T41 respectively. FIMO motif search analysis of RUNX2-SENHs identified 137 RUNX2 putative cooperators in TPC1. Members of JUN, FOS, and TEAD families, previously characterized as RUNX2 regulators, were included in this list and were also identified as RUNX2 direct targets. This finding suggests the existence of regulatory circuits in which oncogenic TFs regulate each other and cooperate on SENHs in TC. All the obtained data were integrated with the TCGA clinical and transcriptomic profile in a computational hierarchical model that recapitulates the TC dependency on RUNX2. 12 discrete disease modules of highly correlated RUNX2 targets were identified. 57 RUNX2-cooperators emerged as key bottleneck regulators of the RUNX2-network. GO enrichment and correlation analyses underlined RUNX2-dependent biological functions associated with TC metastasis.

RAIN ChIRP-seq analysis in TPC1 identified 11,928 genomic sites bound by this lncRNA, with a preferential enrichment for intronic, distal intergenic, and promoter regions. RAIN genomic peaks were assigned to the nearest TSSs, and the obtained list of genes was crossed with the RNA-seq data. 1190 RAIN direct targets were identified by this analysis. Of these, 813 genes were found to be RAIN-specific targets. GO analysis of these genes showed enrichment of processes related to Transcription Regulation. 21 TFs, known for their role in cell differentiation and cancer, were among the RAIN exclusive target genes cross-validated in MDA-T41. 377 genes involved in angiogenesis, transcriptional regulation, and cell motility were found to be commonly and directly regulated by RUNX2 and RAIN, suggesting their functional interaction.

Conclusions

Our data

- map the genomic landscape of RUNX2 in TC, laying the basis for the reconstruction of the complex regulatory network established by this TF in promoting cancer progression
- provide an integrative computational model of the RUNX2 regulatory activity in TC, highlighting the role of this TF on SENHs
- confirm the role of RAIN as chromatin-associated lncRNA with autonomous functions in regulating gene expression
- show the function of RAIN as a regulator of cancer TFs expression

- suggest functional cooperation between RAIN and RUNX2 in transcriptional regulation, introducing additional levels of coding and non-coding functional interplay.

Monica Lispi

CEM Curriculum: Translational Medicine
Tutor: Prof. Manuela Simoni

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

Background

To identify the most efficient treatment for male-factor infertility, it is mandatory to understand thoroughly their mechanism on target cells/organs. Then, the efficacy must be confirmed by clinical trials. Limitations of clinical trials to address efficacy include the lack of objectively assessable clinical endpoint/outcomes. These are the aims of several exploratory studies that should be addressed by a collaboration of research centers and pharma companies, which are interested in building in addressing unmet patients' needs, namely male infertility.
Besides clinical studies *in-vitro* studies can address further topics. One of them is the assessment of Mode of Action (MoA) of molecules used as active pharmaceutical ingredients in drug preparations: hormone choriogonadotropin (hCG), luteinizing (LH) and follicle-stimulating hormones (FSH). LH and hCG bind their common receptor (LHCGR), inducing the synthesis of the androgen dihydrotestosterone (DHT). This event follows the production of different steroids e.g., progesterone and testosterone. However, a "backdoor" pathway has been recently discovered and displays the production of neuro-steroids, such as androsterone, as precursors for testosterone independent DHT synthesis. Finally, little is known about the efficacy of FSH alone or in association with LH in male infertility.

Objectives

Based on available evidence, different scientific questions should be still explored to support efficient strategy for male infertility treatment. Assess MoA of different gonadotropins on target cells; validation of objective clinical endpoint to assess clinical efficacy of gonadotropins; evidence of clinical efficacy of available treatments for different patients' profile, clarification about main male infertility causes and treatment options. This project is aimed at:

- Investigate LH and hCG differential steroidogenesis pathway on target Leydig cells.
- Assess pharmacodynamics and safety of r-hLH (Investigational treatment) and u-hCG (standard treatment) in hypogonadotropic hypogonadal men. The general clinical question is whether LH supplementation could be more efficient than hCG in those cases in which LH-activity is required, such as men with HH or male infertility
- Evaluate the biological/clinical correlation between sDF (DNA Fragmentation), hormonal profile and semen parameters
- Clarify diagnosis of patients' population that could benefit from Gonadotropins' treatment beyond the concept of congenital hypogonadotropic hypogonadism and generate business cases suitable for Pharma Company assessment

Methods

LH and hCG differential steroidogenesis pathway on target Leydig cells

To investigate LH- and hCG-driven, possibly different steroidogenesis pathways, murine Leydig tumor cell lines (mLTC1) and primary human Leydig cells are being used. Cells are treated for 24 h with equipotent

concentrations of hCG and LH (100 and 50 pM, respectively). mLTC1 cells are available in house, while human primary Leydig cells are a relatively rare material to be purchased. An in-house developed panel of steroids will be assessed by liquid chromatography tandem mass spectrometry (LC-MS/MS). Five experimental replicates will be performed and results compared by Kruskal Wallis test followed by Dunne's post-test ($p<0.05$).

Pharmacodynamics and safety of r-hLH (Investigational treatment) and u-hCG (standard treatment) in hypogonadotropic hypogonadal men

To assess pharmacodynamics and safety of r-hLH (Investigational treatment) and u-hCG (standard treatment) in acquired hypogonadotropic hypogonadal men, a multicenter longitudinal, interventional, randomized, open-label, phase II, clinical trial has been designed, approved (EudraCT 2019-004677-12) and initiated. The statistical hypothesis is non-inferiority of the highest LH dose employed compared to hCG. Primary endpoint: serum testosterone levels evaluated by LC-MS/MS. Secondary endpoints: Safety and tolerability will be evaluated. A total of 32 of subjects will be enrolled and randomized (1: 1) following a list of permuted block randomization, either to the study group, treated with r-hLH (Luveris® daily subcutaneous administration) or to the control group, treated with u-hCG (Gonasi HP® two intramuscular administrations per week). In both cases, increasing dosages will be administered at two-week intervals to obtain a dose-response curve of stimulated serum testosterone levels. Patients will be followed up for a further 4 weeks after stopping treatment. During the study, patients will be evaluated twice a week during the treatment phase and every two weeks in the follow-up phase.

Biological and clinical correlation between sDF, hormonal profile and semen parameters

To evaluate the biological/clinical correlation between sDF, hormonal profile and semen parameters a retrospective post-hoc re-analysis is performed on raw data of RCTs in which idiopathic infertile men were treated with FSH and both testosterone serum levels and sDF were reported among primary and/or secondary endpoints. Additional data regarding couple infertility history, age, anthropometric variables, FSH treatment scheme and semen variables were included in a single dataset

Preliminary Results

Preliminary results are related to accomplishment achieved on the first three activities part of this Industrial

PhD up Q1 2022.

LH and hCG differential steroidogenesis pathway on target Leydig cells

Levels of 16OH-progesterone, 11-deoxycortisol, androstenedione, 11-deoxycorticosterone, testosterone, 17OH-progesterone, androstanedione, epitestosterone, dihydrotestosterone, progesterone, androsterone and 17OH-allopregnanolone were within the measurement range. hCG induced higher 17OH-progesterone, androstenedione, testosterone and DHT levels than LH, which resulted to be more effective on progesterone production ($p<0.05$). Interestingly, hCG induced effectively the production of backdoor steroids, such as 17-allopregnanolone, androsterone and androstanediol, while LH reduced the production of some of them (i.e. androsterone and androstanediol) below the basal threshold ($p<0.05$). No differences were found between LH vs hCG-induced production of 17OH-dihydroprogesterone. Our LC-MS/MS method included a broad number of androgens, precursors and metabolites. Most of them were effectively measured in mLTC1 supernatants, however, Δ5 and some 5α and 5α,3α steroids were below sensitivity threshold. We provided a powerful tool to simultaneously characterize Leydig canonical and backdoor pathways.
After a long time of unavailability, human primary Leydig cells from anonymous donors were found and purchased by a Company (Innoprot, Bizkaia, Spain; ref. P10795). These cells will be thawed, cultured and treated with LH/hCG before steroid measurement.
All experiments are still ongoing.

Pharmacodynamics and safety of r-hLH (Investigational treatment) and u-hCG (standard treatment) in hypogonadotropic hypogonadal men

The longitudinal interventional, randomized, open-label clinical phase II trial has been recently started with the aim to evaluate the pharmacodynamics of recombinant luteinising hormone (rLH) therapy in men with hypogonadotropic hypogonadism acquired (HH). The pharmacodynamic profile of rLH is compared with the treatment applied in usual clinical practice, i.e. with urinary hCG. Pharmacodynamics is assessed by measuring testosterone levels in response to treatment with increasing doses of LH, compared with response to increasing doses of hCG. Four patients out of 32 HH men planned have been enrolled and one has already ended the study period.

Biological and clinical correlation between sDF, hormonal profile and semen parameters

In relation to the Individual Patients Data analysis, three RCTs were included accounting for 148 patients (median age 37, 25-52 years). After three months of FSH administration, a significant increase was observed in FSH levels ($p<0.001$), inhibin B ($p=0.012$), sperm concentration ($p=0.003$), total sperm number ($p=0.021$), progressive motility ($p<0.001$) and normal sperm morphology ($p<0.001$). Moreover, an overall sDF index reduction was confirmed after treatment ($p=0.002$). SDF resulted significantly inversely related to sperm concentration both at baseline and after FSH treatment ($p<0.001$ and $p=0.001$, respectively). Interestingly, the sDF index after treatment showed a significant inverse correlation with testosterone serum levels ($p=0.002$). Multivariate stepwise linear regression analyses using sDF index as dependent variable identified testosterone as a predictor for sDF index change ($p=0.005$). Similarly, logistic regression analysis highlighted testosterone and SHBG levels as markers of treatment responders ($p=0.043$ and $p=0.005$, respectively).

Preliminary Conclusion

In the mLTC1 cell line, LH- and hCG-specific steroid synthesis was found, suggesting that the choice treatment of male infertility operated with hCG may not replace qualitatively that one mediated by the natural pituitary hormone.

Combining raw data of published RCTs investigating FSH administration to idiopathic infertile men, a significant amelioration of conventional semen parameters together with a reduction in sDF were confirmed. Intriguingly, a potential correlation between testosterone serum levels and sDF was highlighted for the first time, opening a completely unexplored way in the identification of potential early markers of FSH therapy response in male idiopathic infertility

Annamaria Paolini

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STUDY OF ADAPTIVE IMMUNE RESPONSE TO COVID-19 INFECTION AND VACCINATION

Background

After more than two years from the first reported cases of COVID-19 infection, SARS-CoV-2 is still circulating worldwide. The introduction of vaccines has been crucial for limiting the effects of SARS-CoV-2 infection, decreasing hospitalizations, severe symptoms and deaths. The adaptive immune system, mainly represented by T and B cells, is the main actor involved in controlling most viral infections. The cooperation of B and T cells is fundamental to develop a pool of antigen-specific memory T cells able to provide long term immunity against secondary infection. Phenotype and functionality of B and T cells following COVID-19 infection or vaccination can be different impacting on the effectiveness of the protection against reinfections and severity of symptoms. In addition, combinations of different vaccines can also differently shape the antigen-specific response. Thus, shedding light on the adaptive immune response induced either by COVID-19 infection or by vaccinations could pave the way to improve current vaccination protocols.

Objectives

The aim of the project is to decipher the phenotypic and functional aspects of the adaptive immune response in both COVID-19 recovered patients and vaccinated individuals. The principal objectives of this project are:

- to identify the characteristics of antigen-specific B and T cells in both COVID-19 recovered patients and vaccinated individuals;
- to find unique traits in the phenotype and functionality of antigen-specific B and T cells, typical of the subject populations under investigation.

Methods

Three categories of donors were investigated so far: (i) patients who recovered from COVID-19 enrolled during follow-up visits at the Infectious Diseases Clinics of the Azienda Ospedaliera Universitaria di Modena (REC); (ii) vaccinated donors that received three different doses of vaccine (first dose: ChAdOx1; second dose: BNT162b2; third dose: mRNA-1273; AZPM); (iii) vaccinated donors that received two different vaccines (first dose: BNT162b2; second dose: BNT162b2; third dose: mRNA-1273; PFZM). Antigen-specific CD4+ and CD8+ T cells were identified as those cells expressing CD137and CD69 after 18 hours' stimulation with Wuhan SARS-CoV-2 Spike glycoprotein. In these populations, the expression of these molecules was measured: CXCR5, CCR6, CXCR3, CD45, CD3, CD4, CD8, CD27, CD57, CD279 (PD1), CD28, CCR7, CD45RA, CD69, CD137 and CD95.

The capability to produce different interleukins (IL) simultaneously (polyfunctionality) was assessed by analyzing the percentage of cells producing IL -17, -2, Tumor Necrosis Factor (TNF), interferon (IFN) -γ, and granzyme B (GRZB).

Results

The percentage of antigen-specific CD4+ T cells was similar among the groups. However, a different CD4+ T cells distribution in central memory (CM) and transitional memory (TM) compartments was noted. In particular, REC displayed a lower percentage of CM T helper (Th) 1 and Th2 compared to PFZM. Conversely, REC presented a higher percentage of circulating T follicular helper (cTfh) CD4+ T cells compared to both the categories of vaccinated donors. Finally, REC exhibited a lower percentage of TM Th1 compared to PFZM. Regarding CD4+ T cells functionality, REC displayed a higher percentage of CD4+ T cells producing TNF, IL-2 and IL-17 compared to AZPM. CD4+ T cells from PFZM, in addition, exhibited a higher percentage of CD4+ T cells producing IL-2 compared to AZPM. Polyfunctionality of REC was different from both AZPM and PFZM, which exhibited similar polyfunctional profiles. In particular, REC displayed a higher percentage of CD4+ T cells simultaneously producing IL-2 and TNF compared to AZPM. REC showed a higher percentage of CD4+ T cells producing only TNF or only IL-2 compared to AZPM. On the other hand, both vaccinated groups exhibited a higher percentage of CD4+ T cells producing simultaneously CD107, interferon-γ (IFN-γ), IL-2 and TNF compared to REC. The percentage of antigen-specific CD8+ T cells was similar among the groups. However, different distribution of effector memory (EM) and terminally differentiated effector memory (EMRA) was noticed. In particular, REC displayed a lower percentage of EM Th1 CD57+ PD-1+ compared to both AZPM and PFZM. Regarding EMRA, AZPM presented a higher percentage of EMRA Th1 CD57+ PD-1− compared to both REC and PFZM as well as EMRA Th1 CD57+ PD-1+ compared to REC. No differences were observed regarding CD8+ T cells total production of cytokines nor overall polyfunctionality. However, among polyfunctional subsets of CD8+ T cells, REC exhibited a lower percentage of cells simultaneously producing CD107, IFN-γ and TNF compared to AZPM.

Finally, REC displayed a lower percentage of antigen-specific B cells compared to AZPM and PFZM. Within antigen-specific B cells, REC presented a higher percentage of naïve (N) B cells and lower percentages of memory B cells (MBC) IgG+ CD71+ compared to AZPM and PFZM. However, REC displayed a higher percentage of MBC IgA+ compared to both AZPM and PFZM.

Conclusions

COVID-19 infection and vaccination have a different impact on the immune system, not in terms of magnitude regarding the percentage of antigen-specific memory B and T cells developed, but in terms of phenotype and function, meaning that the creation of the memory pool could follow a different route of

differentiation. Further studies are needed to understand if these differences were due to metabolic characteristics of the cells of interest, and/or to different gene expression profiles.

Pierluigi Di Vinci

CEM Curriculum: Translational Medicine
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THE ROLE OF ENDOGENOUS INOSITOL IN PLACENTA VILLOUS TISSUE IN OBESE PREGNANT WOMEN

Background

Metabolic abnormalities such as gestational diabetes, glucose intolerance, and obesity during pregnancy will affect not only the long-term maternal health, but can expose infants to an increased risk of developing metabolic disorders later in life. The placenta, being the interface between maternal and fetal circulation and overseeing multiple functions to maintain fetal well-being, plays an active role in fetal developmental programming. The alteration of its function by environmental challenges can impact the offspring's health lifelong. In particular, maternal obesity induces changes in glucose metabolism in the placenta. Precisely, trophoblast cells in placental villous tissue are the main players in mediating the effects of nutritional challenges between the mother and the fetus. New therapeutic strategies need to be developed to control NCDs related to maternal obesity. Previously in our lab, we highlighted the benefits of Inositol in such a context. We demonstrated beneficial effects of a diet supplemented with INOs during pregnancy to control glucose homeostasis, improving insulin sensitivity. INOs intake ameliorate obesity related conditions in pregnancy and have a beneficial role for fetus health. Inositols (INOs) are polyalcoholic sugar molecules. Their amount in the body depends on diet intake and endogenously synthesis in the kidney. Inositol are known to be critical regulators of glycogen storage, insulin-glucose and calcium pathways. INOs can enter in the cells via sodium-myoinositol co-trasporter 1 and 2 (SMIT1 and SMIT2), it is synthetized by Inositol-3-Phosphate Synthase 1 (ISYNA1) and Inositol Monophosphatase 1 (IMPA1). INOs acts, also, as a second messenger interacting with Inositol 3 phosphate receptor 1 (ITPR1) which mediates efflux of calcium ions in the cytoplasm modulating cellular functions and gene expression. However, in the placenta, their beneficial effects and their regulation are not clearly understood. Interestingly, in the developmental origins of health and diseases, epigenetics is being described as a bridge between nutrition and health. Thus, alterations in inositol pathway and their epigenetic regulations in the trophoblast cells represent a potential underlying mechanism in the obesity mediated offspring phenotype, and a potential therapeutic target.

Objectives

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

epigenetic mechanisms behind gene and protein alterations. We aim to investigate biomarkers in cord blood related to INOs pathway and metabolic alterations. Knowing the intracellular maternal INOs activity in placenta might help up to develop new non-pharmacological therapy to restore metabolic profile in pregnant obese women.

Methods

The Ethics Committee approved the project in order to collect placentas and cord blood from controls and obese women. Patients included in the study are single pregnancies, age over 18yo and the obesity is defined as BMI ≥ 30 kg/m^2. Trophoblast cells are isolated from placentas based on enzyme digestion and FACS purification. To define trophoblast cell population, MoAb staining procedure for FACS analysis is performed. Trophoblast cells are selected by a positive staining for Cytokeratin7. Total mRNA is extracted with column based methods and quantity and quality are evaluated by spectrophotometer measurements. SMIT1, SMIT2, IMPA1, ISYNA1 and ITPR1 expression in the placenta tissue and in isolated trophoblasts are evaluated at the mRNA and protein level by RT-qPCTR and Western Blot approaches. Epigenetic analysis is performed by the analysis of DNA methylation levels through MEDIP approach; histone modification (Acetylation, methylation) by ChiP experiments, microRNAs analyses through RT-qPCR. Functional assays are performed through the silencing of genes with siRNA approach and stimulating cells with INOs molecules. Data are analyzed with Graphpad v9 and non-parametric t-test was performed for statistical analysis.

Results

For now, we recruited and isolated trophoblast cells from 3 CTRLs and 5 Obese women. In these cells, we did not detect any significant modification in the mRNA levels of SMIT1, SMIT2, IMPA1 and ISYNA1 between obese and control women. In contrast, we found a significant decrease in ITPR1 mRNA levels in the trophoblast cells from obese women compared to lean ones.

Conclusions

With our preliminary data, we could highlight an alteration in the inositol pathway in the trophoblast cells induced by maternal obesity, as revealed by the significant decrease in ITPR1 expression. As found in literature, ITPR1 locus SNPs are correlated with intrauterine growth restriction affecting fetus growth and health, increasing aging stage of placenta. In our study, we described an original finding not described before in other studies. ITPR1 is a receptor located on endoplasmic reticulum (ER) and it is involved in calcium ions releasing from the ER lumen to the cytoplasm. Ca ions in cytoplasm modulate gene expression and cell functions as senescence, apoptosis, and modifications in mitochondrial metabolism. The next approach is to delineate the role of epigenetic regulations in the down-regulation of ITPR1 induced by maternal obesity. We aim to confirm these findings in the next patients that are being recruited. Understanding the intracellular

maternal INOs activity in placenta will help up to develop new non-pharmacological therapy to restore metabolic profile in pregnant obese women, ameliorate fetus health.

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

Background

Combined Pulmonary Fibrosis and Emphysema (CPFE) is a smoking-related interstitial lung disease (ILD) characterized by the coexistence of both defects and accounts for the 35% of total patients with Idiopathic Pulmonary Fibrosis (IPF). The frequent association with pulmonary hypertension negatively affects the natural course of CPFE, whose prognosis is even worse as compared with IPF without emphysema. Emphysema and fibrosis cause different physiologic effects. While emphysema reduces lung elastance and negatively affects maximal expiratory flows causing an obstructive defect, fibrosis reduces lung compliance with a restrictive pattern at the pulmonary function tests (PFTs). Although CPFE patients present a respiratory functional profile with relatively spared lung volumes and flows, given the balance between lung restriction and obstruction, the diffusing capacity of lung for carbon monoxide (DLCO) results dramatically reduced. Furthermore, CPFE patients suffer from symptoms associated with both conditions and, due to the lack of any approved therapy, present an extremely impaired quality of life. As such, inhaled long-acting beta-agonist or anti-muscarinic (LABA or LAMA) bronchodilator therapy for emphysema and chronic obstructive pulmonary disease has proved so far efficacy in reducing symptoms and improving quality of life. However, data are lacking on the effect of these medications in patients with CPFE, who mostly do not show obstructive defects.

Objectives

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

Methods

As a preliminary investigation we did not perform an *a priori* sample size calculation.
30 patients with established diagnosis of CPFE referred to our Center for Rare Lung Disease of the University Hospital of Modena have been consecutively enrolled. Patients showing obstructive defects at the PFTs and aged < 18 years were excluded.

After enrollment, patients received inhaled bronchodilator therapy over 3 consecutive months. Assessment of symptoms and quality of life by the Saint George Respiratory Questionnaire (SGRQ), modified Medical Research Council (mMRC) and COPD Assessment Test (CAT), PFTs (by full spirometry), and functional performance by the 6-minute walking test (6MWD) took place before and 3-month after bronchodilation therapy.

Change of SGRQ, mMRC and CAT over time were considered as the primary study aim, whereas changes in either respiratory function and functional performance were explored as secondary outcomes. Student t test and Fisher test or Chi square test (as appropriate) was used for analysis. Statistical significance was set at 0.05.

Results

30 patients were enrolled consecutively. They were predominantly male (96%). All patients have a history of smoking (median = 30 pack/years). 18% have respiratory failure requiring long-term home oxygen therapy. The main comorbidities found were cardiovascular diseases and gastroesophageal reflux. The most frequent ILD was IPF (79%) followed by indeterminate ILD (14%). Most of the patients were on antifibrotic therapy (53%) with a prevalence in the use of Nintedanib (73%). The most used bronchodilator was Umeclidinium (61%), followed by Tiotropium (14%) and Glycopyrronium (11%).

In this pilot study a significant improvement in quality of life (i.e. reduction of respiratory symptoms such as shortness of breath, cough and sputum production) has been revealed.

Patients showed a significant reduction of the average score in each questionnaire (SGRQ -10.9, mMRC -0.82 and CAT -4.9, $p < 0.0001$). At PFTs Forced Vital Capacity (FVC) and Forced Expiratory Volume in the first second (FEV1) did not show a significant variation (respectively $p=0.7$ and $p=0.8$). While a significance reduction of the Residual Volume (RV) was revealed ($p=0.048$).

Finally, patients showed an increase in distance at 6MWT, although it did not reach statistical significance ($p=0.07$).

Conclusions

Up to now in the few studies, in which bronchodilator therapy was used in CPFE patients, the rationale is based on the presence of emphysema and bronchial obstruction. This pilot study revealed the efficacy of bronchodilator therapy in improving respiratory symptoms and quality of life after 3 months of therapy in a population of patients affected by CPFE with no evidence of bronchial obstruction at the PFTs.

From a functional point of view, a reduction of residual volume was observed; this value is the only independent factor associated with a clinical improvement after starting the therapy. A limit of this study could be the absence of a group of control. This study can be the basis for multicenter studies with a larger population.

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CHARACTERIZATION OF HUMAN MILK GLYCOSAMINOGLYCANS AND DETERMINATION OF THEIR ANTI-VIRUS ACTIVITY AGAINST PEDIATRIC VIRAL INFECTION

Background

Human milk (HM) is known to have many properties and benefits, which are mediated by its multiple nutritional, trophic, and immunological components. The World Health Organization actively promotes breastfeeding as the best source of nourishment and protection against infection for neonates. Although its recognized properties, breastfeeding can cause the mother-to-child transmission of some viral infections. Zika virus (ZIKV) and Usutu virus (USUV) are two emerging flaviviruses transmitted by mosquitoes and found in breast milk. Symptomatic ZIKV and UTUV infections can cause conditions ranging from a mild fever to a more severe neurological involvement.

Breast milk composition is complex, and it depends on individual factors and on the stage of lactation. Glycosaminoglycans (GAGs) are important constituents of human milk. GAGs are a family of complex carbohydrates composed of a variable number of repeating disaccharide units. They are grouped according to their chemical structure and composition: hyaluronic acid (HA), chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS) and heparin (Hep), and keratan sulfate (KS). GAGs are involved in regulating many cellular and physiological processes. HM-GAGs have shown antiviral activity, acting as soluble receptors inhibiting the attachment to the intestinal mucosa.

Objectives

In this study, we analyzed the structural characteristics of glycosaminoglycans in human milk and tested their anti-viral activity to better understand HM-GAGs contribution to human milk anti-viral potential.

First, we focused on analyzing and identifying the GAGs composition of human milk through a qualitative and quantitative characterization of purified samples

Then we investigated the activity of purified HM-GAGs against ZIKV and USUV.

Methods

HM-GAGs were isolated from a pool of human mature milk provided by the University of Turin, according to a standardized protocol. Samples were firstly defatted and then treated with pancreatin. Samples were applied to a column packed with an anion-exchange resin and eluted using low-pressure liquid chromatography. Fractions positive to uronic acid assay were collected. The pooled fractions were precipitated, dried, and lyophilized for the virus inhibition assay performed by the University of Turin. The

HM-GAGs composition was evaluated by electrophoresis on acetate of cellulose. The purity of the extract was evaluated by measuring the protein content (Folin-Ciocalteu test) and the GAGs content (uronic acid assay). For the structural characterization, purified GAGs were digested with enzymes to obtain disaccharides and then tagged with a fluorescent molecule. Tagged disaccharides will be separated and quantified by capillary electrophoresis equipped with a Laser-Induced (LIF) detector.
The antiviral activity of HM-GAGs was evaluated by means of plaque reduction assay (anti-ZIKV activity) and focus reductio assay (anti-USUV assay). The EC_{50} was determined by comparing the treated with the untreated cells.

Results

Quantitative evaluation of the total GAGs in human milk samples confirms previous results. Human milk tested in this study is mainly composed of CS/DS and HS/Hep. Data obtained from the structural characterization analysis show that HM-GAGs are ~55% CS and 1-2% DS, ~40% HS/low sulfated Hep (or fast-moving Hep), ~2% high sulfated Hep (or slow-moving Hep), and trace (1-2%) of HA. Moreover, HM-CS charge density is ~0.35, lower than other known CS. The purified GAGs are tested to have a purity greater than 98%. HM-GAGs are confirmed to be active against ZIKV and USUV. The calculated EC_{50} values are 5.8 mg/ml for ZIKV and 3.3 mg/ml for USUV.

Conclusions

Knowledge regarding the content, structure, and function of breast milk glycosaminoglycans (GAGs) is still scanty. In this project, we investigated both the composition of HM-GAGs and their anti-viral activity. The quantitative and qualitative characterization of HM-GAGs we performed confirms previous data. GAGs fraction in human milk is very complex and is characterized by the presence of an under-sulfated CS with a low charge density value. The results of this study can be useful in expanding knowledge on human milk composition and this information can be used for the improvement of infant formulas.
Acting as soluble receptors, GAGs could contribute to human milk anti-viral activity by inhibiting the binding of both flaviviruses to cells (potentially acting as decoy receptors). According to our results, the anti-viral action of HM-GAGs is not as strong as expected but the EC_{50} values fall within the range of GAGs concentration in preterm and term colostrum. These results indicate that GAGs contribute to the overall anti-viral activity of breast milk, together with other factors. Furthermore, our results highlight the importance of cellular GAGs for the attachment to cells of ZIKV and USUV.
In conclusion, according to WHO recommendations our data support breastfeeding during ZIKV infection and it can also contribute to producing new guidelines for a possible USUV epidemic.

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

Background

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

Objectives

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

In detail, the aims of our project are:

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

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

Methods

The EORP-AF Long-Term General Registry is a prospective, observational, large-scale multicentre registry on AF patients in current cardiology practice held by the ESC and endorsed by the European Heart Rhythm Association (EHRA). The registry enrolled 11,096 AF consecutive patients in 250 centres from 27 participating ESC Countries. Both in- and outpatients were consecutively enrolled when presenting with AF as a primary or secondary diagnosis from October 2013 to September 2016. All patients were ≥ 18 years old and provided a written informed consent form. The qualifying AF event had to be recorded by a 12-lead ECG, 24h ECG Holter, or other electrocardiographic documentation within the 12 months before enrolment. All follow-up was performed at 1 and 2 years after enrolment. All the following incident major adverse clinical events were recorded: (i) all cause death; (ii) any haemorrhagic events (i.e. intracranial bleeding, major bleeding or clinically relevant non-major bleeding); (iii) any thromboembolism (TE) (including stroke, transient ischaemic attack, and any peripheral embolism); (iv) any ACS; (v) CV-death and (vi) any myocardial revascularization (including percutaneous coronary intervention and coronary artery bypass grafting). All data about hospital admissions (any admission, AF-related, CV-related and non CV-related) were also recorded. All-cause death, Major Adverse Cardiovascular Events (MACE, as the composite of any TE/ACS/CV death), any hemorrhagic events will be the primary endpoints of our analyses. All continuous variables were reported as median and interquartile range (IQR) or mean and standard deviation (SD). Among-group comparisons were made using non-parametric tests, Mann-Whitney U or Kruskal–Wallis test where appropriate. Categorical variables were reported as counts and percentages. Among-group comparisons were made using a $\chi 2$ test or Fisher's exact test (if any expected cell count was less than five). Plots of Kaplan–Meier curves for time to outcomes according to different subgroups were performed. Survival distributions were compared using the log-rank test. Adjusted Cox regression analyses were used to evaluate the association between the variable/group of interest and the risk of the outcomes.

Results

A total 11 096 patients were enrolled in the Registry (40.7% female; mean age 69±11years). We performed different post-hoc analyses:

1. Cardiac troponins and adverse outcomes in European patients with atrial fibrillation.

We aimed to assess the factors associated with troponin testing in routine practice and evaluate the association with outcomes.

Main results: Among 10 445 AF patients (median age 71 years, 40.3% females) cardiac troponins (cTn) were tested in 2834 (27.1%). cTn was elevated in 904/2834 (31.9%) and in-range in 1930/2834 (68.1%) patients. Female sex, in-hospital enrollment, first-detected AF, cardiovascular risk factors, history of coronary artery disease, and atypical AF symptoms were independently associated with cTn testing. Elevated cTn were independently associated with a higher risk for MACE (Model 1, hazard ratio [HR] 1.74, 95% confidence interval [CI] 1.40-2.16, Model 2, HR 1.62, 95% CI 1.28-2.05; Model 3 HR 1.76, 95% CI 1.37-2.26) and all-cause death (Model 1, HR 1.45, 95% CI 1.21-1.74; Model 2, HR 1.36, 95% CI 1.12-1.66; Model 3, HR 1.38, 95% CI 1.12-1.71).

2. **Impact of clinical phenotypes on management and outcomes in European atrial fibrillation patients**.

We aimed to describe AF patients' clinical phenotypes and analyze the differential clinical course using hierarchical cluster analysis.

Main results: A total of 9363 were available for this analysis. We identified three clusters: Cluster 1 (n = 3634; 38.8%) characterized by older patients and prevalent non-cardiac comorbidities; Cluster 2 (n = 2774; 29.6%) characterized by younger patients with low prevalence of comorbidities; Cluster 3 (n=2955;31.6%) characterized by patients' prevalent cardiovascular risk factors/comorbidities. Over a mean follow-up of 22.5 months, Cluster 3 had the highest rate of cardiovascular events, all-cause death, and the composite outcome (combining the previous two) compared to Cluster 1 and Cluster 2 (all p <0.001). The adjusted Cox regression analysis showed that compared to Cluster 2, Cluster 3 (HR 2.87, 95% CI 2.27-3.62; HR 3.42, 95%CI 2.72-4.31; HR 2.79, 95%CI 2.32-3.35), and Cluster 1 (HR 1.88, 95%CI 1.48-2.38; HR 2.50, 95%CI 1.98-3.15; HR 2.09, 95%CI 1.74-2.51) reported a higher risk for the three outcomes respectively.

3. **Real-world applicability and impact of early rhythm control for European patients with atrial fibrillation**.

The aim was to evaluate the real-world applicability and impact of an early rhythm control strategy in patients with AF.

Main results: A total of 3774 (34.0%) were analyzed. Early rhythm control was associated with better quality of life, but with greater use of health-care resources. Over a mean (SD) follow-up of 675.4 (181.3) days, a total of 532 (14.1%) primary outcome events (composite of CV death/any stroke/worsening heart failure/acute coronary syndrome) were reported with an overall incidence of 8.9 per 100 patient-years. Death occurred in 321 (8.5%) and 380 (10.1%) experienced MACEs. The primary outcome occurred less often in early rhythm control patients than in those with no rhythm control (13.6% vs. 18.5%, p < 0.001). In the

multivariate adjusted Cox regression model, no significant difference was found between no rhythm control and early rhythm control, for the primary outcome (Model 1 HR 0.83, 95% CI 0.68-1.01; Model 2 HR 0.84, 95% CI 0.66-1.19). No difference in the primary outcome between early rhythm control and 'no rhythm control patients' adherent to Atrial fibrillation Better Care (ABC) pathway' was evident (p = 0.753).

4. **Comparison of HAS-BLED and ORBIT Bleeding Risk Scores in AF Patients treated with NOACs**.

We aimed to compare different bleeding scores (the HAS-BLED and ORBIT scores) in AF patients treated with NOACs.

Main results: A total of 3018 patients (median age 70; 39.6% females) were included: median [IQR] HAS-BLED and ORBIT scores were 1 [1-2] and 1 [0-2], respectively; 356 (11.8%) patients were at high risk for major bleeding using HAS-BLED (≥3) and 123 (4.1%) using ORBIT (≥4). Overall, 60 (2.0%) major bleeding events were recorded, with an incidence of 1.1 per 100 patient-years. Both HAS-BLED and ORBIT were associated with outcome, modestly predicting major bleedings (AUC 0.653, 95% CI 0.593-0.714 and AUC 0.601, 95% CI 0.526-0.677, respectively). Calibration plots showed that both scores were poorly calibrated, particularly the ORBIT score, which showed consistent poorer calibration. Time-dependent reclassification analysis showed a trend towards incorrect lower risk reclassification using ORBIT compared to HAS-BLED.

5. **Impact of malignancy on outcomes in European patients with atrial fibrillation.**

We aimed to evaluate the outcomes of patients with active or prior malignancy in a contemporary cohort of European AF patients.

Main results: A total of 10 383 patients were included. Patients enrolled were categorized into 3 categories: No Malignancy (NoMal), Prior Malignancy (PriorMal) and Active Malignancy (ActiveMal). Of these, 9597 (92.4%) were NoMal patients, 577 (5.6%) PriorMal and 209 (2%) ActiveMal. Lack of any antithrombotic treatment was more prevalent in ActiveMal patients (12.4%) as compared to other groups (5.0% vs 6.3% for PriorMal and NoMal, p <0.001). After a median follow-up of 730 days, there were 982 (9.5%) deaths and 950 (9.7%) MACE events. ActiveMal was independently associated with a higher risk for all-cause death (HR 2.90, 95% CI 2.23-3.76) and MACE (HR 1.54, 95% CI 1.03-2.31), as well as any haemorrhagic events and major bleeding (OR 2.42, 95% CI 1.49-3.91 and OR 4.18, 95% CI 2.49-7.01, respectively). Use of oral anticoagulants was not significantly associated with a higher risk for all-cause death or bleeding in ActiveMal patients.

Conclusions

Our analyses highlighted the complexity of pathophysiology, epidemiology, and clinical management of European AF patients in daily clinical practice. Our results reinforce the importance of comprehensive management in the care of AF patients who may be burdened by poor prognosis and increased risk of

outcomes if not properly treated. These analyses, derived from this large European registry will help to adapt or strengthen European practice guidelines recommendations in “real-world” AF patients identifying where care is not guideline adherent and where treatment gaps exist.

Giacomo Santandrea

CEM Curriculum: Translational Medicine
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PROGNOSTIC AND PREDICTIVE MARKERS IN MEDULLARY THYROID CARCINOMA: A MOLECULAR AND CLINICO-PATHOLOGICAL STUDY

Background

Medullary thyroid carcinomas (MTC) account for 1-2% of all thyroid malignant tumors and carry a rather indolent clinical behavior. Derived from parafollicular C-cells, these tumors may be either sporadic or part of genetic hereditary diseases, namely, Multiple Endocrine Neoplasia (MEN) type 2 and Familial Medullary Thyroid Carcinoma (FMTC) syndrome, all characterized by a distinctive molecular alteration of RET gene. Several histological and immunohistochemical parameters, such as cytology (spindle cell vs epithelioid cell), nuclear pleomorphism, amyloid deposits, RET and RAS mutation, have been investigated as potential prognostic parameters, but none resulted to be significant in a multivariate analysis. A recent paper from Ghossein et al. (2020) found tumor necrosis and mitotic activity to be independent predictors of tumor disease specific survival. However, no study has unified molecular and histo-pathological data in light of new technologies such as Next-Generation sequencing.

Objectives

The aim of the present study is to search for prognostic and predictive bio-molecular and clinico-pathological factors in MTC.

Methods

The databases of the Pathology and Endocrinology Units were retrospectively searched to identify all patients who developed a MTC in the last 30 years. Complete clinical, surgical data and follow-up were collected. All slides and blocks were retrieved from the Pathology archive. Cases were reviewed by two independent pathologists according to the last World Health Organization tumor classification, and according to the most recent literature (Ghossein et al. 2020). Tumors were staged according to the American Joint Committee on Cancer (AJCC) classification system. The most representative block was then chosen and targeted-NGS molecular analysis was performed. The results were analyzed in GraphPad Prism (v5.0, GraphPad Software Inc., San Diego, CA, USA) and SPSS Statics v22.0. Clinical and morphologic data were analyzed in relation to disease specific survival (DSS), loco-regional recurrence free survival (LRFS) and distant metastasis free survival (DMFS) using Fisher exact test. The prognostic significance of each parameter on DSS, LRFS and DMFS was calculated using univariate Cox proportional model for log-transformed post-operative calcitonin levels

and log rank tests for all other variables. Factors significant on univariate analysis will be subsequently subjected to multivariate analysis using Cox proportional hazards model. P values less than 0.05 were considered to be statistically significant.

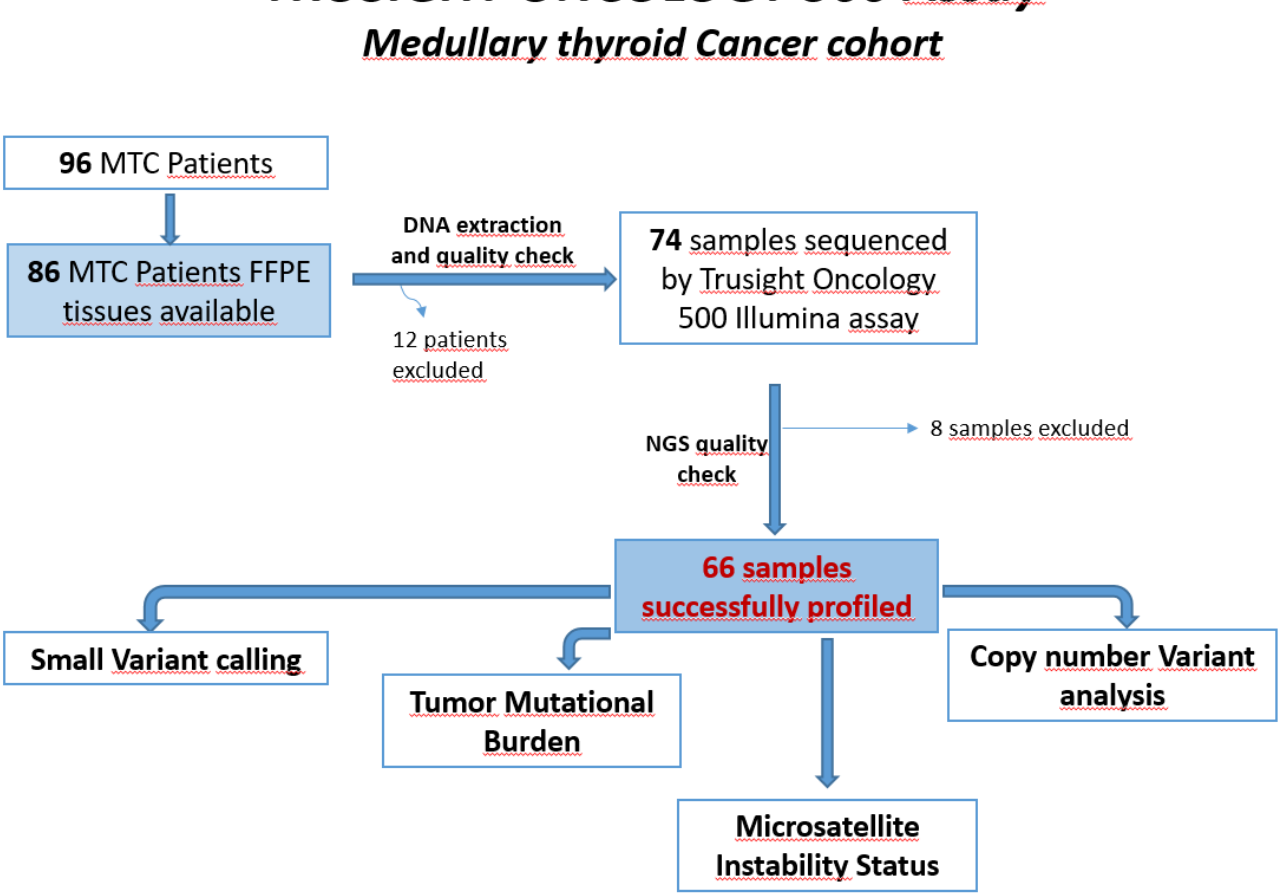
Preliminary results

96 patients (36 females and 60 males) with a diagnosis of MTC were found in our records (median age 61, range 12-84). At histological examination, the vast majority (68 out of 96, 70.8%) of cases showed small, sharply demarcated nodules totally encased in the thyroid gland, stage 1a-1b according to the AJCC tumor staging system. Tumor lymphovascular invasion (p=0.003), thyroid capsule infiltration (p=0.0002) and AJCC staging (p=0.0006) were found significantly correlated with laterocervical metastases. 5 (5.2%) out of 96 patients died of the disease. At the time of the diagnosis RET mutation was found in 18 (18.5%) cases. 14 (14.6%) cases were familial, of which 7 MEN 2a and 7 FMTC.

86 cases had available formalin-fixed, paraffin-embedded (FFPE) blocks, however, 20 failed DNA quality check and NGS quality check resulting in 66 samples successfully profiled. Molecular analysis demonstrated a mutation in at least 1 gene in 91.9% of the patients, with an average of 1-5 genes mutated per case. 86,051 small variants have been found, of which 380 (0.44%) were found with a variant allele frequency ≥3%. Median tumor mutational burden was 2.35 (range 0-20). The unsupervised cluster analysis demonstrated 5 different clusters of patients according to the mutation types. Survival analysis according to the clinico-pathological and molecular data is still ongoing.

Preliminary conclusions

In our study MTC demonstrated indolent behavior in the vast majority of cases. Tumor LVI, thyroid capsule infiltration and AJCC stage was found to be significantly correlated with lymph-node metastasis. The absence of these features suggests a benign clinical course and highlights the role of a careful examination of tumor tissue on microscopy. The multivariate analysis combining molecular and clinic-pathological data is still in progress. Our expected result is to find bio-molecular and histopathological factors with an adverse prognostic and/or predictive role in MTC, focusing on these rare, but rather aggressive, subsets of cases.



MTC: Medullary Thyroid Carcinoma

Arianna Rinaldi

CEM Curriculum: CEM Curriculum: Nanomedicine, Medicinal and Pharmaceutical Sciences
Tutor: Prof. Giovanni Tosi
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OPTIMIZATION OF POLYMER-LIPID HYBRID NANOPARTICLES FOR THE SIMULTANEOUS DELIVERY OF TEMOZOLOMIDE AND SIRNA AGAINST GLIOBLASTOMA MULTIFORME

Background

Glioblastoma multiforme (GBM) is the most aggressive and common malignant brain tumour condemning most patients to a short life expectancy of only 12–18 months after diagnosis, with few efficacious treatment options. In recent years, the combination of two or more therapeutic agents with different mechanisms of action has gained increasing attention to increase therapeutic effectiveness against GBM, help overcome multidrug resistance, and reduce adverse drug effects. Recently, co-administration of the first-line agent temozolomide (TMZ) and small interfering RNA (siRNA) molecules has demonstrated improved efficacy with synergistic effects; however, the differences in their physical-chemical properties represent a major limitation to their simultaneous delivery. In this view, nanomedicine could offer a novel solution to simultaneously deliver both. In recent years, polymer-lipid hybrid NPs (HNPs) have emerged as attractive delivery vehicles, as they combine the advantages of both polymeric NPs and liposomal nanocarriers that are commonly employed to deliver siRNA and TMZ. On the one hand, the polymeric component in HNPs ensures lower toxicity and higher mechanical stability compared to cationic liposomes; on the other hand, the presence of cationic lipids may ensure higher ability to complex siRNA and, as a lipid component, enhance the encapsulation of TMZ compared to polymeric NPs.

Objectives

The current study aimed at developing novel and versatile HNPs that can individually deliver both TMZ and siRNA. The first aim was to test the ability of HNPs to form complexes with siRNA and optimize their physicochemical characteristics and technological properties. The second aim was to select the most promising formulation of siRNA-loading HNPs, in terms of ability to form complexes with siRNA, and test them separately for TMZ loading. The physico-chemical and technological-pharmaceutical properties of siRNA/HNPs complexes were compared to the ones obtained from the complexation of siRNA with polymeric PLGA-based and PLA-based NPs as negative control, or with cationic liposomes as positive control. Following the same approach, TMZ-loaded HNPs were compared to polymeric NPs and cationic liposomes loaded with the drug.

Methods

HNPs, all produced with biocompatible polymers (PLGA, PLA) and lipids (DOTAP, DC-CHOL, DOPE), were prepared by the nanoprecipitation method and then incubated with siRNA to form charge complexes at different nitrogen-to-phosphate (N/P) ratios (ranging from 20:1 to 0.5:1). Then, a complete physical-chemical characterization (size, polydispersity index, zeta-potential and morphology) was performed. A gel retardation assay of the free siRNA by electrophoresis analysis was carried out for each complex to quantify the amount of siRNA complexed on the nanoparticle surface. The most promising HNPs were used in RNase degradation assays to test their ability to protect the absorbed siRNA against RNAse enzymes. The most promising formulations were then optimized for TMZ loading and characterized as previously stated. All analyses were performed and compared to polymeric or lipidic NPs as controls.

Results

Empty HNPs presented size values < 250 nm, low polydispersity indexes (< 0.28) and positive values of zeta-potential (from +37.0 to +46.3 mV). After the addition of siRNA to form complexes, the zeta-potential gradually decreased when increasing N/P ratio for each formulation, while no significant changes in nanoparticle size was observed. siRNA/HNP complexes at the maximum N/P ratio (N/P of 20:1) showed the highest ability to form complexes with siRNA (from 73.0 to 99.0 % siRNA is complexed on the surface of HNPs). RNAse protection assay performed for each formulation at N/P ratio=20 showed that, while naked siRNA was almost completely degraded by RNase, more than 60% siRNA in HNPs was protected against RNase. While the protection ability against RNAse was comparable for all HNP formulations, HNPs showed about 25% and 40% higher ability to protect siRNA compared to cationic liposomes and polymeric NPs, respectively. Formulations were then tested for TMZ loading separately. Optimized TMZ-loaded HNPs formulations exhibited a size ≤230 nm and a low polydispersity index (≤0.28), with a TMZ encapsulation efficiency (EE) of ~10.0% and a loading content (LC) value of ~ 0.4%. These values are higher than those obtained when loading TMZ into PLGA and PLA-based polymeric NPs (EE= 0.6-0.8%; LC= 0.03-0.04 %), but lower than the ones obtained for cationic liposomes loaded with TMZ (EE= 44.0%; LC=3.1%). Further optimization of TMZ-loaded HNPs is ongoing.

Conclusions

In this work, we developed novel HNPs to separately load both TMZ and siRNA, two drugs with different physico-chemical characteristics. These nanoplatforms have been proven to effectively form complexes with siRNA. HNPs showed a higher ability not only to complex siRNA compared to polymeric NPs, but also to protect siRNA against RNAse enzymes compared to both cationic liposomes and polymeric NPs. Further studies are under investigation to assess the maintenance of physico-chemical and technological properties of HNPs after freezing, test their ability to protect siRNA in blood serum and enhance TMZ loading. These

studies set the basis to investigate the possibility of using these novel HNPs as a versatile vehicle for the simultaneous delivery of siRNA and TMZ, with the aim to exploit their synergistic therapeutic effect in GBM treatment.

Filippo Monelli

CEM Curriculum: Translational Medicine
Tutor: Dr. Giulia Besutti

QUANTITATIVE IMAGING APPLIED TO DIAGNOSIS AND RESPONSE PREDICTION AND ASSESSMENT IN PATIENTS WITH SOLID AND HEMATOLOGIC MALIGNANCIES TREATED WITH CHEMO- IMMUNO- THERAPY

Background

The incidence of cancer diseases is constantly increasing in industrialized countries, replacing heart diseases as the main cause of death and this causes a growing adoption of diagnostic imaging for disease diagnosis and monitoring during antitumoral therapies. In this context, a need is emerging to improve the understanding of disease evolution, including imaging features that may be associated with biological characteristics and other known prognostic factors. For example, in the setting of disease monitoring, iRECIST, which is the latest evolution of RECIST (Response Evaluation Criteria in Solid Tumors) criteria, is specifically designed to consider the differences that exist between tumor response to traditional chemotherapy and immunotherapy.

Quantitative imaging methods applied to diagnostic imaging may have the ability to extract hidden information already present in radiological images that is not perceivable by human eyes. Radiomics and deep learning, which are the most used quantitative imaging strategies, have been increasingly used in cancer diagnosis and cancer therapy response prediction and assessment.

Objectives

The objective of the project is to develop and test a methodology that uses radiologic diagnostic images performed in clinical practice to extract radiomic features associated with biological characteristics (biomolecular, histological and genetical) that may help to improve diagnosis, assessment of response to chemo- immuno- therapy, and oncological outcome prediction.

Methods

Preliminarily, we performed a systematic review of the existing literature by analyzing main databases (Embase, Scopus, Cochrane and PubMed) looking for articles that used quantitative imaging methods to assess and predict response to immunotherapy using diagnostic imaging, focusing on CT. On those selected works, data extraction, analysis of the risk of bias using QUADS version 2 tool, as well as analysis of quantitative imaging methodology using Radiomics Quality Score version 1 have been performed.

Clinical ongoing studies are focused on 1) non-Hodgkin lymphoma, 2) neuroendocrine tumor liver metastases, and 3) colorectal cancer liver metastases. 1) Non-Hodgkin lymphoma study includes two cohorts

of patients from two different hospitals (Parma and Reggio Emilia) to evaluate a radiomic model in both training and internal validation sets and in an external test set. Lymph node and spleen radiomic features extracted from baseline total-body portal venous phase CT will be used to predict response at clinic/radiological follow-up. 2) Neuroendocrine tumor liver metastases study includes patients who received both CT-PET and contrast-enhanced CT at baseline and after therapy: radiomic information will be used to assess tumor response in comparison to RECIST criteria. 3) Colorectal cancer liver metastases study is an ancillary study of two multicenter studies, in which radiomic information will be used both for prediction and assessment of response to immunotherapy, and to evaluate the association between radiomic features and liquid biopsy results.

Results

Twentynine articles (from 2014 to 2022) were included in the systematic review, showing promising but largely heterogeneous results. The risk of bias analysis revealed that only 9 studies had a low risk of bias, and the main sources of bias were the type of prediction model (no validation of produced model) and analysis (excessively optimistic results), both frequently caused by the low number of patients included. Radiomics Quality Score analysis revealed an overall low quality of included studies in terms of methodology with a mean score of 12.21±6.06 on a maximum score of 36 (33.92%) with a growing trend in the last years.

1) Non-Hodgkin lymphoma study: 360 patients have been included in the analysis extracting radiomic features of the main lymph node for every nodal station and of the whole spleen. Bioinformatic analysis is currently ongoing to develop and test a predictive radiomic model for therapy response.

2) Neuroendocrine tumor liver metastases study: 66 patients have been included for a total number of 340 liver metastases. Manual segmentation is currently being performed to process the images with quantitative imaging software and extract radiomic features.

3) Colorectal cancer liver metastases study: in a first study we included 62 patients with a total number of 72 liver metastases that will be used for model training. Those models will be further validated and tested on patients from a second multicentre study which is currently ongoing.

Conclusions

Our preliminary systematic review underlined a high variability of methods which harms the generalizability of results, and the adoption of an external validation cohort may not be enough without the implementation of prospective studies. The project includes different studies at different stages of development, with the aim to produce a rigorous methodology that may allow the use of quantitative imaging to improve diagnosis and assessment of response to antineoplastic therapies.

Adriana Scamporlino

CEM Curriculum: Translational Medicine
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SURGICAL LUNG BIOPSY FOR THE DIAGNOSIS OF INTERSTITIAL LUNG DISEASES: PROPOSAL OF A LESS INVASIVE SURGICAL APPROACH

Background

Surgical lung biopsy (SLB) represents the gold standard for the diagnosis of interstitial lung disease (ILD) when radiological features alone do not demonstrate a clear pattern suggestive of a diagnosis. Minimally invasive surgical approach by video-assisted thoracoscopic surgery (VATS) and the multiple lung biopsies in different pulmonary lobes are recommended for SLB.

Objectives

The aim of our study is to propose a less invasive surgical approach for SLB in patients with suspected ILD. A comparison with our standardized technique already in use will be performed. The first goal of the study is to evaluate the diagnostic yield of our novel approach. Secondly, an evaluation of intra-operative and post-operative parameters will be performed to test the short term surgical outcomes of the procedure.

Methods

The first part of the study is a retrospective analysis of patients who underwent SLB for the diagnosis of ILD at the Thoracic Surgery Unit of the University Hospital of Modena from January 2011 to December 2021. These subset of patients will be used as the control group, representing our former standardized technique for SLB. The procedure is performed under general anesthesia with double-lumen intubation and a tri-portal VATS approach. Three pulmonary biopsies at different sites are collected.

The second part of the study is a prospective study including patients undergoing SLB with our novel surgical approach at the same Hospital from May 2021. These patients represent the study group. The new proposed procedure is performed in non-intubated patients under sedation and local anesthesia and a bi-portal VATS approach. Two surgical biopsies at different sites were performed.

In both groups, indication for surgery was approved in the setting of a multidisciplinary team including a pulmonologist, a radiologist, a pathologist and a thoracic surgeon. Surgical lung biopsy was performed on the left lung, with the exception of a predominant right-sided disease on pre-operative imaging.

Results

Ninety patients were included in the control group, with a male to female ratio of 2:1. Mean age at the time of operation was 64 years old (range 34-79). Seventy-four patients (83%) underwent left VATS and 16 patients

(17%) were operated on the right side. Mean operative time was 46 minutes (range 30-110). In all patients a conclusive pathological diagnosis was obtained, with a diagnostic yield of 100%. Usual interstitial pneumonia (UIP) was the definitive pathological diagnosis in 73 patients (81%). Mean drainage time was 1.3 days (range 1-3) and mean length of hospital stay was 2.7 days (range 1-10). Eight patients (8,9%) experience post-operative complications. Thirty-day mortality rate was 1.1%.

Two patients were enrolled in the study group. Male to female ratio was 1:0. Mean age was 54 years old. Both patients were operated on the left side. Diagnosis of usual interstitial pneumonia (UIP) was obtained in both patients with a diagnostic sensitivity of 100%. Mean operative time was 40 minutes. Mean drainage time was 12 hours and mean length of hospital stay was 1.5 days. No post-operative complication was recorded.

Conclusions

Initial results of our study demonstrate high diagnostic yield of the novel surgical approach and a trend toward reduction of drainage time and hospital stay. Further data will be collected and analyzed as the enrollment process proceeds to validate these findings.

XXXVII cycle

Jessica Rossi

CEM Curriculum: Translational Medicine
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CLINICAL, ELECTROPHYSIOLOGICAL, AND BIOMOLECULAR FACTORS ASSOCIATED WITH EPILEPSY IN PATIENTS WITH GLIOBLASTOMA

Background

Epilepsy can manifest in 29-52% of patients with glioblastoma and has an important role in the natural history of the tumor and the quality of life of patients. It is common knowledge that epileptogenesis in glioblastoma is partially related to increased intracranial pressure due to mass effect, edema, hypoperfusion, and neoangiogenesis. However, it is also strongly influenced by specific structural and functional changes in the peritumoral cortex, where the altered permeability of the blood-brain barrier leads to the activation of astrocytes, microglia cells, and recruitment of circulating macrophages. The creation of an inflammatory microenvironment with increased concentrations of cytokines, chemokines, and growth factors contribute not only to epileptogenesis but also to tumor proliferation and invasiveness. Nevertheless, current evidence suggests that the presence of seizures at the onset of glioblastoma could be a possible favorable prognostic factor in patients. The biomolecular mechanisms and the role of the peritumoral microenvironment in favoring epileptogenesis, as well as the role of epilepsy in determining the prognosis of glioblastoma, are not fully elucidated.

Objectives

The primary endpoints of the project are to study the:

- Differences in gene expression patterns in patients with isocitrate dehydrogenase (IDH)-wild type glioblastoma and pre-surgical tumor-related epilepsy, compared to seizure-free patients, focusing on the peritumoral microenvironment.
- Peritumoral molecular markers associated with the development of epilepsy in patients with IDH-wild type glioblastoma.

Secondary endpoints are to study the:

- Molecular markers predictive of improved survival in glioblastoma patients.
- Molecular markers predictive of a better response to radio-chemotherapy.
- Presence of peritumoral molecular markers correlated with increased epileptiform activity on electroencephalogram (EEG).
- Possible correlations between clinical, electroencephalographical, and biomolecular variables and epilepsy severity.

- Possible correlations between clinical, electroencephalographical, and biomolecular variables and the development of status epilepticus.
- Possible correlations between clinical, electroencephalographical, and biomolecular variables and the development of drug-resistant epilepsy.

Methods

In this monocentric retrospective study, 50 patients with glioblastoma who underwent neurosurgery for macroscopically total removal of the lesion between January 2013 and December 2021 will be consecutively analyzed. Twenty-five patients who developed epilepsy before surgery (defined as "EP+" group) and 25 patients with similar clinical and neuroradiological characteristics who did not present seizures before surgery will be included. All histological preparations of patients enrolled in the study will be reviewed. After selection of the peritumoral area, 5 µm-thick FFPE slices and one hematoxylin-eosin-stained slice with the areas to be examined will be cut from the blocks for each case. For the selected samples, total RNA will be extracted by Maxwell® RSC RNA FFPE (Promega) from FFPE slices. RNA quantity and quality will be assessed using NanoDrop2000 (Thermo Fisher Scientific). For suitable samples, the gene expression profile will be assessed using the PanCancer Immune Profiling Panel (NanoString Technologies). This panel includes 770 genes from 24 different immune cell types, covering both adaptive and innate immune response types. The analysis will be conducted using nSolver Analysis Software 3.0 (NanoString). After normalization, differential analysis of expression profiles will be performed comparing cases and controls. Differentiation genes will be sorted by fold change (FC) and false discovery rate (FDR) and only genes with $FC \geq 2$ and $FDR < 0.1$ will be included in subsequent evaluations. Further analyses will be conducted to link identified expression profiles to specific immune pathways and subpopulations.

Expected results

We expect to identify specific molecular determinants which are more represented in the peritumoral microenvironment of patients with epilepsy and may be associated with an increased risk of developing tumor-related epilepsy in patients with IDH-wild type glioblastoma.

Valentina Bergamini

CEM Curriculum: Translational Medicine
Tutor: Prof. Massimo Dominici
CoTutor: Dr. Elena Veronesi

TISSUE ENGINEERING BY 3D PRINTING: CREATION OF BIOMIMETIC MODELS FOR PREDICTIVE MEDICINE AND FOR INNOVATIVE EX-VIVO BIOCOMPATIBILITY TESTS AIMING AT THE REDUCTION OF ANIMAL STUDIES

Background

Biofabrication, bioassembly and bioprinting are three terms that have received great attention in recent years. Bioprinting refers to the use of a computer-aided transfer process for patterning and assembling living and non-living materials with a prescribed 3D organization, to produce bio-engineered structures.

These methods developed concurrently with the technologies used for their implementation, such as inkjet, extrusion, laser-assisted printing and stereolithography, three of the most common 3D printing technologies. In particular, extrusion-printing can be divided into three mechanisms of extrusion: mechanical, pneumatic and rotary screw extrusion. All of which employ a syringe for the deposition of the printing material, while differ in how the process is controlled, for mechanical extrusion a motor push downwardly the piston of the syringe, forcing its content out, pneumatic extrusion form a differential pressure between the inside of the syringe chamber and the environmental pressure to drive the flow of the material whereas for rotary-screw, the angular turn of a graduated screw affects the amount of material extrusion.

In the context of bioprinting, it is important to classify the biomaterials into two groups: hard biomaterials and soft biomaterials. Hard biomaterials are ceramics, bio-glass and synthetic polymers, that generally need high temperature to be printed, that makes those materials inappropriate to encapsulate cells. Soft biomaterials, for example hydrogel, have been employed to embed cells in 3D printing technology.

By using this technology, bio-inks that include hydrogels, cells, and growth factors, can be precisely positioned to create 3D *in vitro* models in order to recapitulate the native tissue architecture, cellular composition and vasculature. For this reason, 3D bioprinting allows the creation of biomimetic tissue models, which can be used for studying disease mechanisms, screening drugs, and personalized and regenerative medicine. The decision regarding which bio-ink, and cell line to insert into the tissue model depends on the type of experiment to be carried out.

Even the printing parameters are fundamental for the correct success of the tissue model and must be set experiment by experiment, accordingly.

Objectives

This project has multiple objectives, first of all to create a platform able to reduce and progressively abrogate animal experimentation with innovative, biocompatible and low industrial impact technologies. 3D printing can be also applicated to obtain healthy tissues to be used for the biocompatibility study of new compounds and new medical devices in order to reduce animal testing. The second aim is to use the platform to test the effectiveness of pre-clinical therapeutic approaches in oncology, in order to test drugs with a view to personalized medicine and to study the efficacy of cell therapies. Different cell responses between 2D and 3D cancer models have been shown in protein and gene expression, cell signaling, migration, morphology, proliferation, viability and drug response. This might be the reason why most anticancer drugs are effective *in vitro* but exhibit low therapeutic effects in clinical trials.

Methods

Three-dimensional healthy and pathological tissue models will be developed using the bioprinting technique, implemented with the extrusion method. The proper cell types will be selected and embedded in hydrogels based on gelatin, collagen, and hyaluronic acid in order to generate the "bio-ink".

The printing parameters, such as speed, pressure and temperature, will be set and chosen based on the type of bio-ink that will be used.

In particular, 3D platforms obtained by extrusion of cells isolated from healthy donors will be used for the development of novel biocompatibility assays for medical devices. Cytotoxicity, sensitization and irritation will be evaluated in a three-dimensional model that will try to reproduce the physiological tissue that will have to come into contact with the medical device.

Three-dimensional platform that reproduces the complexity of the tumor microenvironment will be printed, in which various components play fundamental roles for the progression of the disease and for its invasiveness. In this way we want to go and test anticancer drugs and evaluate their effectiveness on a three-dimensional model, which can also be made up of tumor cells derived from a biopsy of the patient himself.

Expected results

This study faces two issues: the reduction of animal experimentation and the development of personalized oncological therapeutic approaches.

We expect our study to represent a useful tool to be use for the biocompatibility study of new compounds and new medical devices, in order to reduce animal testing by reflecting the ethical principles of the "3Rs".

Moreover, we expect this project to provide valid support in the development of an increasingly personalized cancer therapy, allowing us to study the efficacy of anti-tumor drugs *in vitro* and to permit the clinical to choose the most appropriate drug for his patient.

Giulia Bertani

CEM Curriculum: Translational Medicine
Tutor: Prof. Carlo Salvarani

DYNAMIC STRESS AFFECTS THE BIOLOGICAL BEHAVIOR OF MESENCHYMAL STEM CELL UNDER INFLAMMATORY MICROENVIRONMENT

Background

Human dental pulp stem cells (DPSCs) represent a neuro-ecto mesenchymal stem cell niche localized in the perivascular area of dental pulp and are characterized by low immunogenicity and immunomodulatory/anti-inflammatory properties. Previous studies from my laboratory demonstrated that in standard culture conditions DPSCs modulate the inflammatory microenvironment taking advantage of the crosstalk existing among different immunomodulatory pathways such as PD1/PD-L1 and Fas/FasL. Based on their peculiar embryological origin and their pericyte-like features, DPSCs are able to differentiate into the endothelial lineage and to promote vascularization in vitro and in vivo animal models. Evidence reports that angiogenesis plays a pivotal role in the pathogenesis of autoimmune diseases. Autoimmune disorders are considered a clinical syndrome spanning several diseases, such as rheumatoid arthritis, vasculitis, idiopathic retroperitoneal fibrosis. These autoimmune diseases entail several inflammatory cascades associated with self-reactive CD4+, CD8+ T cells, macrophages which result in tissue damage. Particularly, vascular endothelium participates in the trigger and maintenance of the inflammatory response and vascular dysfunction has been described in the synovial membrane in rheumatoid arthritis patients. The activation of pericytes, endothelial and resident cells is associated with a variety of stimuli, including hypoxia, inflammatory mediators and mechanical stress. Between mechanical stress, dynamic changes determined by blood flow (shear stress), might be among the primary triggers in the initiation and persistence of the inflammatory process.

Objectives

The present study aims to evaluate how DPSCs stemness phenotype is affected by the exposure to an inflammatory microenvironment under dynamic shear stress culture conditions.

Particularly, we plan to investigate:

1. How immune-modulatory properties of DPSCs are affected by dynamic shear stress in standard cultured conditions and under exposure to inflammatory microenvironment
2. How immune cell subtypes, with particular focus on macrophages phenotype, can be modulated by dynamic shear stress conditions before and after co-culture with DPSCs

Methods

The experimental plan of my research will be articulated as follows:

- DPSCs will be isolated from human dental pulp and immune-selected for stemness markers.
- Dynamic shear stress will be generated by using a peristaltic pump connected to a specific cell culture chamber.
- Inflammatory microenvironment will be mimicked by pre-activating peripheral blood mononuclear cells with anti-CD3 and anti-CD28 antibodies (aPBMCs).
- CD14+ monocytes will be isolated and polarization towards M1 and M2 macrophage phenotypes will be evaluated.

All the experimental analyses will be carried out at cellular level, thanks to biomolecular technologies including PCR, Western Blot, Mass Spectrometry and FACS. On a morphological basis, analyses will be performed through immunofluorescence and immunohistochemistry techniques.

Expected results

We expect that differentiation potential of pericyte-like cells are altered by the exposure to dynamic shear stress culture conditions and, mostly, their immunomodulatory abilities are affected consequently to shear stress and to inflammatory microenvironment. Based on our expected results, further investigations on samples from autoimmune disease patients will shed light on the pathogenesis of autoimmune diseases in correlation to their microenvironment and to understand the role of shear stress in autoimmune diseases.

Marianna Tauro

CEM Curriculum: Rigenerative Medicine
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OPTIMIZATION PROCEDURE FOR ISOLATION AND EXPANSION OF ENDOMETRIAL DECIDUAL TISSUE DERIVED MESENCHYMAL STROMAL CELLS USED AS EXTRACELLULAR VESICLES PRODUCER CELL LINE

Background

Mesenchymal stromal/stem cells (MSCs) are adult progenitor cells isolated from several human adult and perinatal tissues. Progenitor isolation frequently requires traumatic procedures. One of the most widely recognized sources of MSCs has been the bone marrow, but MSC progenitors have recently been isolated from lipoaspirates and other tissue sources including bones, placenta, umbilical cord and cord blood. In the attempt to identify a different and more approachable MSC source, we focused our study on endometrial decidual tissue (EDT) obtained from menstrual blood, accordingly to recent literature finding. These have shown that EDT may contain heterogeneous populations including some having MSC-like features. It is well known that EDT-MSCs have immunomodulatory capacity and a strong capability of extracellular vesicles (EVs) production. Notably, EVs mirrored tropism and properties of their secreting cell line. Therefore, we tried to use EDT-MSCs as a producer cell line to obtain EVs.

Objectives

The main objective of the project is to develop Standard Operation Procedures (SOPs) for:

- The isolation and expansion of Endometrial Tissue derived Mesenchymal Stromal Cells (EDT-MSCs) suitable for the production of EVs.
- The production and separation of EDT-MSCs derived EVs.

Methods

Decidual endometrial blood was collected from healthy female donors using a menstrual cup during the first few days of the monthly periods. The blood sample can be processed immediately or it can be stored at +4° but no later than 24 hours after collection for the isolation of EDT-MSC. The sample was processed using a protected isolation protocol and isolated EDT-MSCs were cultured up to cell passage 2 in flasks with a specific isolation culture medium, which was changed every 48 hours. The *in vitro* approach was performed to evaluate the clonogenic potential of EDT-MSCs and cytofluorimetric analyses were also performed on adherent cells to demonstrate the expression of mesenchymal surface markers such us CD73+, CD90+, CD14- and CD45-. After characterization, EDT-MSCs were seeded and expanded in a specific culture medium for EVs

isolation. The conditioned medium was harvested every 48 hours and immediately processed whit specific protocols for EVs separation and purification.

Expected results

EDT derived cells expressed typical MSC markers and *in vitro* cells grown showed a rapid proliferation and high clonogenic potential. These results suggested that EDT-MSCs could be largely expanded *in vitro* for EVs production. It will be of interest to understand if these isolated EVs show the same properties of their producer EDT MSC line

Lorenza Di Marco

CEM Curriculum: Translational Medicine
Tutor: Prof. Erica Villa

CLINICAL, AND THERAPEUTIC MANAGEMENT AND BIOLOGICAL FEATURES OF PATIENTS WITH RECURRENT HEPATOCELLULAR CARCINOMA AFTER LIVER TRANSPLANTATION

Background

Liver transplant (LT) is currently the only really curative treatment for patients with hepatocellular carcinoma (HCC). The criteria for access to LT must be precisely defined to reduce the rates of HCC recurrence (HCC-R) and to increase post-OLT survival. The risk of HCC recurrence has been related with the number and size of tumors as well as other parameters such as alpha-fetoprotein (AFP) or vascular invasion, with recurrence rates ranging from 8 to 20%. HCC-R LT is difficult to manage and often characterized by relevant aggressiveness and extrahepatic localizations. Currently TKIs are the only therapeutic option, but results in terms of overall survival (OS) and progression-free survival are disappointing. Immune checkpoint inhibitors in association with a recombinant humanized monoclonal antibody to vascular endothelial growth factor (VEGF) were to be proved effective in non-LT patients with metastatic hepatocellular carcinoma in determining tumor stabilization and improved survival. However, as in LT- patients immunotherapy may increase the risk of acute liver rejection, such combination therapy has not been used so far.

Objectives

My project aims to analyze clinical, histopathological, and biological features of patients with HCC, the rate of HCC-R and death after LT, in order to identify the predictive factors associated with cancer recurrence and survival. This project also aims to identify expanded therapeutic approaches for HCC-R after LT.

Methods

Biologic features of HCC-R after LT were characterized by immunohistochemistry for Angiopoietin-2. Patients at transplant were staged according to the most used scoring system (Milan criteria; up-to-7 criteria, AFP model). Clinical and demographic features were recorded. As the preliminary histochemical data indicated a marked activation of neoangiogenesis in recurrent LTs, we designed a proof-of-concept study to analyze safety and efficacy of combination of Nivolumab and Bevacizumab in LT patients as treatment for HCC-R after LT. Combination therapy received nominal authorization from the Provincial Medicines Commission. Candidate patients underwent routine blood test (hepatic, renal, and cardiac function), echocardiogram and a total body CT as baseline assessment. Patients with creatinine higher than 1.5 ULN or with ejection fraction lower than 45% were excluded. Five patients who experienced HCC-R 2 to 8 years after LT with pulmonary,

lymph node, adrenal, bones, and hepatic localization and were either intolerant to TKIs or had had progression on TKIs, were included. All recurrent tumors and extrahepatic localizations were characterized by biopsy, the histologic and histochemical results show a high biological aggressiveness as tested by ANGPT2 expression in the HCC-R tissue. Patients received Nivolumab infusion every 14 days at a dose of 240 mg. Total body CT scan was performed every 8 weeks. When progression disease (PD) was detected at CT scan, Bevacizumab was added at a dose of 5 mg/kg every 14 days.

Ongoing results

One patient received a single Nivolumab infusion but was withdrawn because of rapid tumor progression, eventually followed by death. Another patient experienced moderate/severe rejection, which resolved with 1g/day Methylprednisolone i.v. for 3 days followed by Prednisone 50mg/day for 1 week then tapered and stopped. This patient was maintained on double therapy without residual problems. The 4 alive patients are still on double therapy after a mean period of treatment of 36±21 weeks. Disease stability with the combination of Nivolumab/Bevacizumab was achieved in all patients at hepatic and all extra-hepatic HCC localization but bone, where 2/2 patients experienced progression. It should be underlined, however, that progression was very slow and mean survival of these two patients on combination therapy is 54±2 weeks. The Combination therapy of Nivolumab and Bevacizumab is well tolerated, with few side effects throughout the treatment period. These results indicate that the combination Nivolumab/Bevacizumab can be safely used in liver transplant patients. In our initial clinical experience, the risk of acute allograft rejection is minimal and manageable. Bone metastases remain an active and unsolved problem and require a further alternative therapeutic approach. For patients with hepatic or other extrahepatic localization, to date, this combination has achieved a relevant and prolonged stabilization of the disease.

Marco Spadafora

CEM Curriculum: Translational Medicine
Tutor: Prof. Caterina Longo

CLINICAL REAPPRAISAL OF MUCOSAL MELANOMA: A 10 YEARS' EXPERIENCE OF A REFERRAL CENTER

Background

Primary mucosal melanoma (MuM) is an exceptionally rare neoplasm (0.03% of all cancer diagnosis and 0.8-3.7% of all melanomas). MuMs arise in mucosal membranes lining anorectal, vulvovaginal, and head and neck district in order of incidence. Late diagnosis is contributed by occult anatomic site of origin and scarcity of presenting symptoms unless in advanced disease. First line treatment of MMs consists in radical surgical excision and/or radiation therapy. Novel biological therapies have now become available for patients with MuMs. Since MuM is an exceedingly rare neoplasm, demographic, therapeutical and survival records on this topic are scarce.

Objectives

The aim of the current study is to provide a 10-year retrospective clinical re-appraisal of the real-world data on MuMs managed in a tertiary referral center in Italy.

Methods

We included consecutive patients with histopathologically confirmed diagnosis of MuMs from January 2011 to December 2021. Recurrent tumors, unknown primary melanomas, and cases for which histological slides were not available were excluded. Location was documented as 'sinunasal, 'anorectal', 'vulvovaginal'; demographic and relevant data as site of metastasis at diagnosis, date of patient's death or last known follow up were collected. Treatments were categorized as surgery (radical or debulking procedure), radiation therapy and first- and second-line systemic treatment. A survival analysis was performed.

Expected results

Among 33 patients, we found 9 sinunasal, 13 anorectal and 11 vulvovaginal MuMs with median age of 82 years and a higher incidence in female (n= 22, 66.7%); 18 cases (54.5%) presented with metastasis at diagnosis ($p<0.05$); in vulvovaginal subgroup, only 4 patients (36.4%) showed metastasis, all diagnosed in regional lymph node. Sinunasal MuMs were all surgically managed (44.4%) with a debulking purpose, while every case of anorectal and vulvovaginal melanomas underwent a surgical excision with a radical intent (30.8% and 45.5%). 15 patients were managed with systemic biological therapy as first-line treatment ($p<0.05$). Radiation therapy has been used in all MuMs of the sinunasal district ($p<0.05$). Overall survival was

longer in vulvovaginal melanomas (26 months), than sinunasal (14 months) and anorectal MuMs (6 months). Univariate analysis showed that patients with metastasis have an increased hazard ratio for death; the prognostic value of metastatic status was also reported by multivariate model, while administration of a first-line immunotherapy has demonstrated a protective role.

Conclusions

The absence at diagnosis of metastatic disease is the most relevant factor that influence the survival of patients with MuMs. Therefore, efforts should be made to avoid late diagnosis. Moreover, the use of new biological therapies might prolong survival of patients with MuMs.

Clarissa Caroli

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

CANNABIS SATIVA L.: A GREEN AND SUSTAINABLE PARADIGM OF MEDICINAL INTEREST

Background

Cannabis sativa L. is a well-known plant that belongs to the *Cannabaceae* family [1]. Based on its chemical composition, it is classified in recreational-type (marijuana), medical-type and fibre-type (hemp), and it is a source of different bioactive compounds, such as phytocannabinoids, polyphenols and terpenes. Regarding cannabinoids, recreational-type and medical-type *Cannabis sativa* L. plants contain mainly Δ^9-tetrahydrocannabinolic acid (THCA), while hemp has cannabidiolic acid (CBDA) as the prevalent constituent. The native acidic cannabinoids undergo a spontaneous decarboxylation process that converts them into their neutral forms Δ^9-tetrahydrocannabinol and cannabidiol (Δ^9-THC and CBD) [2,3]. Polyphenols in *C. sativa* are mainly represented by flavonoids, mainly by cannflavins A and B, that are methylated isoprenoid flavones. Terpenes are volatile organic compounds responsible for the aroma of the plant, and they can be divided into sesquiterpenes and monoterpenes [3]. All these chemical classes have already demonstrated several biological activities. For this reason, the interest in the enriched fractions and pure compounds from *C. sativa* is increasing [2–4]. Due to the increasing role of sustainability, which has become an important issue, it is fundamental to find an approach to obtain new bioactive compounds in a sustainable way, with a view to a circular economy.

Objectives

The aim of this project is the extraction and fractionation of hemp-by products for the recovery of bioactive compounds to be exploited in the medicinal chemistry ambit. In addition to inflorescences of *C. sativa* (var. Kompolti), industrial waste material, resulting from the extraction of CBD from biomass using different methods, will be used to obtain different enriched fractions of cannabinoids, terpenes and polyphenols and to test their biological activities. Initially, the work has focused on the optimization of the extraction method for polyphenols, with the objective to remove as many cannabinoids as possible. Both a target as well as an untargeted analysis by high-performance liquid chromatography coupled with a diode array detector (UV/DAD) and with a high-resolution mass spectrometer (HRSM) was applied to fully characterize the extracts.

Methods

A new method was developed here for the first time to extract and purify the polyphenolic fraction from hemp inflorescences and waste material. The method was then optimized to remove cannabinoids and to obtain an enriched fraction of the phenolic compounds. To reduce the content of cannabinoids in the extract, a first step of decarboxylation was necessary to eliminate terpenes and to convert CBDA into CBD and, subsequently, to remove it from the extract. This was possible by using a semi-preparative chromatographic system operated under normal-phase conditions, that allowed us to obtain a fraction with a high content of polyphenols, in particular cannflavins, to be used in the biological assays.

Expected results

We expect to identify bioactive compounds present in non-psychoactive *C. sativa* using *in vitro* and *in vivo* models of cancer and neurodegeneration for a new lead identification. Moreover, we expect to obtain the recycle of industrial hemp waste, complying with the sustainable principle of circular economy.

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Carla Pezzuoli

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GREASE II. A PHASE II RANDOMIZED, 24-MONTH, PARALLEL-GROUP, SUPERIORITY STUDY TO EVALUATE THE EFFICACY OF A MODIFIED ATKINS DIET IN ADPKD PATIENTS

Background

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is characterized by the formation and enlargement of multiple renal cysts that cause progressive renal function decline. To date, the only therapeutic option available for ADPKD is Tolvaptan, but its use is limited due to side effects. In recent years, deregulation in glucose metabolism in ADPKD has been identified. In particular, the data suggest that cystic cells shift their energy metabolism from oxidative phosphorylation to aerobic glycolysis. In PKD animal models the induction of ketosis, through various dietary manipulation techniques led to beneficial effects in terms of reduction of cystic size, interstitial fibrosis, and disease progression. The University of Modena and Reggio Emilia conducted a study involving three ADPKD patients treated with a ketogenic modified Atkins diet (MAD) that confirmed the feasibility of a clinical trial testing the effect of a ketogenic diet in an ADPKD population. Following the positive phase I results, this phase II aims to evaluate the efficacy of a MAD protocol compared to a balanced normocaloric diet (BND).

Objectives

The objective of this project is a clinical trial that will evaluate the effect on Total Kidney Volume (TKV), safety, and tolerability of MAD in a selected ADPKD population. The trial will have, as a secondary objective, the slowing of renal function decline and the evaluation of the MAD impact on the variation of exploratory prognostic biomarkers (urinary markers β2MG and MCP-1).

Methods

The study will be conducted as a multicentric trial involving the Nephrology Divisions of the University Hospital of Modena (Modena, Italy) and the University Hospital of Bologna IRCCS Sant'Orsola-Malpighi Polyclinic (Bologna, Italy). We will conduct a 24-month randomized, parallel-group, two arms, superiority trial with 1:1 allocation to evaluate the efficacy of a MAD compared to BDN on ADPKD patients. Patients eligible for the trial must comply with the following randomization: i) subjects of both sexes, ii) aged 18 - 60 years, iii) renal function larger than 24 ml/min/1.73m^2 according to the CKD-EPI formula, iv) Mayo score 1C-1D-1E (TKV calculated by MRI). The patient's eligibility criteria will be assessed during a first screening visit during which consent to the trial will be obtained. An MRI will be programmed to calculate the TKV. At the same

time, the patient will start a twice-daily assessment of glycemia and ketonemia by digit puncture. During the allocation visit, 30 days after the screening visit, 90 patients will be randomized in equal proportions between MAD and BND. The MAD will be plant-forward, to prevent dyslipidaemia, with the following bromatological breakdown of total energy intake: 4-6 % carbohydrates (20 g), 25-30 % proteins, and 60-70 % lipids. A sugar-free multivitamin supplement will be provided. The BND composition will be in line with the recommendations for the Italian population. K or Mg Citrate supplement will be provided to all patients to decrease the risk of renal stone formation. In both arms, the calorie intake will be adjusted during the study to obtain stable body weights. During the treatment period, patients will be evaluated with monthly visits, during which vital signs, anthropometric measurements, and blood pressure will be collected, and laboratory tests will be performed. Patients will be provided with personalized recipes and will meet dietitians monthly to verify compliance with nutritional therapy and to identify strategies to overcome encountered difficulties. A specific phone app (KETAPP, Copyright by University of Modena and Reggio Emilia) will help patients follow their diet. To enhance the validity of data, multiple methods will be used to assess diet adherence, including evaluation of data collected by KETAPP and evaluation of glycemia and ketonemia levels. After the post-allocation treatment time of 24 months, a final visit will be scheduled no later than 14 days after the end of the treatment. No later than 30 days after the end of the treatment the second MRI for TKV calculation will be performed.

The Primary Endpoint TKV, which has a high sensitivity to ADPKD progression, will be evaluated by MRI. Tolerability will be assessed by a Dietary Satisfaction Questionnaire. Safety will be assessed by monitoring serious adverse events/adverse events classified according to the Common Terminology Criteria for Adverse Events (CTCAE) Version 4.0. Efficacy will be evaluated by the comparison of the variation of kidney function expressed as eGFR, according to the CKD-EPI formula, between the two randomized arms of the study. The Biomarker Exploration will evaluate if the diet has any impact on the variation of exploratory prognostic biomarkers (urinary markers ß2MG and MCP-1). Percentages, means (with standard deviations), and medians (with I–III quartile range) will be used for descriptive purposes, as appropriate. For the evaluation of the treatment effect on the primary efficacy endpoint a t-test for the comparison of the pre-post diet mean delta calculated as the difference between the kidney volume observed at 24 months minus the volume at baseline will be applied (two-sided $\alpha = 0.05$). The same parametric approach (t-test) will be used for the analysis of secondary endpoints of efficacy while, when this is not appropriate, a non-parametric test (Wilcoxon signed-rank test) will be considered.

Expected results

We expect to obtain a reduction of the TKV in the group of patients treated by the MAD protocol. Preliminary data obtained in the feasibility study have suggested excellent tolerability in the three-month period. We believe the same good tolerability can be replicated in this extended study.

Ilaria Ferrigno

CEM Curriculum: Translational Medicine
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CROSSTALK BETWEEN INFLAMMATION, AUTOIMMUNITY AND SOLID CANCERS

Background

Some studies have reported an association between cancers and autoimmune diseases, but the mechanisms underlying this association are largely unknown. Dermatomyositis (DM) is the autoimmune disease with the highest association with cancers. It is a systemic inflammatory disorder, which affects skeletal muscle, skin, and other organs (e.g. lungs). Specific autoantibodies against intracellular antigens have been detected with a different frequency in patients with DM and cancers. Nevertheless, the increased risk of cancer is not yet fully defined in patients with DM since patients negative for all known autoantibodies can also have an increased risk.

During cancer progression, immune checkpoint molecules are crucial for the cancer-cell escape from immune surveillance. Immune checkpoint inhibitor (ICI) drugs able to block these molecules (antibodies directed against CTLA-4, PD-1, or PD-L1) have shown efficacy in some cancers. However, they often have off-target effects resulting in immune-related adverse events (irAEs) e.g. inflammatory and autoimmune reactions. Risk factors for the development of irAEs during ICIs still need to be identified.

Inflammation plays a promoting role in cancer development, especially in tumors of the gastrointestinal tract. Inflammatory cytokines in the tumor microenvironment and drugs used in cancer therapy can alter the expression of immune checkpoints. However, extensive knowledge on their effects is still lacking.

Objectives

The purposes of the project are: 1) to identify novel antigens and immunological pathways which can drive cancer-associated autoimmune diseases, particularly DM; 2) to identify risk factors for the development of irAEs in patients treated with immune checkpoint inhibitors; 3) to evaluate the effects of cytokines commonly present in tumor microenvironment and the effects of drugs commonly used in gastrointestinal cancer on immune checkpoints in immune system cells as well as in tumor cells.

Methods

Objective 1. Analyses will be focused on the detection of antibodies against plasma membrane proteins, which are the ones that can be promptly targeted by antibodies in vivo. Antibodies in sera will be used as primary antibodies in flow cytometry assays using viable cancer cell lines of different histotypes and skeletal myoblasts (commercial cells), followed by incubation with anti-IgM and anti-IgG secondary antibodies

labeled with Phycoerythrin (PE). In addition, they will be used as primary antibodies in ELISA assays using viable cell monolayers. In case a different reactivity among groups of subjects is found, to identify the antigens, they will be used in immunoprecipitation assays using cell lysates enriched in plasma membrane proteins. Immunoprecipitated proteins will be identified by mass spectrometry. In addition soluble immune checkpoint molecules (e.g. BTLA, GITR, HVEM, IDO, LAG-3, PD-1, PD-L1, PD-L2, TIM-3, CD28, CD80, CD137, CD27, CD152) will be quantified in sera with multiplex bead-based assays.

Objective 2. Soluble immune checkpoint molecules will be profiled in sera from patients. Cell-based assays will be used to evaluate T cell activity in peripheral blood mononuclear cells (PBMCs).

The following groups of patients will be compared:

Objective 1) patients with cancer-associated DM *versus* DM patients without cancers. Serum samples will be collected before chemotherapy and any biological therapies.

Objective 2) patients treated with ICIs who develop irAEs *versus* patients treated with ICIs who do not show irAEs. Serum samples and PBMCs will be obtained before ICIs and after therapy.

A cohort of healthy subjects will be included as reference.

Objective 3. PBMCs or cell lines of gastric, colon, pancreatic and hepatocellular carcinoma will be treated with inflammatory cytokines commonly present in the tumor microenvironment or with drugs used in gastrointestinal cancer therapy (e.g. corticosteroids, folinic acid, 5-fluorouracil, oxaliplatin). Then, levels of immune checkpoints will be evaluated by flow cytometry. In addition, cells will be sequentially treated with cytokines and drugs to identify which therapies can revert the immune checkpoint modifications induced by cytokines. When data indicate a crosstalk between cytokines and immune checkpoints, their levels will be also determined in tumor specimens stored in the biobank at the Azienda Unità Sanitaria Locale-IRCCS in Reggio Emilia by immunohistochemistry.

Expected results

We expect to identify circulating markers: autoantibodies and soluble immune checkpoints differentially expressed in patients with and without cancer-associated DM. This could help identify those patients who mostly need cancer surveillance. We expect to identify soluble immune checkpoints and T cell features associated with the risk of irAEs following ICIs. Moreover, we expect to increase the knowledge on the effects of ICI therapy. We anticipate to identify circulating markers with different levels between DM patients and healthy subjects, and patients with cancers and healthy subjects. Finally, we expect identifying which cytokines and drugs are able to alter immune checkpoint molecules in the tumor microenvironment, and in which manner. This will deepen the knowledge on the effects of inflammation and drugs on cancer immune surveillance.

Francesca Rodà

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ADVANCED PRODUCTION AND CHARACTERIZATION OF NANOMEDICINE FOR THE TREATMENT OF BRAIN DISEASES

Background

The research for successful treatment for brain diseases is a grand challenge due to the presence of the blood brain barrier (BBB) that limits drugs access to the brain. Indeed, the success rate of drugs against brain disorders in clinical trials is one of the lowest in the field of drug discovery. In this framework, nanomedicines (NMeds), thanks to their tunable characteristic and the drug -protective behavior, represent potential nanocarriers for an effective delivery of anticancer agents or gene materials to the central nervous system. The pharmaceutical, chemico-physical, technological and morphological characterization of these NMeds represents a crucial step to have a comprehensive overview of the physical, chemical, and biological features and to evaluate the efficacy and safety in biological systems. Hence the need of properly and interdisciplinary characterization approach to facilitate comparison across NMeds, correlate their features to biological effects, and predict the therapeutic outcomes

Objectives

The principal aim of the project is the development of three NMeds of different origins (polymeric, lipid, hybrid) for the delivery of drugs for central nervous system diseases. The NMed composition and/or the presence on the surface of ligands for selected cellular receptors will enable to cross the BBB, by a receptor or non-receptor mediated manner, and thus reach the disease site. Specific aims will be:

Aim1: Optimization of NMed synthesis

Aim2: Physico-chemical characterization

Aim3: Drug release kinetics and binding affinity analysis

Methods

Aim1: Optimization of NMed synthesis

NMeds will be formulated by nanoprecipitation or double emulsion technologies in the view of taking advantage of the microfluidic technology. In the first case, all the composition and surface engineering technologies will be carefully evaluated and optimized. In the latter case, parameters such as the flow rate ratio, temperature, total flow rate will be optimized to produce NMeds with the desired characteristics. The choice of the type of polymers and lipids FDA approved, biocompatible and biodegradable used will be

evaluated to assure the maximum advantage in terms of loading capacity and activity towards selected active molecules. Surface engineering will be applied in pre or post formulation modality, in function of the nature of the ligand to be used, and proper active molecules will be loaded into NMeds.

Aim2: Physico-chemical characterization

The particle size, polydispersity index and the surface charge will be determined using a Zetasizer Nano ZS, whereas the particle concentration will be assessed by Nanoparticle Tracking Analysis.

The amount of ligand bound to NMeds and the resulting functionalization degree will be calculated by suitable technologies, based on ligand chemical properties. In parallel, proper analytical methods (i.e. HPLC, UV, MS, NMR) will be used for the drug quantification. Morphology will be assessed using high resolution microscopy techniques, such as Transmission Electron Microscopy, or Atomic Force Microscopy that enable the visualization of single particles.

To check quality and reproducibility of NMed production, Raman Spectroscopy will be exploited. Drug-loaded NMeds and single formulation components will be analyzed on a CaF2 disk. The single components of NMeds will be acquired both on powder and on the vehicle buffer to mimic the physiological condition. Moreover, the NMeds will be tested for stability to lyophilization. As cryoprotectant, trehalose will be added at the formulations in different concentrations. Samples will be frozen, then lyophilized and characterized to evaluate the maintenance of their chemical properties.

Aim3: Drug release kinetics and binding affinity analysis

To evaluate the kinetics of drug release from NMeds, at various time points an aliquot will be withdrawn from the suspension and purified to separate the drug leaked. The drug loading will be evaluated at each timepoint and compared with the initial formulation.

The binding affinity of NMeds and encapsulated drugs to their relative targets will be evaluated by Surface Plasmon Resonance imaging (SPRi). In particular, ligand targets will be immobilized on a SPRi gold chip to verify NMed binding, whereas to test the interactions and the integrity of the drugs to corresponding targets, after disruption, NMeds will be incubated on a SPRi gold chip where the drug receptor has been immobilized. The spotting procedure for ligands immobilization will be performed using iFOUR dispensing system, and parameters like ligand concentration, buffer and spot diameter will be optimized in order to obtain the most reproducible results.

Expected results

The project will develop and characterize innovative and tailored NMeds for neurological diseases, complying with the needs of industrial development and application in a clinical setting.

Therefore, the final outcome of the project will be to develop and characterize multifunctional nanoparticles that will be tested in vitro and in vivo tests (in a future project) in order to be potentially used for facilitating the management of brain diseases and improve the patients' prognosis.

Valeria Vannini

CEM Curriculum: Public Health
Tutor: Prof. Paola Ferri

THE EFFECTS OF ONLINE TEAM-BASED LEARNING ON UNDERGRADUATE NURSING STUDENTS' PERFORMANCE, ATTITUDES AND ACCOUNTABILITY DURING COVID-19 PANDEMIC

Background

The COVID-19 pandemic affected all educational systems worldwide. Due to social distancing requirements, many institutions decided to move their teaching methods, including Team-Based Learning (TBL) activities, from face-to-face to an online format.

Team-based Learning is an active learning and small group instructional strategy that can be applied to large classes. It provides students with opportunities to apply conceptual knowledge through a sequence of activities that include individual work, teamwork and immediate feedback. Recent systematic reviews support the use of TBL with evidence of positive outcomes in terms of student experience and academic achievement. In particular, this student-centred learning strategy promotes the development of communication and teamwork skills, problem-solving and critical thinking skills and professional behaviours. In recent years, before the advent of COVID-19, several TBL practitioners have been developing methods for implementing TBL approaches for online delivery but, after the outbreak of the pandemic, more and more institutions have adopted online TBL.

Objectives

The primary aim of this study is to examine the performance of undergraduate nursing students on Team Readiness Assurance Test (TRAT) and Individual Readiness Assurance Test (IRAT) during online TBL.

The secondary aims of this paper are to evaluate the students' attitudes toward online TBL and their accountability, preferences and satisfaction with this learning methodology.

Methods

The study employed a one-group pretest-posttest quasi-experimental design. Fifty-four first-year undergraduate nursing students from the University of Bologna, Italy, attended nine online TBL sessions in the Clinical nursing course during the 2020/21 academic year. To deliver the different steps of the TBL strategy, Microsoft Teams and a Moodle Learning Management System (LMS) platform were used.

The primary outcome of the study was evaluated by comparing the average scores obtained by the students in the IRAT (pretest) versus the TRAT (posttest) in each online TBL session.

The students' attitudes towards TBL, before and after the training intervention, were measured through the anonymous questionnaire designed by Parmelee et al. (2009). Finally, only after the training intervention,

students' accountability, preferences and satisfaction were collected through the questionnaire Team-Based Learning Student Assessment Instrument (TBL-SAI) (Mennenga, 2012).

Results

A statistically significant improvement was identified in students' performance between IRAT and TRAT in all the nine online TBL sessions (TBL 1 t-test=-4.91, p<0.001; TBL 2 t-test=-6,38, p<0.001; TBL 3 t-test=-5.19, p<0.001; TBL 4 t-test=-10.50, p<0.001; TBL 5=-8.68, p<0.001; TBL 6=-8.66, p<0.001; TBL 7=-7.31, p<0.001; TBL 8=-8.93, p<0.001; TBL 9=-11.32, p<0.001).

Cronbach's alpha for Parmelee et al.'s questionnaire was 0.91. A comparison of overall mean scores for statements in the category "Satisfaction with team experience" showed a statistically significant increase after the online TBL sessions (m=3.78±0.6 vs m=4.19±0.8, t-test=-3.10, p=0.003). Participants appreciated the experience of peer review and considered it to be fair (m=3.34±0.6 vs m=4.02±0.8, t-test=-4.72, p<0.001), even though they did not consider it as a factor encouraging them to study more (m=3.57±0.9 vs m=3.02±1.2, t-test=2.27, p=0.028). Finally, students felt that being part of a team made them more competent in clinical reasoning and in making the right decisions (m=3.32±0.7 vs m=3.98±0.9, t-test=-4.06, p<0.001).

Cronbach's alpha for TBL-SAI was 0.76. The mean scores for the accountability (m=30.02±3.7) and preferences for TBL (m=51.78±6.3) were higher than their neutral (N) values (N=24; N=48). Students' satisfaction was neutral (m=27.87±5, N=27).

Conclusions

Institutional restrictions due to the COVID-19 pandemic should not be an obstacle to conducting student-centred active learning programs. Our study results show that online TBL represents a valid alternative for fostering students' competencies development in nursing academic education. Teamwork improves the group performance compared to individual results and is appreciated by the participants. In addition, the online TBL has a positive effect on the accountability of students who prefer it to frontal lectures. To our knowledge, this is the first study of online TBL among nursing students in Italy.

Among the future developments of this line of research, it is worth mentioning the assessment of the validity and reliability of the Italian version of the two questionnaires administered in this study (Parmelee et al. and Mennenga).

Federica Veneri

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Tutor: Prof. Luigi Generali

ADVANCED TECHNOLOGIES IN ORAL MEDICINE: OZONE APPLICATIONS IN PEDIATRIC DENTISTRY

Background

Ozone is a powerful oxidizing agent, which has broad-spectrum antimicrobial properties and can be found in nature as a trivalent oxygen molecule (O_3). Among other formulations, medical ozone can be used as gaseous ozone and ozonized water, thus being especially recommended for oral applications since it has proven safe and easy to use.

Dental caries is a multi-factorial pathological process which affects approximately 90% of the world population and its prevalence is remarkably high in childhood. This disease has an extremely adverse impact on health, quality of life both of children and their families and it is considered a serious public health concern.

Ozone application has been suggested as an alternative approach to achieve an ultra-conservative and minimally invasive treatment of dental caries in young patients as it can be used for the direct decontamination of oral tissues and of dental hard tissues, possibly in association with agents that promote surface remineralization. Recent technologies have remarkably improved medical ozone efficiency, thus allowing an extremely conservative management of both early and deep carious lesions.

Objectives

The aim of this research project is to evaluate the effectiveness of dental ozone application and to identify the most effective protocols for the clinical management of caries in pediatric dentistry, by assessing (a) the specific antibacterial activity, (b) the promotion or remineralization and (c) the clinical effectiveness in treating carious lesions in primary dentition

Methods

Preliminary specific reviews of the literature have been conducted to identify potential research gaps and to implement the most suitable protocols available to date to investigate the addressed study topics. Humadent (Humares® GmbH**,** Germany) and Cytozone (Hänsler Medical GmbH, Germany), which are recent devices dedicated to oral and dental applications, will be used as medical ozone generators for gaseous ozone and ozonized water, respectively.

The specific antibacterial activity will be investigated through an *in vitro* comparative analysis (a) of different antiseptic agents against cariogenic bacterial strains in polymicrobial biofilms. Cariogenic biofilms will be

obtained from dentin samples taken from carious lesions both of permanent and deciduous teeth. Gaseous ozone (40 μg/mL), ozonized water (10 μg/mL) and chlorhexidine gluconate (CHX) 0,2% will be tested as antiseptic agents. Qualitative and quantitative assessment of the antibacterial activity will be performed through: (i) direct morphometric analysis and count of the colony forming units (CFU) of bacterial cultures on petri plates exposed to the antibacterial agents under study; (ii) planktonic growth inhibition assay on 96-well microtiter plates cultures, assessing optical density (OD) and absorbance through spectrophotometric technique.

The promotion of remineralization will be investigated through an *ex-vivo* comparative analysis (b) of the effectiveness of different remineralizing systems, such as fluoride-calcium-phosphate varnish, and casein phosphopeptide and amorphous calcium phosphate (CPP-ACP) compounds, used alone and in combination with gaseous ozone and ozonized water. A sample of 36 extracted sound teeth (e.g. impacted third molars) undergoing a controlled and standardized demineralizing procedure will be used. Remineralization will be assessed after treatment on representative tooth specimens through: (i) direct examination under scanning electron microscope (SEM) of the depth and morphology of remineralization; (ii) fluorescence variation analysis assessed through laser technology; (iii) Raman spectroscopy techniques.

The clinical effectiveness will be investigated through a randomized controlled clinical trial (c), to evaluate ozone application within the selective carious tissue removal procedure in primary molars. According to the performed sample size calculation, 16 teeth among those meeting inclusion criteria will be included in each treatment group. After the selective removal of carious dentin, (i) gaseous ozone (40 μg/mL) or (ii) ozonized water (10 μg/mL) will be applied for 60 seconds directly on residual affected dentin. Control group (iii) will undergo no further treatment. Restoration will be performed with bioactive composite resins. Clinical and radiological follow-up will be carried out at 3, 6 and 12 months through the evaluation of multiple success criteria, including restoration retention, vitality of the tooth and absence of pain. Treated teeth will be extracted just prior to natural exfoliation to histologically assess the amount and the morphological features of residual affected hard tissues.

Expected results

The results of this research project will support the introduction of reliable and effective protocols to prevent and treat early and deep carious lesions with ozone applications. As a minimally invasive, safe and easy-to-use approach, ozone-therapy will provide a greater acceptance of dental treatments by pediatric patients, allowing an alternative management in less compliant and uncooperative patients as well.

Sara Alberti

CEM Curriculum: Public Health
Tutor: Prof. Paola Ferri
CoTutor: Prof. Sergio Rovesti and Dr. Loris Bonetti

PATIENT INVOLVEMENT IN NURSING EDUCATION

Background

Patient involvement in healthcare is assuming increasing importance as a key to building a partnership. Scientific evidence suggests that patient involvement leads to better outcomes in health, quality and experience of care for patients.

Patient involvement in healthcare education, in particular, is considered a powerful tool to help future health professionals develop communication and relational skills that are imperative to care quality, safety, equity and experience of care. However, preliminary research found a lack of evidence about these effects, especially in nursing education. The latest international conference on patient involvement in the healthcare professionals' education "Where's the patient's voice?" held in Vancouver in 2015 highlighted the lack of outcomes data and proposed a goal the provide further evidence on the short- and long-term effects of patient involvement in education, including patient benefits, learning outcomes, and the process by which they are acquired.

At the University of Modena and Reggio Emilia, the "Educare" project is actively aimed at promoting patient involvement in education and healthcare facilities. In this context, patients and healthcare professionals are trained together in a postgraduate course entitled "Didactic methodology for teaching medicine with patient-partners" in the first semester of the academic year 2021/22 to carry out lessons in partnership and implement a new teaching method.

Objectives

The overall objective of the research project is to promote patient involvement in undergraduate nursing education at the University of Modena and Reggio Emilia and evaluate its effects. According to the Implementation Science Framework, the first development phase for implementing evidence-based educational practice, such as teaching with patient partners, includes a literature review to understand the evidence and decide on outcomes to be examined when this new teaching method is implemented, and exploring the readiness of educators.

Therefore, the specific objectives of the first phase of the research project are:

1. To investigate the effects and experiences of patient involvement in nursing education through a systematic review.

2. Describe the experience of healthcare professionals who participated in a postgraduate course for teaching with patient-partners at the University of Modena and Reggio Emilia through a qualitative study.

Methods

To respond to the first research objective a mixed-method systematic review protocol inspired by the methodology of the Joanna Briggs Institute and the PRISMA 2020 updated guidance was developed. The sources that will be searched are Cochrane Library, PubMed, CINAHL, PsycINFO, Scopus, ERIC, Embase and Google Scholar; reference lists of all reports and articles will be searched for additional studies. The screening process and methodological assessment of included studies will be conducted independently by two researchers. The convergent integrated approach will be adopted to analyze and synthesize data.
The protocol was submitted for PROSPERO Registration. Preliminary searches and piloting of the study selection process have already been begun.

A qualitative study with an ethnographic approach will be conducted to investigate the experience of educators in training with patient partners. Ethnography is a research methodology that studies personal experiences and behaviors in a specific context to gain a deep understanding of the phenomenon in its natural setting. The eligible sample is represented by healthcare professionals who participated in the postgraduate course "Didactic methodologies for teaching medicine with patient-partners" in the 2021-2022 academic year at the University of Modena and Reggio Emilia. Data will be collected using single interviews, follow-up focus groups, participant observation and course documentation. Data will be analyzed through thematic analysis.
The protocol of this study was approved by the independent ethics committee of Area Vasta Emilia Nord.

Expected results

From the literature review, we expect to obtain integrated results about effects and experiences helpful in identifying process and outcomes measures to use in evaluating patient involvement in education.
From the qualitative research, we expect to identify elements of learning and relationship of health professionals with patient partners and possible perceived gains for professional practice useful for the implementation of the new practice in nursing education at the University of Modena and Reggio Emilia.

Lara Baschieri

CEM Curriculum: Translational Medicine
Tutor: Prof. Livio Casarini

NON-HORMONAL (NH) CONTRACEPTION BY NANOBODY-MEDIATED MODULATION OF OVARIAN GPCRS

Background

In some low- and middle-income countries, contraceptives methods are not commonly available to the population, due to practical, social, cultural and religious issues. In order to provide a safe alternative to those women who cannot or do not want to use hormonal contraceptives, we are looking for a non-hormonal method based on the use of nanobody fragments (VHH). VHH derive from camelid heavy-chain-only antibodies (HCAbs) and have already demonstrated their ability to pharmacologically modulate various G protein-coupled receptors (GPCRs) acting as allosteric modulators. An allosteric modulator is a ligand binding to a receptor site distinct from that of the endogenous agonist, regulating the hormone-specific signal. The control of ovarian function is exerted by two gonadotropin GPCRs, i.e. follicle-stimulating hormone receptor (FSHR) and luteinizing hormone/choriogonadotropin receptor (LHCGR) and G protein-coupled estrogen receptor (GPER); many signaling pathways may be activated by these receptors and was demonstrated that is possible to exert pathway-specific effects on receptor signaling by using biased allosteric modulators (BAM).

Objectives

I aim to evaluate the impact of nanobodies on FSHR-, LHCGR- and GPER-mediated intracellular signaling. These VHH were identified by collaborators from the Institut national de recherche pour l'agriculture, l'alimentation et l'environnement (INRAE) using validated synthetic libraries. First, the effect on FSHR signaling was evaluated in FSHR-expressing cells *in vitro* by using three specific nanobodies.
Goals of our experiments are: a) ensure that the VHH modulates the activity of the receptor; b) characterize nanobodies that can inhibit FSHR-mediated intracellular proliferative signals without completely depleting steroidogenesis.

Methods

Experiments were performed using two cell models: 1) transfected human granulosa-like tumor cell line (KGN), overexpressing the human FSHR (KGN/FSHR) and 2) human ovarian primary granulosa cells (hGLC) collected from donor women undergoing oocyte retrieval for assisted reproduction, which are naturally expressing the endogenous FSHR.

Cells were treated with increasing concentrations of FSH, in absence and in presence of rising doses of the three nanobodies (0-100 nM). Cyclic adenosine monophosphate (cAMP) production and β-arrestin 2 recruitment, which are intracellular signaling endpoints of FSH action, were evaluated using bioluminescence resonance energy transfer (BRET). Moreover, the impact on cell viability was assessed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Finally, the production of progesterone and estradiol was measured by homogeneous time resolved fluorescence (HTRF).
Statistics was performed using Kruskal Wallis test followed by Bonferroni post-test ($p<0.05$), implemented in the GraphPad Prism 9.0 software.

Expected results

First, I have evaluated cAMP production, as a key second messenger for steroidogenesis, modulation of pro-apoptotic signals, and regulation of mitogenic signals. It was found that one of the antibody fragments available (F8-ExpCHO) effectively inhibits FSH-induced cAMP in KGN/FSHR cells.
The second outcome was to evaluate β-arrestin 2 recruitment, which mediates FSHR internalization and may activate late-term signaling, resulting in proliferative and steroidogenic signals. Among the three nanobodies tested, only one (F8-Fc-ExpCHO) resulted to be effective in inhibiting β-arrestin 2 recruitment in KGN/FSHR cells.
Afterwards, cell viability was evaluated in KGN/FSHR cell line through MTT assay: all three compounds did not affect cell health, both in the presence and in the absence of FSH. Interestingly, 30 nM FSH decreased cell viability in the absence of VHH. However, this concentration is not physiological and is known to be harmful for cell viability. Moreover, control experiments were performed upon cell treatment with thapsigargin.
Finally, estradiol and progesterone levels were measured on hGLC using HTRF. Ideal candidates should slightly inhibit the synthesis of progesterone without decreasing levels below those of unstimulated cells. All three nanobodies did not significantly reduce the production of progesterone. Estradiol is fundamental for the progression of folliculogenesis and ideal VHH candidates should inhibit the synthesis of this sex steroid. Cell treatments were performed in the presence of androstenedione in the cell media, as a substrate for the aromatase enzyme, allowing estradiol production by granulosa cells. The highest F8-Fc-ExpCHO concentration significantly reduced FSH-induced estradiol production. More replicates will be performed to improve the statistical strength of results.
By now, F8-Fc-ExpCHO seems to be the best candidate since it shows FSHR-signaling modulation by inhibiting β-arrestin 2 and not interfering with cAMP accumulation, and it does not reduce cell viability. Future experiments will clarify its effect on steroidogenesis.

Elisa Micalizzi

CEM Curriculum: Translational Medicine
Tutor: Dr. Anna Elisabetta Vaudano

THE AMYGDALA INVOLVEMENT IN AUTONOMIC MANIFESTATIONS OF FOCAL SEIZURES AND IN PATIENTS AT HIGH RISK OF SUDEP

Background

The overall risk rate of SUDEP ("Sudden Unexpected Death in Epilepsy") is approximately 1 per 1000 patients per year in the general epilepsy population. It is known that GTCS (generalized tonic-clonic seizures) frequency is the strongest risk factor associated with SUDEP. This risk is significantly higher when GCTS occur more than three times per year. Moreover, several studies have hypothesized a clinically relevant relationship between ictal autonomic manifestations in focal seizures and the SUDEP risk. Recently, some authors have utilized advanced neuroimaging techniques to investigate brain morphometry, structural and functional connectivity modifications in SUDEP and in high-risk SUDEP patients. Many cerebral structural and functional alterations were found, involving cortical and subcortical regions. In this scenario, the amygdala seems to play a key-role due to its involvement in volumetric and connectivity significant modifications in this category of epilepsy patients. The amygdala, specifically the basolateral complex (BLA), appears also to be involved in ictal autonomic manifestations occurring in focal seizures, such as ictal central apnea (ICA) and heart rate modifications. This observation raises the hypothesis that the amygdala, and particularly the BLA, might represent a biomarker of ictal autonomic manifestation. To date, however, neuroimaging studies addressing the role of the amygdala in patients with autonomic symptoms with respect to the SUDEP risk are lacking, despite the known relationship between those events. As one possible explanation, while the heart rate is constantly recorded by electroencephalogram (EEG) during ictal seizures, the respiratory monitoring is frequently missed in most Epilepsy Monitoring Units. In this project we propose to investigate the morphometric and functional engagement of the amygdala in patients with ictal autonomic manifestations and patients at high risk of SUDEP. Thanks to advanced methodological approach, the amygdala will be segmented in subnuclei/complexes, in order to deeply investigate the specific role of each amygdala region.

Objectives

The aims of the present project are: 1. to reveal which amygdala nucleus/complex is involved in autonomic ictal manifestations (like ICA) and in high-risk SUDEP patients 2. to provide additional knowledge regarding the brain circuitries engaged by ictal apnea; 3. to establish an imaging-guided link between autonomic symptoms during seizures and the SUDEP risk.

Methods

The present proposal will adopt advanced morphometric (estimation of cortical thickness and volumes of subcortical structures, structural connectivity) and functional imaging approaches (resting state connectivity study). Study population will include, both retrospectively and prospectively, patients with focal seizures referring to our Epilepsy Monitoring Unit and Epilepsy Center for diagnostic purposes. As regards the patients with ICA, inclusion criteria will be: (i) age ≥ 14 years; (ii) a diagnosis of focal epilepsy; (iii) having at least one seizure recorded with video-EEG combined with cardiorespiratory polygraphy during the admission at our epilepsy monitoring unit; (iii) having performed a volumetric 3D brain MRI; (iv) having performed an EEG-fMRI study (optional). For the high-risk SUDEP patients, inclusion criteria will be: (i) age ≥ 14 years; (ii) a diagnosis of focal epilepsy with GTCS recurrence more than 3 per year; (iii) having performed a volumetric 3D brain MRI (iv) having performed an EEG-fMRI study. For both populations, the brain MRI 3D volumetric scans will be post-processed with FreeSurfer (version 7.1) to extract cortical thickness and cortical and subcortical volumes. Additionally, a segmentation of the amygdala subnuclei and a graph analysis of structural connectivity between the amygdala and the rest of the brain will be performed. The fMRI data will be analyzed in order to verify the whole-brain functional connectivity pattern of the amygdala (as region of interest, ROI) and its nuclei in ICA and high-risk SUDEP patients. Structural and functional data from a population of healthy subjects will be collected and analyzed with the same methodological approach and treated as controls.

Expected results

We expect to observe amygdala morphometric and structural and functional connectivity modifications in SUDEP/high-risk SUDEP patients, thus confirming and expanding the literature data. We also anticipate we will find structural and functional connectivity alterations involving the amygdala also in the subgroup of ICA patients, allowing us to identify useful neuroimaging biomarkers of ICA in relation to the SUDEP risk.

Giulia Rovesti

CEM Curriculum: Translational Medicine
Tutor: Prof. Massimo Dominici

DEVELOPING A PLATFORM FOR IMMUNOTHERAPY OF GLIOBLASTOMA MULTIFORME USING GENETICALLY ENGINEERED TCR-T CELLS: IDENTIFICATION AND IMMUNOGENICITY ASSESSMENT OF CANDIDATE NEOANTIGENS

Background

Glioblastoma multiforme (GBM), the highest grade (WHO IV) astrocytoma, is the most prevalent type of primary central nervous system tumor in adults. With a 1-year survival rate of 37.2% and a 5-year survival rate of 5.1% and with standard treatments limited both in number and in efficacy, GBM eagerly compels substantial therapeutic advances. In the context of cancer immunotherapy, which aims at boosting or restoring the immune system function against the tumor, adoptive cell therapy (ACT) with genetically modified lymphocytes redirected towards cancer cells is nowadays emerging as a novel promising strategy to treat brain cancers. Specifically, TCR (T cell receptor)-T cells are T lymphocytes that are genetically modified to express a TCR, a physiologically occurring protein able to confer recognition to a broad range of targets, with cancer neoantigens being the most relevant targets for a successful TCR-T cell based therapeutic approach. Neoantigens are, indeed, “foreign”, tumor-specific antigens, arising when a non-synonymous mutation or a structural change in the DNA sequence generate a modified peptide that is then processed and presented by Major Histocompatibility Complex (MHC)/Human Leukocyte Antigens (HLA) molecules. Peptide-MHC complexes that are able to induce TCR activation and T cell proliferation upon recognition, thus eliciting an immune response potentially resulting in tumor cells killing, are considered immunogenic and represent the ideal targets towards which genetically modified, adoptively transferred T cells should be redirected.

Objectives

Aims of the study are to analyze the tumor mutational landscape of GBM samples, to identify candidate neoantigens that can be targeted by TCR-T cells, to test their immunogenicity using T cells from healthy blood donors and, possibly, to isolate neoantigen-reactive T cell clones.

Methods

This is a collaborative project between the University of Modena and Reggio Emilia (Italy) and Karolinska Institutet (Sweden). Five patient-derived primary GBM cell lines, established in the Laboratory of Cellular Therapies at the Department of Medical and Surgical Sciences for Children and Adults (University of Modena and Reggio Emilia) upon receiving of surgical samples, are provided to preGMP (pre-Good Manufacturing

Practice) facility at ANA FUTURA in Campus Flemingsberg (Karolinska Institutet). Tumor and matched normal counterpart whole exome sequencing (WES) and tumor RNA sequencing (RNAseq) are carried out by Fulgent Genetics with Illumina Miseq. The variant calling from WES and RNAseq data to identify tumor-specific DNA alterations is performed through two approaches, depending on whether normal reference genome (GBM patient's peripheral blood mononuclear cells, PBMCs) is available or not: PIOR software, developed by NEOGAP Therapeutics (Center for Molecular Medicine, Karolinska Institutet), or support from bioinformatics company Ardigen, respectively. The immunogenicity assessment of the identified candidate neoantigens is performed on healthy donors' peripheral blood lymphocytes (PBLs), by means of co-culturing neoantigen-loaded antigen presenting cells (APCs), in the form of, specifically, peptide-loaded B cells, with their respective T cells, and checking the T cells for upregulation of 41BB (CD137) activation marker. Cultures positive for 41BB are then sorted using a flow cytometer and sorted cells are expanded to T cell clones before TCR sequencing is performed.

All planned procedures are approved by the institutional review board of involved countries.

Expected results

This study faces two current needs: to deepen the knowledge in regard to the immunological landscape of glioblastoma multiforme and to offer an alternative treatment for GBM patients that now have little to no options.

We expect our project to provide the proof of concept that it is possible to identify candidate neoantigens through a careful tumor sequencing data mining and to isolate neoantigen-reactive T cell clones from PBLs of HLA-matching healthy donors, after *in vitro* stimulating them with tumor neoantigens. These represent the initial essential steps that will lead to subsequent isolation of neoantigen-specific TCRs from 41BB+ T cells and to their functional validation. In the long term, we expect to continue with preGMP process development and GMP production of a TCR-T cell based therapeutic product and initiation of a clinical trial for GBM patients.

Eleonora Forabosco

CEM Curriculum: Translational Medicine
Tutor: Dr. Vittorio Checchi

INSTRUMENTAL AND VISUAL COLOR MATCH EVALUATION OF SINGLE SHADE COMPOSITE RESINS IN RESTORATIVE DENTISTRY: AN IN VITRO STUDY

Background

The most complicated challenge in restorative aesthetic dentistry is being able to obtain a correct color match between composite restorations and the surrounding tooth. Color matching depends on various chromatic aspects related to both composite and tooth, which are responsible for the difficulty of achieving this match. These properties are value, chroma and hue, opalescence and translucency, transmission and diffusion of light, and surface texture. Recently, innovative single-shade composites have been introduced in the market, being supposedly able to match all VITA Classical shades, from A1 to D4.

Objectives

The aim of this in vitro study was to evaluate the instrumental and visual color match with the surrounding tooth of four different single-shade resin-based composites used to restore artificial cavities performed on human sound extracted teeth.

Methods

Eighty human extracted posterior sound teeth were used in this study. On each buccal side of the teeth, 2mm above the cemento-enamel junction (CEJ), a standardized V class cavity (2mm depth, 2mm high, and 4mm width) was performed. Teeth were randomly divided into 4 groups, 20 teeth in each group, and were restored through a single increment of the corresponding one-shade composite resin. Group A: Omnichroma (Tokuyama Dental, Tokyo, Japan), group B: Venus Diamond One (Kulzer, Hanau, Germany), group C: Clearfil Majesty ES-2 Universal (Kuraray Medical Inc., Tokyo, Japan), and group D: Essentia Universal (GC Corporation, Tokyo, Japan), with their correspondent adhesive systems: Universal Bond (Tokuyama), iBond Universal (Kulzer), Clearfil Universal (Kuraray) and G2 Bond Universal (GC). VITA color, CIELAB color coordinates (L, a, b), chroma, and hue were recorded using an intraoral spectrophotometer (VITA Easyshade V, VITA Zahnfabrik, Bad Sackingen, Germany) 24 hours after restoration both in the center of the restoration and on the tooth 1.5mm away from the margin of the restoration. ΔE_{ab} and ΔE_{00} after 24h were calculated and subjected to statistical analysis. After instrumental evaluation, visual color assessments were carried out by 16 dental-professional observers. The color differences between each tooth and restoration were graded from 0 to 4, using the scale based on a previous study where level “0” means excellent match; 1, very good

match; 2, not so good match (border zone mismatch); 3, obvious mismatch; and 4, huge (pronounced) mismatch.

Expected results

Following the instrumental analysis performed through the use of a spectrophotometer, we expect the VITA color, CIELAB color coordinates, chroma, and hue values related to the four tested composites to be as close as possible to those related to the teeth color on which the reconstructions were performed. We also expect that the color differences between teeth and composite restorations (ΔE_{ab} and ΔE_{00}) fall within the threshold of acceptability and perceptibility defined in the literature.

Following the visual analysis instead, we expect to obtain values from 0 to 1 from the most experienced observers, those with more years of clinical experience.

Michela Lai

CEM Curriculum: Translational Medicine
Tutor: Prof. Caterina Longo

DERMOSCOPIC AND REFLECTANCE CONFOCAL MICROSCOPY FEATURES OF NODULAR MELANOMA: A PRELIMINARY STUDY

Background

Nodular melanoma is one of the most aggressive variants of cutaneous melanoma. It accounts for about 15-30% of all melanomas but it is responsible for almost 40% of all melanoma deaths. Clinically it may lack the classical ABCDE (Asymmetry, irregular Borders, Diameter >6 mm, multiple Colors, Evolution) rule features, being able to mimic numerous other skin lesions both benign and malignant.

Non-invasive diagnostic techniques, such as dermoscopy and reflectance confocal microscopy (RCM), have been proven to be able to improve diagnostic sensitivity and specificity for the early diagnosis of cutaneous melanoma. Numerous diagnostic features and algorithms have been validated for the dermoscopic and RCM diagnosis of the most common variants of cutaneous melanoma, such as superficial spreading melanoma or in situ melanoma, but few data are available, to date, for the diagnosis of nodular melanoma, particularly for its hypopigmented or amelanotic variants.

Objectives

The aim of our study is to describe clinical, dermoscopic and RCM features of pigmented and hypopigmented/amelanotic cutaneous nodular melanoma, and their histopathological correlates.

Methods

A retrospective, observational, cohort study was performed at the Skin Cancer Center of Reggio Emilia. All cases of nodular melanoma were retrieved from our database from January 1, 2011 to December 31, 2021. Only cases with complete clinical, dermoscopic and RCM images were included.

Patients records were reviewed and demographical and clinical data were obtained. These data include: age at diagnosis, sex, comorbidities, tumor anatomical location, diameter, clinical, dermoscopic, RCM and histopathological features, clinical stage according to the 8th edition of the AJCC-TNM classification, treatment, follow up time and prognosis.

Standard descriptive statistics will be used to summarize the data. Descriptive data, expressed in mean values and percentages, will be generated by pooling data of the included patients. Statistical analysis will be performed using IBM SPSS 27.0 package (Statistical Package for Social Sciences, IBM SPSS Inc., Chicago, III.) and Microsoft Excel 2019 were used for all statistical analysis.

Expected results

The results of our study confirm that RCM can provide diagnostic clues for nodular melanoma, especially when dealing with clinically difficult-to-recognize hypopigmented variants. Further studies are needed to validate our results and to determine the diagnostic accuracy of the identified RCM features.

Angela Contri

CEM Curriculum: Public Health
Tutor: Dr. Stefania Costi
CoTutor: Dr. Isabella Campanini

CANCER SURVIVORS: THE CHALLENGE OF CHRONICITY IN PHYSIOTHERAPY AND THE NEW FRONTIER OF ASSISTED SELF-CARE

Background

Recently, there has been a spate of interest in how to improve the quality of life of Cancer Survivors (CSs), whose number is constantly growing in Italy and the other Western Countries. Thanks to the numerous advancements in therapy and diagnosis, many forms of cancer, previously considered "incurable diseases", have acquired a life expectancy not very different from general population, sex and age matched.

The healthcare needs associated with cancer survival are often complex and require specific and personalized approaches. These involve a wide range of individualized interventions, delivered in various settings and by different health care professionals working in an integrated way in what has been defined a defined a “Survivorship Care Plan” (SCP).

Nonetheless, the adoption of SPCs adapted to our context is still a sporadic phenomenon.

Objectives

The aim of this project is to investigate the existing Survivorship care organizational models and the health needs of CSs in order to:

1 - Identify the local ones applicable in our context

2 - Derive an SCP that is based on the health needs of the CSs

3 - Define the involvement of health care providers, and of Physical Therapists in particular.

There will also be a special focus on Breast Cancer Survivors and their possibly post-surgery sequelae, such as lymphedema and web axillary syndrome, to identify a clinical surveillance model for early detection of secondary lymphedema, analyse treatment options and develop training materials for self-management of this sequelae.

Methods

This project represents the first phase of development a complex intervention, according to the guidance proposed by the Medical Research Council.

The setting of the study is the Local Health Authority - IRCCS of Reggio Emilia and its province.

The population involved in the qualitative data collection is represented by the CSs in follow-up at the USL-IRCCS of Reggio Emilia structures, by their caregivers, the professionals involved in their care (medical doctors, health professionals, etc ..) and by members of voluntary associations in the oncology field.

We started from an assessment of the rehabilitative needs of the CSs, realised through a literature review of systematic reviews to detect the most valid evaluation tools for the healthcare needs of CSs. Analysing their dimensions, domain assessed and psychometric properties, we will identify the instruments that will better fit a target population of CSs suffering from non-cutaneous oncological disease with a 5-year survival greater than or equal to 65% (breast, prostate, thyroid, colorectal cancer, lymphomas, multiple myeloma diagnosed at an early stage).

A qualitative data collection will be made to verify the completeness of the identified tools, in relation to the health needs that will be expressed by the CSs participating in the study. The qualitative data collection will be made through conducting focus groups with patients, caregivers, and professionals.

At the end of this stage, we will compare and integrate the qualitative data collected in the local context and these highlighted by the literature review to define a list of relevant needs of CSs.

Parallel to this, a specific focus on the rehabilitative needs of Breast Cancer Survivors will be made. Starting from a literature review to describe the current rehabilitative treatment for common post-surgery issues, such as web axillary syndrome and lymphedema, the frame of a clinical surveillance model for early detection of secondary lymphedema and its treatment options will be depicted.

Expected results

The primary result of this project will be represented by the development of the complex intervention (the organizational model of survivorship care), potentially applicable to the local context, described in its components, processes and potential outcomes, the feasibility of which will be verified in a following phase.

Lorenzo Tagliazucchi

CEM Curriculum: Nanomedicine, Medicinal and Pharmaceutical Sciences
Tutor: Prof. Maria Paola Costi

DEVELOPMENT OF SELECTIVE MOLECULES AGAINST TEAD TRANSCRIPTION FACTOR ACTIVATION AND IMPACT ON THE UP-STREAM EFFECTOR KINASES OF THE HIPPO PATHWAY TO HALT CANCER CELL GROWTH

Background

The Hippo pathway is a finely regulated kinase cascade which controls cell growth and apoptosis, whose dysregulation generates uncontrolled cell hyperproliferation triggered by up-regulation of antiapoptotic genes. hTEAD1-4 (human Transcriptional Enhancer Associated Domain) - the downstream effector of the path – is frequently mutated in several solid tumors, causing its nuclear permanence and the up-regulation of anti-apoptotic and proliferative genes [1]. Five main kinases modulate TEAD transcription factor activity: MST, SAV, MOB1A/B, LATS and YAP, the last one responsible for YAP:TEAD (YT) complexation that suppresses gene transcription. Recent efforts have been pulled out to investigate YT mechanism of association, and drug discovery attempts in designing strategies to halt the Hippo Pathway. However, protein-protein disruption is a difficult task, no clear description of the kinase modulation in the network exists, and still no clinical candidate has been generated [2]. Thus, a better description of the kinome around LATS may lead to the identification of more specific chemical probes and drug candidates that can increase YAP phosphorylation with anti-TEAD compound, but also from upper stream effectors.

Objectives

The general objective of this work is to identify newer and more specific inhibitors for the downstream of TEAD transcription factor, able to prevent the onset of metastatic cancer.

Three specific objectives of this study include: i. Characterization of the Hippo phosphoproteome modulation through targeted approaches, focused on the six above-mentioned proteins, by LC-MS and bottom-down phosphoproteomics; ii. Mechanism of drug:target interaction and cellular activity of the best dimer disrupters of YT, that will be obtained from the molecular optimization of two well established hits; iii. Characterization of the MOB1A/B protein as potential new target for the aim. The overall project is part of the National Grant entitled “HipTargeting-TEAD ligands prevent YAP binding and combat colorectal cancer growth and resistance”, assigned to Prof MP Costi.

Methods

A medicinal chemistry program has started to synthesize a library of indazole and oxadiazole-like compounds from the primary hTEAD hit derivatives (S049 and D361 respectively, from the Drug Discovery and

Biotechnology Lab) previously obtained from molecular modelling studies. hTEAD4 protein (434aa) displays a highly lipophilic surface in which three main sites can be distinguished according to their physical chemical characteristics. Interface 3, as being able to dimerize with Yap Binging Domain (YAP*) Ω-loop*, is the most interesting from the pharmaceutical aspect, and its YBD is already available as 6-His-Tagged recombinant protein.

An isobaric labelled protocol with peptide enrichment will be applied to in vitro lysates of HCA46 (5-FU sensitive) and HT29 (5-FU resistant) cells to identify and quantify the phosphorylation sites by HRMS (Nano-LC Orbitrap Exploris, UniMORE), after the administration of our molecules. TEAD-ligand interaction has been examined with molecular modelling approach (PyMol and Maestro) and characterized with analytical and biophysical assays. A Forster Resonance Energy Transfer (FRET) displacement experiment between fluorescein labelled protein and a rhodamine tagged YAP-like peptide is helping to determine the compounds affinity, along with isothermal microcalorimetry titrations (micro-ITC), HPLC-ELSD and top-down MS experiments. Preliminary studies on FRET assay set-up, suggest that the fluorescence resonance method and the investigation of TEAD:ligand interactions through anisotropy fluorescence is feasible and already applicable to the recombinant protein.

Expected results

Two main pharmacophores have been developed as cores for molecular modifications of the hits targeting hTEAD4 interface 3 (D and S series, respectively). Their cellular activity has been demonstrated by synthesizing a small library designed on the hits, which was tested on A2780 cells. Five compounds demonstrated an IC50 >50% after 40uM single administration, and most of the molecules were able to decrease the expression of YAP and hTEAD genes (qPCR mRNA direct quantitation). We have achieved biophysical screening to test their *Kd's,* which suggests they can bind hTEAD in the low-micromolar range, and actively disrupt YAP-TEAD dimer.

Next steps will include performing a total cell proteomics and phosphoproteomics experiment to identify the kinases directly affected by our molecules, the development of an orthogonal microcalorimetric assay to confirm their affinity, and description of the thermodynamic binding modes of the hits.

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Maria Elena Nizzoli

CEM Curriculum: Translational Medicine
Tutor: Prof. Stefano Luminari

THE ROLE OF LIQUID BIOPSY IN FOLLICULAR LYMPHOMA

Background

Follicular lymphoma (FL) represents the most common form of indolent lymphoma and the second most frequent subtype of nodal lymphoid malignancy in western countries. Despite relevant improvements in overall survival (OS), a significant proportion of patients still experiences a dismal outcome with a 5y OS of less than 50%. Recent evidences have revealed that FL is featured by a complex genetic background and displays both spatial and temporal heterogeneity.

Liquid biopsy of circulating tumor DNA (ctDNA) consists in the isolation of tumor-derived components from peripheral blood and their genomic assessment through polymerase chain reaction (PCR)-based or next-generation sequencing(NGS)-based technologies. These techniques enable either a wide genomic profiling in order to evaluate molecular alterations with a yet unknown prognostic meaning, or a targeted assessment of a limited number of molecular alterations. Requiring a non-invasive sampling, these analyses can be carried out throughout the disease course, representing an attractive tool to investigate clonal patterns and determinants of poor outcome during disease evolution.

Objectives

The study aims firstly at evaluating ctDNA concentrations in FL, investigating potential correlations with clinical features. We will further assess the ability of ctDNA to resemble the mutational profile of paraffin embedded tumor biopsy. Lastly, the study aims at identifying additional genomic information derived from ctDNA, thus evaluating the role of liquid biopsy in representing the whole disease burden in FL.

Methods

BIOLIQUID_2021 is an ongoing observational monocentric study enrolling patients diagnosed with FL at the hematology department of IRCCS Arcispedale Santa Maria Nuova (Reggio Emilia). The study received approval by the local ethical committee on 27th May 2021. Both newly diagnosed patients and relapsed patients are recruited. Among inclusion criteria, the availability of formalin-fixed paraffin embedded tumor biopsy at diagnosis/relapse together with clinical and radiological data is mandatory. Patients with FL grade 3B or concurrent active viral infection (e.g. HIV, HCV, HBV) are excluded.

The enrollment of 70 patients is expected within two years. Peripheral blood (PB) is collected in BCT tubes within 6 months from tissue biopsy and before any therapeutic intervention. Both ctDNA and T-lymphocytes are promptly isolated from PB, whereas FFPE-DNA is extracted from paraffin embedded tissue biopsy. ctDNA is firstly quantified through ProNex DNA QC assay system. This quantification is necessary to establish median ctDNA concentrations in plasma and to define the sensitivity of the assay.

gDNA derived from T-lymphocytes is employed to distinguish somatic from germline mutations. ctDNA, FFPE-DNA and gDNA are analyzed through CAPP-seq (Cancer Personalized Profiling by deep-Sequencing) with a personalized panel (SeqCap EZ Choice Library, Roche Nimblegene). cfDNA and FFPE-DNA are firstly compared to gDNA and subsequently compared to each other in order to assess mutational concordance rate and potential additional mutations in ctDNA.

Expected results

A total of 48 patients have been recruited so far. Paired samples of ctDNA, FFPE-DNA and gDNA have been stored and clinical data have been collected. After an accurate literature review, a personalized CAPP-seq panel analyzing 164 genes has been designed and next generation sequencing analysis are planned.

Agnese Razzoli

CEM Curriculum: Translational Medicine
Tutor: Dr. Chiara Marraccini

BIOCHEMISTRY APPROACES TO INVESTIGATE BREAST CANCER PHENOTYPE

Background

Breast cancer is a complex and multifaceted disease that includes neoplastic disorders affecting the breast tissue and showing heterogeneous morphological, molecular and clinical attributes. After histological assessment, the tumor is graded according to its differentiation and staged using the Tumor-Node-Metastasis (TNM) system, which uses both clinical and pathology information such as tumor size, lymph node status and spread to metastatic sites. Furthermore, the evaluation of four biomarkers is used at breast cancer diagnosis: the expression of estrogen receptors (ER), progesterone receptors (PG), over-expression of human epidermal growth factor receptor 2 (HER2) and levels of a nuclear non-histone protein (Ki-67) associated with cellular proliferation. Based on these markers it is possible to identify some main breast cancer subtypes: luminal A (highly ER and PG positive with low Ki-67 levels); luminal B HER2-positive (ER and PG positive, HER2 positive with high Ki-67 levels); luminal B HER2-negative (ER and PG positive, HER2 negative with high Ki-67 levels); and triple negative (ER, PG and HER2 negative). Showing high proliferative index, poor responsiveness to therapy, high frequency of distant metastasis and probability of relapse in the first five years after surgery, luminal B HER2-negative represents one of the most aggressive subtypes. Clinical treatment consists of surgical resection that can be followed by adjuvant hormone therapy and chemotherapy (taxanes and anthracyclines), whose real advantage in the early stages is still a subject of debate. In fact, no clinical characteristic accurately predicts disease aggressiveness, therapy response, the chance of recurrence or metastasis, or long-term patient outcome; therefore, it is extremely challenging for clinicians to predict which patients would benefit from a specific intervention, minimize over-treatment and provide the appropriate therapy. Although some gene expression profiling tests have been developed to support both the prognosis and the definition of the treatment regimen (e.g. Oncotype DX and Mammaprint), they are neither economical nor practical. Luminal B HER2-negative biochemistry studies may provide not only novel more powerful protein biomarkers, easily and cost-effectively assessable by immunohistochemistry, but also information about cancer molecular mechanisms not accessible by gene expression analysis.

Objectives

This project aims at:

- Identifying specific and effective immunohistological markers to be routinely used in clinic to stratify the risk of recurrence in luminal B HER2-negative patients and help discriminating those that would benefit from the administration of the adjuvant therapy;
- Discovering novel targets for innovative luminal B HER2-negative pharmacological treatments, which would help to reduce the risk of relapse;
- Understanding the luminal B HER2-negative heterogeneity between patients at a molecular level.

Methods

Tissue samples from four groups of luminal B HER2-negative patients, with homogeneous diagnosis and positivity to the main predictive markers but having different prognosis, will be retrieved from the Biobank of the AUSL-IRCCS di Reggio Emilia: i) patients not treated with the adjuvant therapy and recurrence-free ten years after the surgery; ii) patients not treated with the adjuvant therapy that developed distant metastasis; iii) patients treated with the adjuvant therapy and recurrence-free ten years after the surgery; iv) patients treated with the adjuvant therapy that developed distant metastasis.

Tissues will be compared through untargeted proteomic and phosphor-proteomic approaches. The proteins and pathways found to be differentially expressed between the four groups will be validated first with a targeted proteomic approach and then with immunohistochemistry, immunofluorescence and RNA-scope on contiguous sections from the same tissues. Next, the proteins emerging from the targeted analysis as potentially useful to stratify the patient's groups will be analyzed by immunohistochemistry in a larger cohort of stage I-IIIc Luminal B HER2-negative patients.

In parallel, an *in vitro* study on a breast cancer cell line and a non-tumorigenic epithelial breast cell line will be performed to validate the findings and elucidate the molecular mechanisms involved through up/down-regulation of the identified proteins and pathways.

Expected results

This study will provide in-depth information about the cellular mechanisms that characterize Luminal B HER2-negative breast cancer and its heterogeneity. It will allow the identification of a protein panel correlating with the different phenotypes, which will be useful to stratify patients based on the risk of recurrence and to identify those who should be treated with the adjuvant therapy. The study will also point out novel targets for innovative pharmacological therapies.

Jacopo Francesco Imberti

CEM Curriculum: Translational Medicine
Tutor: Prof. Giuseppe Boriani
CoTutor: Prof. Gregory YH Lip

SEARCH AHRE 4 STROKE
proSpective resEARCH on Atrial High Rate Episodes for STROKE reduction

Background

Atrial High Rate Episodes (AHRE) are a relatively new clinical entity currently defined as episodes of atrial tachycardia lasting ≥ 5 minutes (min) with an atrial rate ≥ 175/min detected through the capability of continuous monitoring and data storage of cardiac implantable electronic devices (CIEDs) with atrial sensing, occurring in patients with no history of clinical atrial fibrillation (AF) or AF related symptoms and with no AF at routine 12-lead ECG. As more patients will have CIEDs, AHREs will be more frequently recognised and, consequently, will represent a significant future management challenge. Their estimated incidence approaches 20-30% after 1 year of average follow-up and their prevalence is expected to increase with population aging and comorbidities. Moreover, AHREs are associated with a 2.5-fold higher risk of thrombo-embolic events, carrying significant morbidity and mortality and high social costs. This thrombo-embolic risk increases with increasing burden of AHREs, but robust and precise data on risk stratification parameters are limited. The clinical management of AHREs, in particular with regard to prophylactic treatment with oral anticoagulants, remains uncertain and heterogeneous, highlighting the need for newer, more solid scientific evidence.

Objectives

- To investigate the clinical epidemiology of AHREs in large clinical centres in Italy and UK.
- To create risk stratification models for incident clinical AF, systemic thromboembolism, and all-cause death.

Methods

Initially, we will run a retrospective, single center, observational study enrolling consecutive patients with a CIED detected AHRE presenting at our Institution. Inclusion criteria will be as follows: 1) permanent PM, ICD, or CRT-P/D (but not loop recorder) capable of detecting AHRE, 2) at baseline assessment, documentation of at least one episode of AHRE of 5min-23h 59min in duration with average atrial rate ≥ 175/min documented by the CIED at any time prior to enrollment visit, 3) age ≥ 18 years. CIED-recorded AHRE tracings will be visually inspected and adjudicated by two cardiologists independently to avoid false positives; disagreement will be resolved by consensus-based discussion. The only exclusion criteria will be clinical AF documented by

surface ECG (12 lead ECG) or an ECG strip (Telemetry, Holter) lasting ≥ 30 seconds. We will collect patient baseline characteristics, echocardiographic and ECG data, baseline and follow-up device interrogations and clinical outcomes (clinical AF, death, stroke/systemic thromboembolism, major bleedings, and death). Data will be obtained from electronic and paper medical records, including PM/ICD follow-up charts and documented printout of device interrogations and will be stored in a customized, prespecified database. Then, we will merge our database with a similar one enrolling patients form Liverpool, UK. Risk stratification models will be created using clinical and machine learning approaches.

Expected results

A greater understanding of AHREs epidemiology and the development of robust risk stratification models will identify high-risk patients on which more resources for closer follow-up and aggressive treatment may be worth investing in. The prospective evaluation of different disease management strategies (particularly oral anticoagulants) on patients' outcome will contribute to identify the best clinical approach to care for these patients, with implications for morbidity and mortality. This registry will be useful also to verify and implement guideline-directed medical therapy. During the first months of PhD, we already published a preliminary analysis on 104 retrospectively enrolled AHRE patients. In our paper, we report on the clinical characteristics and clinical course of patients with AHRE and risk factors for transition to long-lasting AHRE and/or progression to clinical AF.

Luca Spaggiari

CEM Curriculum: Public Health
Tutor: Prof. Eva Pericolini

MODULATION OF THE MULTISPECIES RESIDENT MICROBIOTA: AN INNOVATIVE AND PERSONALIZED THERAPEUTIC STRATEGY FOR THE TREATMENT OF VAGINAL INFECTIONS DUE TO *CANDIDA ALBICANS*

Background

Vulvovaginal candidiasis (VVC) is a common infection of vaginal mucosa due to several species of *Candida*, most frequently caused by *C. albicans*. This infection can become recurrent (RVVC) and severely impair the quality of life of susceptible women. Overreaction of the immune system together with the mucosal damage induced by *C. albicans* leads to local inflammation and symptomatic disease. The current therapeutic approach involves the use of antifungal drugs that relieve the symptoms but are unable to eradicate the problem. It is well known that *Candida* can also colonize the vaginal mucosa of healthy women as part of the resident microbiota without causing pathology. Hence, the event that triggers the switching of *C. albicans* from a harmless commensal to a virulent pathogen, and consequently the onset of VVC, is yet unraveled. Molecular studies by Ravel et al. (2011) have allowed to cluster the vaginal microbiota of healthy adult women into several community-state-types (CST). Even though the exact number of these groups has not been well defined yet, it is clear that the microbial species isolated are mostly Lactobacilli, in particular the species *L. crispatus*, *L. gasseri*, *L. iners* and *L. jensenii*. According to the available data, vaginal microbiota of European women is dominated by *L. crispatus* (45.4% - CST group I), *L. gasseri* (8.2% - CST group II), *L. iners* (26.8% - CST group III), no Lactobacilli (10.3% - CST group IV), and *L. jensenii* (9.3% - CST group V). Notwithstanding the absence of a vaginal microbiota dominated by the *Lactobacillus* species does not directly imply a disease condition, it has been demonstrated that *Lactobacillus spp.* provide a barrier against the invasion of pathogenic microorganisms such as *Candida*. Therefore, a *Lactobacillus*-dominated microbiota could be considered a good biomarker of vaginal eubiosis and the altered composition of a lactobacilli-dominated microbiota could favor the *Candida* to switch from a harmless commensal to a virulent pathogen. However, specific studies concerning the role of a lactobacilli-dominated microbiota in counteracting the onset of VVC are still lacking.

Objectives

My PhD project aims to evaluate the *in vitro* capacity of typical vaginal lactobacilli and of an artificial lactobacilli-dominated microbiota (produced according to the CST described by Ravel in healthy women) to counteract *Candida* virulence and its ability to induce damage and inflammation of vaginal epithelial cells. This will provide useful information to understand *Candida*-epithelium interplay in the specific microbial

ecosystem and possibly provide new insights on the pathogenic mechanisms responsible for the VVC onset. In particular, the specific objectives of this project are:

Aim 1 – "Analysis of the interaction of lactobacilli with Epithelial cells" (FIRST YEAR OBJECTIVE): to evaluate the *in vitro* capacity of the four different lactobacilli to adhere, grow, and be tolerated by the epithelium and to maintain/improve the epithelial barrier integrity.

Aim 2 – "Host response to *Candida* in the presence of the lactobacilli and the artificial vaginal microbiota": to evaluate the capacity of the four different lactobacilli and of the artificial vaginal microbiota to modulate the epithelial response to *Candida in vitro* (cell damage, loss of barrier integrity, epithelial shedding, production of cytokines and chemokines, inflammasome activation and neutrophils chemotaxis).

Aim 3 – "*Candida* virulence in the presence of the lactobacilli and the artificial vaginal microbiota": evaluate the capacity of the four different lactobacilli and the artificial vaginal microbiota to counteract *Candida* virulence *in vitro* (*Candida* adhesion capacity, translocation, growth, hyphae production, releasing of Quorum Sensing molecules, β-glucan unmasking, expression of virulence genes and co-aggregation capacity with *Lactobacillus*).

Aim 4 – "RNA-sequencing analysis during epithelial cells infection with *Candida* in the presence of the artificial vaginal microbiota": identification of possible intracellular pathways differentially modulated after epithelial cells infection with *Candida* in the presence of the artificial vaginal microbiota.

Methods concerning preliminary experiments

The *in vitro* experiments were performed by establishing an epithelial cell monolayer starting from the A-431 vaginal epithelial cell line (ATCC CLR-1555). The epithelial cells monolayer was infected with the four different *Lactobacillus* spp (*L. crispatus* ATCC 33820, *L. iners* ATCC 55195, *L. jensenii* ATCC 25258, *L. gasseri* ATCC 33323). Then, bacterial adhesion and growth were analyzed by means of colony forming units (CFU) counts after 90 min and 24-48h of incubation, respectively, at 37°C plus 5% CO_2. Cell-damage was kinetically evaluated by spectrophotometric quantification of lactate dehydrogenase (LDH) release in culture medium by specific kit (Abcam).

Preliminary Results

Our preliminary results show that all the lactobacilli tested are well tolerated by the vaginal cells. Indeed, none of the lactobacilli were able to induce cell damage. Moreover, all the lactobacilli have been shown to adhere/colonize the vaginal cells monolayer and consistently grow. Collectively, our preliminary results suggest that we have established a feasible experimental protocol that will allow us to produce a more complex microbial ecosystem ultimately allowing us to analyze the role of the lactobacilli-dominated microbiota in counteracting *Candida* virulence.

Leonardo Bernal

CEM Curriculum: Nanomedicine, Medicinal and Pharmaceutical Sciences
Tutor: Prof. Giulio Rastelli

DRUG REPURPOSING AGAINST ADVANCED-STAGE PROSTATE CANCER THROUGH BIG DATA ANALYSIS AND ARTIFICIAL INTELLIGENCE

Background

With more than 1.5 million new annual cases, prostate cancer (PC) is the second most common type of cancer in men.[1] Especially at the initial stage, therapeutic treatments based on androgen deprivation have shown promising results. Recently, drugs such as docetaxel and cabazitaxel with therapeutic benefits for castration-resistant prostate cancer (CRPC) have also been approved.[2] However, most of the currently available treatments showed limited efficacy in patients with advanced CRPC, mainly due to drug resistance.[3] Development of a new drug is a time-consuming and highly costly process. In recent years, drug repurposing has emerged as an alternative strategy to the identification of new medications, allowing to circumvent some of the issues related to the early stages of drug discovery. In this context, computational approaches have been playing a central role, being able to exploit the enormous amounts of data daily reported in chemical and biological databases.[4] The application of computational approaches such as artificial intelligence (AI) algorithms (*e.g.*, machine learning, ML) to the analysis of such information, has demonstrated to: i) provide valuable insights on the mechanism of action of drugs; ii) help a better understanding of the targets involved in diseases; (iii) help identifying valuable candidates for drug repurposing.[5] On these premises, the application of AI approaches should facilitate the discovery of novel treatments against PC among already reported compounds.

Objectives

In the first year of my Ph.D., I aimed at developing a computational workflow that allows efficient exploitation of data reported in different public databases. The generated data will be processed through machine learning, chemoinformatic, and molecular modeling approaches for the repurposing of compounds against PC. Collaborations necessary for the project development are already in place.

Methods

Bioactivity data reported for a set of selected PC cell lines were downloaded from **ChEMBL**, retaining only those expressed as IC_{50}, EC_{50}, or GI_{50} for a total of 54871 unique compounds. Then, the dataset was filtered to retain **ChEMBL** compounds with bioactivity values between 0 to 1 μM on at least one PC cell line, and molecular weight between 180 and 850 Da (2706 unique ligands). Besides, compounds reported in the **DrugBank** database were downloaded and associated with activity data, therapeutic and target indications

from **ChEMBL** and **Therapeutic Target Database** (TTD). At this stage, the **UniProt** identifiers were associated with the established targets of the **DrugBank** compounds. After data integration, MACCS and ECFP4 fingerprint-based similarity estimations were performed between the subset of **ChEMBL** compounds and **DrugBank** ligands. Similarity calculations were performed by using a python script implementing the OpenEye toolkits, and the results were filtered to retain records with similarity values of MACCS ≥ 0.8 and ECFP4 ≥ 0.3. **ChEMBL** and **DrugBank** compounds showing significant 2D similarities were prepared with OMEGA2 by using default parameters and their 3D shape-based similarity was estimated with ROCS (OpenEye). Pairs of **ChEMBL** and **DrugBank** compounds with significant shape similarity and good superimposition of the atom types (*i.e.*, TanimotoCombo ≥ 1.5) were retained to be further investigated. Together, these analyses allowed us to identify a dataset of 276 **DrugBank** ligands that are similar to molecules known to be active against PC cell lines. The bioactivities of these compounds are currently being investigated for their potential repurposing against PC.

Expected results

105 of the identified **DrugBank** ligands have been already tested against *PC-3*, *DU-145*, and *LNCaP* cell lines, a result that represents a retrospective validation of the adopted approach. Besides, the remaining 161 molecules were never tested on PC cell lines. Target bioactivities of these compounds were further investigated to identify putative candidates for PC repurposing. Remarkably, some of these compounds have already reported bioactivity data on established PC-related targets, therefore are candidates in which repurposing is more likely to have success. Conversely, several other compounds have activity on targets not directly related to PC, and represent situations in which more novelty is expected to come into play. The compounds that emerged from these analyses are currently evaluated for optimal multi-target activities and reported safety profiles. Once valuable candidates are identified, they will be purchased and experimentally tested thanks to collaborations established on purpose. The information related to ligands, targets, and therapeutic indications collected so far will be also exploited for the development of especially devised ML workflows, potentially useful for facilitating PC drug repurposing.

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