University of Padua
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Medical and Biomedical Sciences

Università degli Studi di Padova

PhD STUDENT RESEARCH PROJECT DAY MEDICAL AND BIOMEDICAL SCIENCES (XXXVIII Cycle)

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PhD COURSE "ARTERIAL HYPERTENSION AND VASCULAR BIOLOGY"

COORDINATOR: PROF. TERESA MARIA SECCIA

STATIC BALANCE AND SENSORY NERVE CONDUCTION IN PATIENTS WITH PRIMARY ALDOSTERONISM: CASE-CONTROL, PROSPECTIVE STUDY

Ph.D. Student: Dr. Eleonora BERTOLI
TUTOR: Prof. Gian Paolo ROSSI
Ph.D. Course: Arterial Hypertension and Vascular Biology

Background
Raised blood pressure is a major risk factor for coronary heart disease, stroke and it leads to brain silent infarction and dementia, moreover it increases the risk of falling in hypertensive patients. Primary aldosteronism is the most frequent and curable disease between secondary hypertension. The human equilibrium is synchronized through the visual, vestibular, and proprioceptive regulatory systems containing afferent signals from the periphery to the central nervous system. The damage of one systems starting from periphery reaching to the brain centers might lead to stability disturbances. We herein describe a study protocol to assess the static balance and lower limb sensory nerve conduction in patients with primary aldosteronism (PA) in their pre- and post-adrenalectomy state with control group as hypertensive patients without PA. This prospective, case-control study aims to estimate the risk of falls, as well as to explore the association between postural balance, peripheral sensory nerve conduction, and serum potassium level in patients with PA.

Material and Methods
Thirty patients with primary aldosteronism and hypertensive patients without PA in each group respectively were enrolled in this prospective study. PA patients were evaluated in their pre and post-operative state. Participants underwent an assessment of postural balance on the stabilometric platform with eyes open (EO), eyes closed (EC), and dual-task (DT) conditions with lower limb electroneurography.

Results
We observed: a significant reduction of medio-lateral (ML) sway in eyes open and eyes open head-extended state (p 0,01) between washout and follow-up (FU) state; no differences between wash-out and FU state, except sural nerve latency (p=0,03 WO vs PO); peroneal nerve ankle amplitude higher in wash-out compared to mineralcorticoid receptor antagonists (MRA) state (p=0,005), as well as peroneal fibula amplitude (p=0,01). Postural balance was associated with PA and PA cured status. CoPv (Centre of pressure velocity) in closed eyes condition was significantly higler in wash out state. Patients affected by PA may have higher postural sway, especially without vision control, compared with the same patients biochemically cured from PA. CoPv correlated with sural nerve amplitude and with serum potassium level (p=0,04, p=0,02).

Conclusions
This study demonstrate that PA patients have worse postural performance in comparison to the same patients after cure from PA, especially in closed eyes condition. Predictors for postural balance improvement after adrenalectomy were sural nerve amplitude and potassium level. There was no significant difference of sural nerve amplitude between PA patients before and after adrenalectomy.

CONGESTION GUIDED HEMODIALYSIS: A RANDOMIZED ASSESSMENT

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TUTOR: Prof.ssa Raffaella MOTTA – CO-TUTOR: Prof. Filippo CRIMI'
Ph.D. Course: Arterial Hypertension and Vascular Biology

Introduction
Coronary sinus (CS), the largest cardiac vein draining into the right atrium (RA), can be easily visualized by echocardiography in practically all patients with a permissive acoustic window. CS caliper is minimally affected by respiration, because of its intra-pericardial locations, and it can accurately correlate with fluid overload conditions and right atrial pressure. CS maximum caliper is accurate for estimating fluid congestion and decongestion and for predicting survival in end-stage kidney disease (ESKD) patients undergoing to hemodialysis (HD). Post-HD CS caliper, particularly if > 9 mm, strongly correlate with ESKD-patient survival. **Aim** To investigate if congestion guided HD based on CS dimensional evaluation by echocardiography after HD session in ESKD patients is related to survival and acute events (hypo-hypertension, IMA, TIA, stroke, fistula's thrombosis, infections, hospital admission).

Methods
Single Center Single Blinded Randomized Trial in Padua University Hospital including ESKD patient (M+F, 18-85 years) from October 2023. Exclusion criteria: severe heart valvular disease, h/o cardiac surgery and cardiac transplantation, major congenital heart disease, persistent left superior vena cava, advanced atrium-ventricular block. CS caliper, inferior vena cava (IVC) with collapsibility index, Portal Vein (PV) calipers, portal and hepatic veins (HEV) doppler were assessed by a bedside echocardiography immediately after HD stop. US data were integrated by bio-impedance analysis. Assessments were performed basally and every 3 months. In treatment branch, Patient's dry weight was decreased by the nephrologist in case of CS>9mm.

Results
Thirty Patients (F=11, 64y, IQR 56-75) were enrolled. ESKD main cause: diabetic nephropathy and nephroangiosclerosis. HD time: 4.6 y (4.6-5.1y). Min-Max dry weight reduction: 0.5-2L. CS caliper positively correlates with IVC caliper ($p<0.0001$) and extra-cellular water ($p=0.025$) and negatively correlates with IVC collapsibility index ($p<0.0001$). Four patients (13%) were lost at FU. One patient died for sepsis (CS always >9 mm) and 1, affected by CAKUT, underwent to kidney transplantation. A total of 29 events were recorded (48%- in treatment branch), infections being the most frequent (31%). There were no significant differences in events between treatment and control branch.

Conclusions
There were no differences in events in patients undergoing congestion guided HD according to CS caliper as a principal estimator of hemodynamic congestion. Population's enlargement to confirm these preliminary results was planned in the next months.

ETIOPATHOGENETIC CLASSIFICATION OF ABDOMINAL AORTIC ANEURYSMS: NEW DISCOVERIES AND EVOLVING CONCEPTS

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CO-TUTORS: Proff. Vito D'ANDREA, Claudio LETIZIA
Ph.D. Course: Arterial Hypertension and Vascular Biology

Background

Abdominal Aortic Aneurysm (AAA) is 3 to 4 times higher in men than in women at age > 65 (1) and the risk of AAA incrase by 40% every 5 years after the age of 65 years (2). Several AAA are secondary to other diseases, such as atherosclerotic disease, trauma, connective tissue disease (Marfan Syndrome, Ehlers–Danlos Type IV), infectious disease (tuberculosis, syphilis, bacteria, fungi), and inflammatory diseases. (3). Current classification of AAA is uncomplete because arteriomegaly and other causes of AAA are not included.The purpose of my research is a new etiopathogenetic classification of AAA.

Materials and Methods

The medical records of patients with AAA admitted to "Azienda Policlinico Umberto I di Roma" since January 2nd, 2010 through today will be critically revised to achieve the aim of my research to propose a new classification of AAA.

Results

The expected result of my research is a new etiopathogenetic classification of AAA, including Arteriomegaly and other causes of AAA.

Conclusion

Most AAAs are asymptomatic and are detected either incidentally while screening for other conditions or in the event of their rupture (4). A new etiopathogenetic classification of AAA, including Arteriomegaly, is the purpose of my reasearch.

RIGHT VENTRICLE TO PULMONARY ARTERY COUPLING IN ACUTE HEART FAILURE PATIENT AND ITS IMPLICATION IN INTENSIVE CARDIAC CARE UNIT

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TUTOR: Prof.ssa Susanna SCIOMER
Ph.D. Course: Arterial Hypertension and Vascular Biology

Introduction
Heart failure is the inability of the heart to provide sufficient cardiac output to meet the metabolic needs of the body under normal ventricular filling pressures, needing hospitalization. It is a major cause of hospitalization in individuals over 65 and it is associated with high rates of in-hospital and post-discharge mortality and readmission. The prognostic significance of right ventricular (RV) dysfunction, alongside left ventricular dysfunction, age, and mean systolic pressure, remains unclear. Evaluating the coupling between the right ventricle and the pulmonary artery (RV-PA coupling) using the echocardiographic TAPSE/PAPS ratio may be prognostically important in these patients, although it is not fully engaged in acute heart failure and in cardiological intensive care.

Study Objectives
This study aims at evaluating the prognostic role of right ventricular function assessed by means of the TAPSE/PAPS ratio in acute heart failure patients, investigating its correlation with in-hospital mortality, six-month follow-up mortality, the need for inotropic support or mechanical circulatory support (MCS) during hospitalization, and readmission due to heart failure exacerbation within six months post-discharge.

Materials and Methods
This prospective study evaluated 100 consecutive acute heart failure patients admitted to the cardiological intensive care unit. Patients were assessed at admission, discharge, and after six months, including clinical, laboratory, and echocardiographic evaluations. Echocardiographic parameters (LVEF, RVEDD, SV, S', TAPSE, TAPSE/PAPS) were analyzed to investigate correlations with mortality and the use of inotropic drugs or MCS.

Results
The study population included 62 men and 38 women, with an average age of 71 ± 12.5 years. Patients generally had a slight overweight condition (BMI 25 ± 3.2), with 56% being smokers and presenting various comorbidities such as hypertension (66%), hyperlipidemia (66%), diabetes (37%), COPD (23%), and chronic kidney disease (21%). Analyzing in-hospital mortality, significant correlations were found with mean arterial pressure ($p = 0.03$), stroke volume ($p = 0.038$), S' ($p = 0.016$), and TAPSE/PAPS ($p < 0.001$), but not with LVEF ($p = 0.091$). There was a significant association between the TAPSE/PAPS ratio and mortality in patients treated with inotropes and MCS ($p = 0.018$ and $p = 0.013$, respectively). TAPSE/PAPS was also a significant prognostic marker for post-discharge mortality ($p = 0.033$).

Conclusions
The study suggests that RV-PA coupling, assessed by TAPSE/PAPS at admission, is a significant prognostic factor for in-hospital mortality and is associated with the need for inotropic support during hospitalization. A reduced TAPSE/PAPS ratio at discharge is a negative prognostic indicator for post-discharge mortality. Thus, RV-PA coupling could be used as an additional prognostic tool in cardiological intensive care for heart failure patients. These findings should be validated by a larger multicentric cohort and longer follow-up.

MULTIMODALITY IMAGING FOR DIFFERENTIATION BETWEEN ADRENAL ALDOSTERONE-PRODUCING ADENOMA AND NOT-SECRETING ADENOMA IN PRIMARY ALDOSTERONISM PATIENTS

Ph.D. Student: Dr. Chiara ZANON
TUTOR: Prof. Gian Paolo ROSSI
Ph.D. Course: Arterial Hypertension and Vascular Biology

Background
High-resolution computed tomography (CT) has increased the incidental detection rate of adrenal masses. Most such cases are benign, although up to 10% of cases are hormone-producing and require surgery. Distinguishing aldosterone-secreting from non-secreting adenomas based on conventional imaging remains challenging because of the absence of established imaging criteria. Texture Analysis (TA). Functional imaging of type 2 and 5 somatostatin receptors using a 68-Gallio-labelled ligand (DOTA-TOC) coupled to positron emission tomography (PET-CT) can help differentiate functionally active tumors, and particularly aldosterone-producing adenomas from non-secreting adenomas.

Material and Methods
24 patients [17 male (70%); mean age, 57 (SD, 10] years) with a confirmed in patients with primary aldosteronism (PA) were included in this prospective study. Two expert radiologists in consensus delineated adrenal volumes of interest (VOI) on unenhanced, venous scans, delay scans CT images and extracted first- and second-order textural features using LifeX software. Additionally, using P-mod software adrenals VOI were delineated on PET CT scans, assessing Metabolic Tumor Volume (MTV) , SUV max, SUV mean on early phase (3 frames of 3 minutes each, starting at injection) and late phase (1h post injection).

Results
To date we have collected data on 24 patients and prepared a database that will serve to determine the accuracy of TA and DOTA-TOC functional imaging using the final diagnosis of aldosterone-producing adenoma as determined by the 5 corners criteria after unilateral videolaparoscopic adrenalectomy as gold reference.
A preliminary analysis of the data has evidenced statistically significant differences among groups in 3 texture features for unenhanced scans (MORPHOLOGICAL_Compacity(IBSI:No) [2372 ± 1130], INTENSITY-BASED_90th(HU)IBSI:8DW [2445±1142], GLCM_ClusterShade(IBSI:7NFM) [815±380] ($p < 0.03$) , 3 texture features for venous scans (INTENSITY HISTOGRAM_MaximumHistogramGradient(HU)IBSI:12CE [2445±1142], GLCM_NormalisedInverseDifferenceMoment(IBSI:1QCO) [104±0.87]+ GLSZM_ZonePercentage(IBSI:P30P) [95±21] ($p<0.04$), and 1 texture feature for delay scans (MORPHOLOGICAL_Sphericity(IBSI:QCFX) [111±21] ($p<0.04$).
Moreover, functional imaging results have evidenced a wide heterogeneity across tumours, whose nature is under investigation.

Conclusions
This study will fill an unmet need in the field of imaging and functional imaging for the characterization of adrenal masses in patients presenting with arterial hypertension.

PhD COURSE "BIOMEDICAL SCIENCE"

COORDINATOR: PROF. ORNELLA ROSSETTO

THE ROLE OF MITOCHONDRIAL CATIONS HOMEOSTASIS IN THE CONTROL OF THE INFLAMMATORY RESPONSE

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Ph.D. Course: Biomedical Science

Background

The immune system is activated in response to pathogenic infections and it relies on the innate immune system's initial response mechanisms. Among these, the assembly of the NLRP3 inflammasome serves as a pivotal step in inducing innate immunity, leading to the release of pro-inflammatory cytokines (IL-1β/IL-18) and promoting pyroptosis. While a consensus model for NLRP3 activation is yet to be established, recent research highlights the significance of mitochondria in maintaining ion homeostasis, particularly involving Ca^{2+} signaling. Our study investigates how changes in mitochondrial Ca^{2+} and K^+ signaling influence NLRP3 inflammasome activation. In detail, Ca^{2+} enters the mitochondrial matrix through the mitochondrial calcium uniporter complex (MCU) where it regulates metabolism, autophagy and cell death. In addition, K^+ fluxes across the inner mitochondrial membrane determine the organelle water content, thus regulating matrix volume. In this scenario, MitoK_{ATP}, one of the channels mediating mitochondrial K^+ entry, plays an essential role

Material and Methods

To achieve this goal, we induce the NLRP3 inflammasome assembly and activation in bone marrow-derived macrophages (BMDMs) by combining different stimuli mimicking the physiological signals necessary for the transcription of its components and its activation. We take advantage of both pharmacological and transgenic models to reduce or abrogate mitochondrial K^+ fluxes or Ca^{2+} uptake and investigate the role of cation fluxes in the control of the inflammatory response.

Results

Our results show that in bone marrow-derived macrophages the inhibition of mitochondrial K^+ and Ca^{2+} fluxes across the inner mitochondrial membrane attenuates NLRP3 inflammasome activation by a variety of inflammatory stimuli. We also show that ion fluxes correlate with the release of mtDNA, which has been proposed to be an important mechanism linking mitochondrial dysfunction to inflammasome engagement.

Conclusions

Altogether, these data corroborate the role of mitochondria in inflammasome signaling routes and highlight MCU and MitoK_{ATP} channels as novel promising drug targets in inflammation.

INVESTIGATING A NOVEL STRATEGY TO IMPROVE BOTULINUM NEUROTOXIN THERAPY

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CO-TUTOR: Prof. Ornella ROSSETTO, Dr. Mickaël MACHICOANE
Ph.D. Course: Biomedical Science

Background

Botulinum neurotoxins (BoNTs) are protein exotoxins responsible for the onset of botulism, a severe, and potentially lethal, disease characterized by flaccid paralysis of skeletal muscles and autonomic nerve terminals.

BoNTs are the most potent neurotoxins discovered so far. However, due to its long-lasting but reversible neuroparalytic effect, BoNT/A is largely used in clinic and aesthetic medicine to treat neuromuscular conditions characterized by the hyperactivation of cholinergic nerve terminals.

Although BoNT/A therapy is highly effective, its pharmacological action is hampered by a slow onset, which takes 2-3 days after the injection to become visible, and at least 2-3 weeks to reach its peak activity. A strategy to speed up the BoNT/A effect after the injection would have major beneficial consequences on the therapeutic use of this toxin.

Material and Methods

Commercial preparations of BoNT/A were combined with fast-acting excitation-contraction coupling inhibitors (ECCIs), and the effect of their co-injection on BoNT/A pharmacological activity has been evaluated via the digit abduction score assay, electrophysiology and imaging analyses in rats and mice. The effect of ECCIs alone on the presynaptic element has also been investigated with electrophysiological techniques.

Results

BoNT/A-ECCIs combinations markedly accelerated the onset of muscle relaxation in a rat model of local paralysis in the hind limb muscle, as expected. Surprisingly, ECCIs co-injection also potentiated the peak effect and extended the duration of BoNT/A pharmacological neuroparalysis.

This synergic effect required both the presynaptic and the postsynaptic elements, and was also visible when ECCIs were coupled with different commercial preparations of BoNT/A.

Mechanistically, ECCIs increased the number of BoNT/A molecules entering the motor-axon terminal by possibly opening alternative pathways for BoNT uptake in the motoneuron.

Conclusions

BoNT/A is routinely used in human therapy to relieve neurogenic muscle hyperactivity. This study shows the first strategy to speed up the onset of its action through the co-injection with fast-acting myorelaxants. This combination efficiently anticipates muscle relaxation and, surprisingly, potentiates BoNT/A-mediated neuroparalysis maintaining its safety profile.

Future studies are needed to understand the specific mechanism behind this potentiation, which may unravel unknown details of BoNT/A mechanism of action useful to further improve its therapeutic use.

PhD COURSE "CLINICAL AND EXPERIMENTAL ONCOLOGY AND IMMUNOLOGY"

COORDINATOR: PROF. ANTONIO ROSATO

CLINICAL IMPACT AND BIOLOGICAL CHARACTERIZATION OF BREAST CANCER-RELATED BRAIN METASTASES

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TUTOR: Prof. Maria Vittoria DIECI – CO-TUTOR: Dr. Gaia GRIGUOLO
Ph.D. Course: Clinical and Experimental Oncology and Immunology

Background
Breast cancer represents the most common cancer diagnosed in women worldwide and one of the leading causes of cancer death, with over 680 000 deaths estimated each year. Brain metastases (BMs) are a common complication of advanced BC, typically associated with poor prognosis. Despite remaining an unmet medical need, the locoregional and systemic management of BMs has significantly evolved over time; however, the clinical impact of these changes remains partially unexplored.
As part of the clinical side of the project, we assessed the temporal evolution of clinicopathological characteristics and treatments of patients with BCBMs as well as outcomes and prognostic factors over the last two decades in a multicentric real-world setting.
Material and Methods
Patients diagnosed with BCBMs at three Institutions (Istituto Oncologico Veneto - Padova, Montpellier Cancer Institute - Montpellier and Center Antoine Lacassagne - Nice) were divided in 3 groups according to year of BMs diagnosis: 2000-2007 (group A), 2008-2014 (group B) and 2015-2022 (group C). Clinicopathological features and treatments were collected from medical charts. Overall survival (OS) was defined as time from BMs diagnosis to death.
Results
Among 779 patients identified, 241 were included in group A, 323 in group B and 215 in group C. According to BC subtype, 33.5% were HR+/HER2-, 21.2% HR+/HER2+, 22.0% HR-/HER2+ and 23.3% HR-/HER2-, with a progressive increase over time of the proportion of patients with HR+ BCs ($p=0.012$).
A significant increase in stereotactic radiotherapy ($p<0.001$) and a decrease in whole-brain radiotherapy use was observed over time ($p=0.010$). Among HER2+ BC patients, significantly more received anti-HER2 therapy after BM diagnosis in recent years (64.8%, 76.1%, 83.5% in group A, B, C, respectively; $p<0.011$), with more recent patients receiving a higher number of anti-HER2 therapy lines ($p=0.002$).
No significant OS improvement was observed in the overall cohort (median OS 8.2 months, 8.7 months, 8.4 months in group A, B, C, respectively; $p=0.260$). A significant OS improvement was selectively observed only in patients with HR-/HER2+ BC (median OS 8.7 months, 10.1 months, 23.7 months in group A, B, C, respectively; $p=0.002$).
While HER2-positivity was not prognostic in group A ($p=0.958$ at univariate analysis), it became a significant prognostic factor in group C ($p<0.001$ at multivariate analysis).
Conclusions
Over two decades, locoregional and systemic therapy of patients with BCBMs has significantly changed. However, in this large real-world cohort, a significant OS improvement was only observed in patients with HR-/HER2+ BCBMs, potentially due to increased availability of anti-HER2 therapies with intracranial activity. In this evolving scenario, it is crucial to reassess prognostic factors, also considering the potential impact that the implementation of proactive brain imaging for HER2-positive and triple negative metastatic BCs might have in the future years.

NEW INSIGHTS INTO THE RELATIONSHIP BETWEEN p53 AND FAK PROTEINS IN CLL

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Ph.D. Course: Clinical and Experimental Oncology and Immunology

Background
We previously found that the focal adhesion kinase (FAK) is present in the cleaved/phosphorylated and activated form in CLL (chronic lymphocytic leukemia) patients, mainly IGHV unmutated (UM), through a mechanism involving other proteins that increases the aggressiveness of the disease. Activated FAK is present also in the cell nucleus where it might be directly involved in survival and proliferation. Studies in solid tumors highlighted how FAK could interact with p53. Since p53 can bind to FAK gene promoter and considering that *TP53* is inactivated in about 15% of CLL patients accounted for unfavorable prognosis, the investigation on FAK-p53 interaction needs to be further explored.

Material and Methods
CD19+/CD5+ cells from untreated CLL patients and normal B cells from age-matched healthy subjects were purified through density gradient centrifugation. Basal expression of p53, full-length (fl) FAK and cleaved (cl) FAK has been assessed by western blotting (WB) in 54 CLL patients and 10 controls. Nucleus-cytosol protein extraction has been performed in 7 CLL patients to analyse FAK and p53 subcellular distribution. p53 expression following FAK inhibition with 5 μM defactinib has also been assessed by western blotting at different time-points in 5 samples.

Results
WB analyses revealed a significant overexpression of p53 in patients versus controls ($p<0.0001$). Moreover, p53 showed to be higher expressed in UM-IGHV patients with respect to mutated ones ($p<0.05$). When we correlated p53 with activated cl-FAK, we found a significant positive correlation ($p<0.0001$, $r=0.57$), being p53 more expressed in patients with more active FAK, preferentially in UM ones. Accordingly, when we analysed the mutual expression of FAK and p53 within the same CLL samples, we found that in the cohort of patients with $p53^{high}$/fl-FAK^{low} there were mainly UM cases, differently from the cohort expressing $p53^{high}$/fl-FAK^{high}; in this latter group UM patients were less represented. In our case study we observed that p53 protein is mostly present in the nucleus of CLL cells (65% ± 0.05 of total protein) with also a fair amount of protein present in the cytosol (35% ± 0.05). Finally, we observed that defactinib is in some way able to modulate p53 expression in CLL; this latter aspect will require further investigation.

Conclusions
Our results provide a snapshot of the expression of p53 and FAK as well as their correlation in patients with CLL. Defactinib has previously been demonstrated to be more effective in unmutated patients and its action can modulate p53 expression, this indicating once again a possible interaction of FAK with p53 in CLL. The presence of both proteins in the nucleus of CLL cells is also interesting since FAK has been indicated as a scaffold protein for p53 itself.

CORRELATION BETWEEN TREATMENT WITH BEVACIZUMAB AND TISSUE MOLECULAR ALTERATIONS IN MENINGIOMA AND GLIOMA

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Ph.D. Course: Clinical and Experimental Oncology and Immunology

Background
Bevacizumab is a recombinant, humanized, monoclonal antibody directed against vascular endothelial growth factor (VEGF)-A. Available literature shows a benefit in progression free survival (PFS) but not in overall survival (OS) with bevacizumab in glioma and relapsed malignant meningioma (grade 2 and 3). [1,2] To date, no predictive tissue molecular biomarker of response have been identified. Based on such data a study was designed, aiming at identifying possible tissutal molecular markers predictive of response to treatment with bevacizumab in patients affected by relapsed meningioma and glioma.

Material and Methods
Tissue samples of relapsed atypical and anaplastic meningioma and by glioma, treated with bevacizumab, were analyzed. Next generation sequencing (NGS) data have been obtained through Foundation Medicine genomic tests. TSO500 tests are to be used as well. Survival analyses were carried out by Kaplan-Meier method; the log-rank test was used to compare the survival curves. The analyses were carried out with R software, version 4.3.1. The level of significance was set at 5%.

Results
NGS data for 20 patients with meningioma treated with bevacizumab were analyzed. The most common genomic alteration detected was *NF2* (14/20 patients, 70% of patients). Patients with a pathogenic *NF2* mutation were compared with patients without such mutation. No statistically relevant difference was detected in OS; the mOS for patients with pathogenic *NF2* mutations was 22.3 months, the mOS for patients without a *NF2* pathogenic mutation was not reached (p-value 0.091). On the contrary, the difference in PFS was statistically significant, with patients with a pathogenic *NF2* mutation having a reduced mPFS of 9.4 months and those without any *NF2* pathogenic mutation having a longer mPFS of 28.8 months (p-value 0.017). Other molecular alterations detected are: *CDKN2A/2B* loss (7/20 cases), *MTAP* (4/20), *PTEN* (3/20), *SUFU* (2/20), and *ARID1A, POLE, ALK, TERT* promoter, *JAK3* (each 1 case).
Another 12 tissue samples of meningioma and 15 tissue samples of gliomas are currently under analysis.

Conclusions
The results obtained so far seem to show a detrimental trend for patients affected by malignant meningioma carrying *NF2* mutations and treated with bevacizumab. Such trend is detectable in the PFS but not in OS. Further developments include first, increasing the number of samples and second, consider possible genomic signatures instead of single genes.

TARGETING HTLV-1-TRANSFORMED CELLS VIA MODULATION OF REDOX BALANCE

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TUTOR: Prof. Vincenzo CIMINALE– CO-TUTOR: Prof. Donna M. D'AGOSTINO
Ph.D. Course: Clinical and Experimental Oncology and Immunology

Background

Human T-lymphotropic virus type 1 (HTLV-1) is a retrovirus that infects 10-20 million people worldwide. The virus causes T-cell immortalization and gives rise to adult T-cell leukaemia/lymphoma (ATLL), an aggressive malignancy of mature CD4+ T-cells. There is no vaccine for HTLV-1 infection, and infected individuals cannot eliminate it. Our laboratory is searching for strategies to target HTLV-1-transformed cells by treating them with drugs that alter redox homeostasis and expression of microRNAs involved in cell death/survival pathways.

Material and Methods

The study employs an *in vitro*-HTLV-1-transformed cell line (C91PL) and ATL-derived cell lines (ED, MT1). Experiments carried out thus far investigated the effects of the mTORC1 inhibitor Everolimus, the Glucose-6-phosphate Dehydrogenase (G6PD) inhibitor DHEA (dehydroepiandrosterone), and the BCL2 inhibitor Venetoclax. The effects of the drugs on cell death were measured by staining cells with propidium iodide, followed by flow cytometry and calculation of specific cell death (SCD). Changes in reactive oxygen species (ROS) levels were measured using the fluorescent probes MitoSOX Red and CellROX Deep Red (to detect mitochondrial and cytoplasmic ROS levels, respectively) and flow cytometry. The mean fluorescence intensity (MFI) values in the live-cell gate were determined. Changes in ROS were expressed as Fx/F0 ratios, where Fx corresponds to the MFI of each sample, and F0 is the MFI of the "no-drug" control. microRNA mimic and siRNA transfections were carried out using a Neon NxT Electroporation System. RNA was extracted and analyzed by qRT-PCR to identify coding transcripts and microRNAs whose expression was altered by the treatments. Western blotting was performed to analyze protein expression.

Results

The panel of cell lines showed variations in their response to the single drugs. DHEA increased the cytotoxic effects of Everolimus and Venetoclax in all the cell lines. A 48-hour kinetics study of ROS production showed that treatment with Everolimus led to a substantial increase in MitoSOX Red and a moderate rise in CellROX Deep Red fluorescence in the 3 cell lines. When combined with DHEA, the levels of fluorescence further increased considerably. Among the three cell lines, MT1 showed the greatest sensitivity to Everolimus.

Quantification of miR-31-5p, a microRNA known to be downregulated in ATL cells, showed that Everolimus drastically increased its levels in MT1 (also confirmed by genetically silencing of mTOR using FRAP1 siRNA) and by combining Everolimus and DHEA in C91PL and ED cells. Transfection of MT1 cells with miR-31-5p resulted in downregulation of a predicted target of microRNA named GCLM (glutamate-cysteine ligase modifier subunit), which codes for a subunit of glutamate-cysteine ligase, an enzyme crucial for glutathione synthesis. In the project's next phase, we will evaluate the mechanisms and consequences of microRNA- and ROS alterations in response to mTOR inhibition by Everolimus.

Conclusions

The study's findings suggest that the combination of Everolimus or Venetoclax and DHEA could be a promising treatment strategy for ATL. Future research will further investigate the role of drug-induced microRNAs and their mRNA targets in cell survival/death pathways and redox hemostasis, potentially paving the way for novel treatment approaches.

MODULATING GLIOBLASTOMA MYELOID MICROENVIRONMENT TO ENHANCE CURRENT THERAPY

Ph.D. Student: Dr. Diana Marcela HERNÁNDEZ PALOMINO
TUTOR: Dr. Ilaria MARIGO
Ph.D. Course: Clinical and Experimental Oncology and Immunology

Background

Glioblastoma (GBM) is the most common primary and aggressive brain tumor with a median survival of less than 21 months with limited treatment options. Current therapies, including surgical resection followed by radiotherapy and oral delivery of temozolomide[1–3], have not significantly improved patient prognosis. Moreover, immunotherapy is still challenging due to various resistance mechanisms such as the tumor's location, blood-brain barrier obstruction, tumor heterogeneity, and the highly immunosuppressive tumor microenvironment (TME)[4]. This project aims to modify the TME using nanosystems to target immunosuppressive pathways with RNA inhibitors and deliver immune-stimulating chemokines directly to the tumor site. The strategy will be combined with radiotherapy and immune checkpoint inhibitors to enhance the effects of individual treatments.

Material and Methods

The gene expression of targeted genes is analyzed in *in vitro*-differentiated macrophages using the TaqMan system. The gene silencing is performed with the commercial siRNA and transfectant from Dharmacon™ for transfection optimization, and the *in vitro* downregulation is evaluated at the mRNA level. Our primary RNA delivery system will be polymeric nanoparticles (NPs) provided by our European partner. Three prototypes have been challenged for cytotoxicity and RNA transfection. The siRNA transfection is followed with the fluorescent-siRNA control (siGLO) by flow cytometry (FC). The mRNA transfection is followed by the reporter mRNA GFP or mCherry by FC, and the IL-12/CXCL9 chemokine expression is analyzed on the cell supernatants by ELISA.
In vivo, we use the SB28 or GL261 orthotopic mouse model to study tumor growth and TME modulation after NPs treatment. The bulk tumor supernatants are also used to test the chemokines by ELISA.

Results

The first goal was to silence *in vitro* the genes involved in the immunosuppression induced by myeloid cells. The silencing of the targeted genes *LGALS9*, *VISTA*, *cEBPβ*, and *LGALS1* was first optimized with the commercial transfectant and siRNA control at different concentrations using *in vitro*-derived bone marrow macrophages. We found that 25nM of siRNA with DharmaFECT 3 is the optimized transfection condition with the least impact on macrophage phenotype. Later, we optimized the transfection of NPs prototypes in our *in vitro* cultures, and we found that 25nM of siRNA was the dose with low toxicity and good transfection efficiency for all three prototypes.
The silencing efficiency was tested with the positive control siGAPDH (housekeeping gene *GAPDH*). After 48h of treatment, Prototype 2 showed the highest gene downregulation and was selected for the following experiments. Instead, Prototype 1 induced the highest IL-12 and CXCL9 expression *in vitro* on transfected macrophages and GBM cell lines.
Our first *in vivo* experiments with mRNA-loaded NPs encoding IL-12 and CXCL9 chemokines in tumor-bearing mice did not show significant effects on TME. Therefore, we are optimizing the timing of the readout for TME evaluation and chemokine production.

Conclusions

The optimized *in vitro* screening allowed us to select the prototypes for the RNA delivery. Our first *in vivo* experiments show that the immune stimulation used by injecting the mRNA-encoding chemokines alone is not enough to induce an effect on the tumor microenvironment. Currently, we are performing the first *in vivo* silencing experiment to modulate the immunosuppressive pathway in the microenvironment.

CLINICAL RELEVANCE OF DDR MUTATIONS AND RESISTANCE MECHANISMS IN METASTATIC PROSTATIC ADENOCARCINOMA TREATED WITH CHEMOTHERAPY AND HORMONE THERAPY, AND ROLE OF LIQUID BIOPSY

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Ph.D. Course: Clinical and Experimental Oncology and Immunology

Background
Prostate cancer (PC) is the 2nd leading cause of cancer-related death in men. Nearly 20% of PC cases exhibit DNA damage repair (DDR) alterations, particularly BRCA1/2 mutations (over half are somatic), that are a predictive marker for PARP inhibitor response. Testing for BRCA mutations in tumor tissue is recommended for all metastatic castration-resistant (mCRPC) patients. However, formalin-fixed paraffin-embedded (FFPE) material presents challenges mainly due to storage time. Liquid Biopsy (LB) is a minimally invasive way to molecularly profile PC patients, and provides real-time insights into tumor heterogeneity. Still, LB faces technical and interpretation issues: alterations in FFPE material may not be highlighted in LB due to low ctDNA concentration, leading to false negatives.
Objective: to assess diagnostic performance of a commercial NGS panel for evaluating BRCA1/2 and other DDR mutations in ctDNA samples obtained from PC patients' plasma.
Material and Methods
We identified 47 patients with metastatic PC who had previously undergone DDR testing on FFPE material, and collected plasma cell-free DNA between 2023 and 2024 during treatment using Maxwell LV PROMEGA kit. Sample quality control was delivered with TapeStation System. Targeted sequencing was performed on cfDNA with NGS panel (Sophia HRS, Sophia Genetics).
Results
We retrieved 47 LB. Most pts presented with high volume (HV) disease (31/47), of whom 31% had visceral metastases. Median extracted cfDNA was 13.9 ug/mL (IQR 10.8-18.8), with median quality of 74.5%. Median levels of cfDNA were higher (23.41 ug/mL, IQR 2.98-24.7) in pts that presented with progressive and HV disease. We found no significant correlation between cfDNA levels and PSA levels (r=0.1), hormone sensitivity status (r=0.19), disease volume (r= 0.1) or BRCAmutations (r=0.21).
We performed DNA targeted sequencing on 11 samples (6 BRCA2mut, 1 ATMmut, 2 CHEK2mut, 2 TP53mut). The concordance for mutation detection in matched samples was 54%. Combined ctDNA and FFPE analysis identified BRCA1/2 mut in 81% of the samples, with 3 new BRCA2 mutations not previously found, with a median VAF of 40%).
Conclusions
Our data is preliminary, but shows that LB can provide additional information to FFPE analyses, suggesting that either is suitable for molecular subtyping PC pts. The optimal approach should use both tissue and LB, as neither captures clinically relevant somatic alternations in all patients.

TERRA AS A SENESCENCE AND PROGNOSTIC BIOMARKER IN PERIPHERAL BLOOD MONONUCLEAR CELLS: FINDINGS FROM COLORECTAL CANCER PATIENTS AND IN VITRO STUDIES

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Background
Aging is associated with several diseases, including cancer. Evaluating aging biomarkers in peripheral blood is crucial for minimally-invasive monitoring of frail patients. TElomeric Repeat-containing RNA (TERRA), a class of long noncoding RNAs transcribed from telomeres, plays multiple roles at telomeres and may impact cellular senescence. We aim to investigate the role of TERRA in peripheral blood mononuclear cells (PBMC) as a senescence and prognostic biomarker in colorectal cancer (CRC) patients and to explore the role of TERRA transcripts in response to senescence stimuli in PBMC in vitro experiments.
Material and Methods
TERRA transcripts from chromosomes 1q-2q-10q-13q (TERRAch1-2-10-13), 15q (TERRAch15), 20q (TERRAch20), and XpYp (TERRAchXY) were assessed in 47 elderly (≥70 years) CRC patients at surgery (baseline) and at one year after tumor resection (follow-up). TERRA levels were quantified by RT-PCR and analyzed in relation to other senescence biomarkers (T-cell immunophenotype, thymic output, telomere length, denervation biomarkers, and senescence-associated secretory phenotype, SASP) and clinical outcomes. For in vitro experiments, phytohemagglutinin-activated PBMC from healthy donors were treated with sublethal concentrations of the senescence-inducing drug doxorubicin for 24, 48, and 72 hours (h). Cellular senescence was assessed by evaluating cell growth rate, telomere length, and markers of senescence-induced cell cycle arrest, such as CDKN1A, CDKN2A, and LMNB1 expression levels. TERRA transcripts were monitored during treatments.
Results
Results from CRC patients confirmed previous data obtained from a smaller group: at baseline, TERRA levels tended to correlate with telomere length. Higher levels of TERRAch15 significantly reduced the risk of adverse events (relapse, progression, or death) with a hazard ratio of 0.369 ($p=0.028$). Additionally, elevated TERRAch15 levels were associated with lower numbers of CD8 T cells, including senescent ($p=0.014$), naïve ($p=0.035$), and recent thymic emigrant ($p=0.026$) cells, as well as a higher CD4/CD8 ratio ($p=0.038$). At follow-up, TERRA levels inversely correlated with SASP markers IL8 and CXCL-1. In vitro results showed that treatment of activated PBMCs with 40nM of doxorubicin for 72h induced a senescence phenotype compared to the untreated controls, evidenced by a 68% reduction in growth rate, significant telomere shortening ($p=0.007$), increased expression of CDKN1A and CDKN2A (mean fold change (FC) 3.70±1.30, $p=0.023$, and 1.78±0.52, $p=0.061$, respectively), and decreased LMNB1 transcripts (FC=0.74±0.01, $p<0.001$). This senescence-induced phenotype was associated with an up-regulation of TERRAch1-2-10-13 (FC=1.82±0.92, $p=0.197$), TERRAch15 (FC=2.56±1.12, $p=0.074$), TERRAch20 (FC=1.60±0.95, $p=0.33$), and TERRAchXY (FC=1.89±0.06, $p<0.001$) expression levels.
Conclusions
Data from patients suggest that TERRA levels in PBMCs could be useful for monitoring "biological aging" and represent a promising prognostic marker for elderly CRC patients. In vitro data indicate that drug-induced cellular senescence appears to enhance TERRA levels, suggesting their potential role as a defence mechanism in the cellular senescence process.

UNRAVELING THE CROSSTALK BETWEEN T-CELL IMMUNOTHERAPIES AND THE TUMOR MICROENVIRONMENT

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Background

In recent years, adoptively transferred T-cell based immunotherapies (ACT) have gained significant attention due to their clinical success; however, they have several drawbacks that limit their use such as T-cell exhaustion and cytokine storm. Moreover, although it is well known that the main mechanism of action is direct cytolysis, their ability to modify the cytokine milieu and reshape the TME isn't fully understood. We realized that by unravelling the interactions between therapeutic T-cells and immune cells in the Tumor Microenvironment (TME) we could gain useful insight for all these knowledge gaps, with the goal of providing a biologic basis for the development of safer and more effective T-cell therapies.

Material and Methods

In this project we are using genetically encoded in vivo enzymatic labelling approaches (LIPSTIC and uLIPSTIC) to understand how immune interactions elicited upon $CD8^+$ ACT shape the antitumoral response. To do so, we rely on a murine lymphoma cell line transduced with OVA as a tumor antigen model. Donor-derived, OVA-specific, ex vivo activated OT-I cells serve as ACT.

Results

We observed that ACT infiltration into the TME and dLN is time-dependant, plateauing at 72 hours. At this timepoint, the TME was drastically reshaped, mainly through an increase of $Ly6C^+$ myeloid populations. When assessing intercellular contacts, we consistently observed that ACTs interacted with several myeloid cells, including macrophages, monocyte-derived cells and classical dendritic cells (cDCs). Through Luminex assays, we showed an upregulation of IFNγ, IL-12, IL-1β and IL-15; consistent with T-cell activation, cDC costimulation, and macrophage activity. Accordingly, flow cytometry data showed that most myeloid populations upregulate iNOS upon treatment. To unravel the nature of ACT:myeloid interactions, we replicated the setup using the OVA-negative parental cell line of our model. Surprisingly, although ACT infiltration was conserved regardless of the absence of tumor antigen, the intercellular interactions were considerably abrogated, possibly implying a role of cross-presentation of myeloid cells to support ACT activity. Finally, preliminary data shows that upon treatment, endogenous T-cells also interact with B-cells in the dLN, although further validation is needed for this observation. We are currently working on scRNAseq experiments to understand the transcriptional differences between interacting and non-interacting cells of a given population. Moreover, we are planning to perform ex vivo analyses of sorted myeloid populations to understand their individual effect on T-cell cytotoxicity and proliferation. Finally, we will test our findings on T-cell therapy models with different mechanisms of actions, mainly CD19 CAR T-cells.

Conclusions

Our findings suggest that ACT significantly reshapes the immune landscape of the TME and engage in intercellular contacts with several myeloid populations that could support their activity through tumor-antigen cross-presentation. Further downstream investigation on these interactions is still needed to confirm the nature of these observations.

BIOLOGICAL AND CLINICAL CHARACTERIZATION IN PATIENTS WITH BONE METASTASES FROM CARCINOMA: ANALYSIS OF OUTCOME IN RELATION TO METABOLIC FACTORS AND BIOLOGICAL AGGRESSIVINESS OF THE TUMOUR

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Bone metastases (BMs) are now considered a disease with a high social impact, for their frequency and for the impact on the prognosis and quality of life.
BMs are frequent findings of several solid tumors, especially breast, prostate, lung, kidney and thyroid cancers and is linked to a poorer prognosis dramatically reducing patient survival.
The management of patients with BMs is challenging and requires a multidisciplinary approach.
BMs rapresent a complex pathophysiological process between host and tumor cells that results in cell invasion, adhesion migration and stimulation of osteoblastic and osteoclastic activity.
This study aimed to investigate the presence and the role of bone marker's metabolism in early diagnosis and monitoring of BMs in 45 patients we enrolled in Orthopaedic Clinic of the University of Padova from October 2022 and evaluated until now (age range : 55-85 years; female predominant, most common site was femur (71.4.1 %) followed by arm-shoulder (25.0 %) and spine (3.6%) Eligibility criteria were as following:1) Bone metastases; 2) Impeding or pathological fractures 3) patients who provided informed consent.
Markers of bone resorption [N-telopeptide-NTx; pyridinoline-PYD] and formation [C-terminal collagen propeptide-CICP; bone alkaline phosphatase-BAP] were measured with serum biomarkers in particular, from our preliminar data, albumin was significantly lower in patients with BM from lung cancer compared to all the other primary tumors ($p=0.014$).
Therefore, it will be possible to compare biomarkers levels between patients with or without BMs in order to find potential predictive and prognostic biomarkers (control group).
These criteria demonstrate that the early identification of biomarkers in bone microenviroment could be used for early identification of patients at high risk of BM development and as good prognostic factors for the survival of patients with BM.

PROGNOSTIC BIOMARKERS IN DEDIFFERENTIATED LIPOSARCOMAS AND THEIR CLINICAL IMPACT ON PATIENTS' MANAGEMENT

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Background
In the era of precision medicine, there is an unmet need of new effective therapies for patients with dedifferentiated liposarcoma (DDLPS), an aggressive subtype of soft-tissue sarcoma. DDLPS has an overall complex genomic profile, which may sustain its heterogeneous clinical behavior. To date, the most important prognostic factor is the anatomical location, with the extremities being a more favorable site than the retroperitoneum. A few studies evaluated the prognostic value of the morphological differentiation in retroperitoneal DDLPS, finding myogenic expression correlated to a worse survival. However, specific molecular markers associated to different clinical and/or morphological features have not yet been identified.

Material and Methods
Data of adult patients with DDLPS treated at Veneto Institute of Oncology between 2018 and 2023 were retrieved from a prospectively maintained database. The impact of age, gender, tumor location, grading, differentiation, size, quality of surgery and peri-operative chemo/radiation-therapy on disease-free survival and overall survival was analyzed for patients who underwent surgery. The effects on progression-free survival and overall survival of the tumor location, grading and differentiation were also studied for patients treated with first-line chemotherapy. The primary objective was the identification of potential prognostic clinical and/or morphological biomarkers, that may then guide the research of the underlying molecular mechanisms.

Results
A total of 61 patients were eligible. Of these, 58 patients underwent surgery. Median age at diagnosis was 67.8 years (range 43–88 years); median follow-up was 28 months (range 22-46 months). The most frequent anatomical location was retroperitoneum (59%). Eighteen per cent of all tumors had myogenic differentiation, 3.3% rhabdomyoblastic elements, 59% a not-otherwise-specified (NOS) differentiation, with the remaining 19.7% having other types of differentiation. In patients who underwent surgery, a high tumor grade was associated with both worse median DFS (14 Vs 33 months for grade 3 and 2, respectively, p=0.001) and median OS (40 Vs 106 months, p=0.001). Also, myogenic differentiation was associated with better DFS than other types of differentiation (median DFS: NA Vs 3, 25 and 62 months for myogenic, rhabdomyoblastic, NOS and other types of differentiation, respectively; p=0.002). No statistically significant differences in terms of survival were seen for the other analysed variables.

Conclusions
The rarity of DDLPSs is further complicated by their heterogeneity, which makes their management very challenging. Our study confirms certain morphological features, such us the type of differentiation, may have a prognostic value. Next steps include studying the molecular mechanisms underlying tumorigenesis and differentiation, in order to improve prognostic stratification and therapeutic planning for these patients.

MOLECULAR CHARACTERIZATION AND CLINICAL IMPLICATIONS OF MITOCHONDRIAL DNA VARIANTS IN COLORECTAL CANCER

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Background
Mitochondrial DNA (mtDNA) variants are among the most common genetic events in tumors and potentially impact metabolic homeostasis. Previous research established that mitochondrial DNA variants are found in about 30% of colorectal cancer (CRC) patients. Although mitochondrial variants have been reported in CRC, they have not been further characterized either at the molecular level or in terms of their clinical impact. The purpose of this project is to study a collection of frozen tissue samples of CRC to detect mtDNA variants and to investigate the association of mtDNA alterations with metabolism alterations and with patients' clinical overall survival and relapse free survival.

Material and Methods
Fresh-frozen CRC samples of II-III stage and their relative healthy counterpart have been obtained from the biobank of the General Surgery III unit (Prof. Salvatore Pucciarelli, Azienda Ospedaliera di Padova). DNA was obtained from 30 mg of frozen tissue, sectioned at 20 µm at the cryostat. DNA was extracted from frozen samples using the AllPrep DNA/RNA Mini Kit (Qiagen). DNA concentration was quantified using Nanodrop and DNA quality analyzed by Genomic DNA ScreenTape Analysis (Agilent). Eight µm sections were fixed with cold acetone, and either subjected to hematoxylin and eosin (H&E) staining or stained with anti-MCT4 antibody, using Bond III stainer (Leica Biosystems). H&E sections were reviewed by a pathologist and tumor area was used for normalization of variant frequencies. Mitochondrial DNA sequencing was performed in collaboration with Dott. Leonardo Caporali (University of Bologna).

Results
Genetic analysis disclosed the presence of 25 mtDNA variants in 18 CRC samples. Among all variants, 12 (48%) were present at high frequency (VAF>50%), and 7 of them were predicted to be pathogenic. We found that the most affected genes are MT-ND5 and MT-CO1, together accounting for 40% of the detected variants. To evaluate tissue expression of MCT4, a lactate extruder reportedly associated with increased glycolysis, immunohistochemistry and subsequent digital pathology analysis of tissue sections were performed. The majority of CRC samples showed low MCT4 expression; in any case, MCT4 expression was detected in the matched healthy tissue counterpart. Notably, MCT4 expression was found to be higher in tumor tissue compared with the matched healthy counterpart in 5 out of 18 CRC samples, including 4 samples bearing mtDNA variants.
Altogether, no association between MCT4 expression and the presence of mitochondrial DNA variants was observed. Moreover, although in the literature the presence of mtDNA variants has been associated with improved survival, our preliminary data at present do not confirm this.

Conclusions
These results show that high frequency mtDNA variants affecting the respiratory complex I and complex IV are found in a large proportion of the CRC samples. Further investigations on a larger number of colon cancer samples will contribute to the elucidation of the metabolic alterations and clinical implication associated with the presence of mtDNA variants.

PhD COURSE "CLINICAL AND EXPERIMENTAL SCIENCES"

COORDINATOR: PROF. ROBERTA RAMONDA

Curriculum "CLINICAL METHODOLOGY, METABOLISM, ENDOCRINOLOGY, NEPHROLOGY AND EXPERIMENTAL EXERCISE"

THE ROLE OF MITOCHONDRIAL CATION CHANNELS IN THE CONTROL OF LUNG EPITHELIAL INFLAMMATION

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Background
Mitochondria are recognized to not only be the site of aerobic metabolism, but also signaling hubs that receive several physiological and pathological inputs. They regulate diverse processes such as apoptotic, necrotic cell death, and inflammation induction. Indeed, mitochondrial dysfunction has been demonstrated to play a key role in the pathogenesis of a variety of pathological conditions, including neurodegeneration, diabetes, and cancer. The overall mitochondrial cation homeostasis has been linked to a surfeit of disorders involving inflammation both acute and chronic. Notably, maintaining Ca^{2+} and K^{+} homeostasis is one of the crucial tasks carried out by these organelles and which also has potential roles in pathophysiological conditions upon aberrations in their fluxes.
In this context, a key role is played by mitochondrial ion channels, and in particular the Ca^{2+} and K^{+} channels that were identified by our group, MCU and mitoKATP. The present study explores the intricate interplay between mitochondrial cation channels and inflammation in the context of pathological conditions in lung epithelia. In the lungs, epithelial cells serve as the frontline defense against inhaled pathogens and environmental insults. Under pathological conditions, such as chronic obstructive pulmonary disease (COPD) or asthma, mitochondrial dysfunction exacerbates inflammation, leading to tissue damage and impaired lung function. Dysregulated mitochondrial cation channels, and in particular MCU-mediated Ca^{2+} overload, have been proposed to enhance tissue damage, perpetuating the inflammatory cascade. To get insight into these processes, we take advantage of recent advancements in imaging techniques, such as the Split-GFP (SPLICs) technique, to enable real-time monitoring of mitochondrial DNA (mtDNA) release from damaged mitochondria during inflammation.
Material and Methods
We utilized BEAS 2B cells, derived from human bronchial epithelia and SPLICs-based new probes to visualize using confocal microscopy and quantify mtDNA release in response to a variety of inflammatory stimuli. We adapted various molecular biology approaches as well such as immunoblotting, cytosolic and mitochondrial Ca^{2+} measurement and ELISA for IL-6 release.
Results
Together our collective data suggests a prominent role of MCU in the induction of Inflammation in epithelial cells and the release of IL-6 as a sequential outcome of the inflammatory process. Inhibiting MCU, shows a prominent decrease in the release of such pro-inflammatory cytokines upon exposing the epithelial cells to environmental insults.
Conclusions
The combination of these new probes, together with mitochondrial Ca^{2+}, membrane potential and morphology imaging probes, allows us to investigate the pathogenic mechanism triggered by environmental air-borne agents, highlighting mitochondrial ion channels as new pharmacological targets to address the growing number of airway disorders.

ADIPOGENESIS AND ADIPOCYTE METABOLISM: A FOCUS ON PROTEIN KINASE CK2

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Background
Adipose tissue (AT) is able to rapidly expand by both increase in size of existing adipocytes (hypertrophy) or formation of new adipocytes through differentiation of resident precursors (hyperplasia). CK2 is a constitutively active Ser/Thr protein kinase, composed of 2 catalytic (α/α') and 2 regulatory (β) subunits. We previously identified CK2 up-regulation as a hallmark of AT pathological expansion in patients with obesity and diabetes. Moreover, high CK2 expression and activity have been reported in human mesenchymal stem cells and decreased with their differentiation into fat cells. The standard *in vitro* model for adipogenesis is given by 3T3-L1 murine pre-adipocyte cell line, able to differentiate in mature adipocytes after treatment with a specific adipogenic induction medium (MAD), that could be supplemented with Rosiglitazone (MIR), ligand of the master regulator of adipogenesis PPARγ. In this PhD project, we aim to further characterize the role of CK2 in adipogenesis, as well as its involvement in the regulation of adipocyte metabolism.
Material and Methods
We exploited the role of CK2 in adipogenic differentiation targeting its subunits in 3T3-L1 mouse preadipocyte cell line through RNA interference (RNAi) and gene editing approaches. Silencing of CK2 α or β was performed two days before induction with adipogenic medium and confirmed by WB quantification of CK2 subunits and activity. CRISPR-Cas9 technology and FACS sorting were used to generate several 3T3-L1 preadipocyte CK2β Knockout (KO) clones characterized by the almost complete ablation of CK2β and the resulting reduction of CK2-specific activity. Effects on adipogenesis were evaluated by morphological analysis, OIL-RED-O staining and Western Blot for adipogenic markers. Metabolomic and lipidomic analysis were performed on 3T3-L1 mature adipocytes treated with CK2 inhibitors (CX-4945 and SGC-CK2-1) for 24 hours. At the end of the treatment, cell lysis was performed in cold methanol and lysates were analysed with LC/MS (Agilent 1290 II coupled to a time-of-flight mass spectrometer).
Results
The silencing of CK2 α or β highlighted a decreased adipogenesis, particularly marked in cells treated with siRNA for CK2β. In addition, the majority of CK2β KO clones showed a reduced proliferation capacity, a cell size enlargement and the inability to differentiate into mature adipocytes upon standard adipogenic induction (MAD), while we observed a rescue of adipogenesis in clones treated with Rosiglitazone (MIR) for the first three days of differentiation. The Principal Component Analysis (PCA) efficiently separated CK2 inhibitors-treated adipocytes from controls in metabolomic but not in lipidomic experiments. Mature adipocytes treated with both CK2 inhibitors displayed a significant accumulation of Branched Chain Amino Acids (BCAAs) valine, leucine and isoleucine. In contrast, control adipocytes showed an enrichment in Fatty Acid esters of Hydroxy Fatty Acids (FAHFAs) and carnitines.
Conclusions
Our results disclose a fundamental role for CK2β regulatory subunit in adipogenesis supported by inability of CK2β KO clones to differentiate in standard adipogenic medium. However, the capability of Rosiglitazone treatment to rescue clones' adipogenic differentiation suggests mechanisms to investigate. In this way, CK2β KO clones characterization and transcriptomics analysis will be performed in order to identify the specific adipogenic pathways affected by CK2β loss. Different statistical analysis on enriched polar metabolites and lipids, integrated with literature data, highlight that CK2 pharmacological inhibition in mature adipocytes could associate with insulin resistance conditions via the accumulation of BCAAs and Phosphatidylcholines (PC). In contrast, CK2 activity could improve insulin sensitivity and exert anti-inflammatory effects, probably mediated by carnitine and FAHFAs accumulation.

PREVALENCE OF METABOLIC-ASSOCIATED FATTY LIVER DISEASE IN PATIENTS WITH PRIMARY ALDOSTERONISM

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Ph.D. Course: Clinical and Experimental Sciences
Curriculum "Clinical Methodology, Metabolism, Endocrinology, Nephrology and Exercise"

Background

MAFLD, a new definition of metabolic dysfunction-associated fatty liver disease, affects almost a quarter of the world's adult population. Despite the well-known role of obesity and insulin resistance in determining metabolic disturbances such as liver steatosis, there are important relationships between MAFLD and endocrinopathies. PA has been reported to be associated with a higher prevalence of fatty liver disease and metabolic syndrome, compared to normotensive patients or patients with essential hypertension. A close relationship between overt cortisol excess in CS, subclinical cortisol excess in MACS and autonomous aldosterone secretion with higher cardiovascular risk, is reported.

Therefore, we tried to assess the impact of hormonal excess on hepatic steatosis, thus considering the concept of EAFLD in which aldosterone/cortisol excess was considered to have equal weight in MAFLD determination, compared to the other known metabolic criteria.

Material and Methods

Hepatic steatosis was assessed by liver/spleen (L/S) ratio from unenhanced abdomen computed tomography images (reference value <1.1) in a cohort of 41 patients with PA, 20 monolateral (PA^{mono}) and 21 bilateral (PA^{bilat}), 50 nonfunctioning adrenal incidentalomas (NF-AI), 48 mild autonomous cortisol secretion (MACS) and 10 adrenal Cushing Syndrome (CS). We proposed a new definition of Endocrine Adrenal associated Fatty Liver disease (EAFLD), including hormonal excess (aldosterone or cortisol) as a diagnostic criterion.

Results

Hepatic steatosis was increased at PA diagnosis: L/S ratio was lower in PA than NF-AI (1.10 vs 1.25, $p < 0.001$) and MACS (1.10 vs 1.21, p 0.007), but was similar to adrenal CS (1.10 vs =1.15, $p = 0.147$). Longitudinal evaluation before and after PA treatment showed that the median L/S ratio at diagnosis was 1.10 (1-1.22) and the median L/S ratio post-treatment was 1.26 (1.13-1.36), indicating a reduction of liver steatosis ($Z = -4.017$, $p< 0.001$) either after medical or surgical treatment. MAFLD prevalence was higher in PA compared to MACS (49% vs 25%, $p<0.05$) and NF-AI (49% vs 14%, $p<0.001$), but similar to CS (49% vs 45%, $p=0.61$). PA^{mono} patients had higher prevalence of both MAFLD and EAFLD compared to PA^{bilat} group (71% vs 25% and 76% vs 35%, respectively).

Conclusions

We reported a considerable prevalence of MAFLD in PA, higher in PA^{mono} compared to PA^{bilat} patients, probably reflecting lower potassium levels. We also described a significative difference between PA and NF-AI/MACS concerning MAFLD prevalence, while MAFLD prevalence in PA and adrenal CS was comparable. Finally, our definition of EAFLD for patients with adrenal hormone excess (cortisol or aldosterone) demonstrated to increase sensibility in the identification of patients at high risk of MAFLD who don't fulfill classical diagnostic criteria but present hormonal excess with known metabolic implications.

INTERPLAY BETWEEN SKELETAL AND CARDIOVASCULAR HEALTH IN ENDOGENOUS CUSHING'S SYNDROME

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Background

Endogenous Cushing's syndrome (CS) is associated with significant cardiovascular comorbidities, including hypertension, atherosclerosis, cardiac remodeling, thromboembolic events, and metabolic dysregulation. Skeletal complications are common in CS patients as well, encompassing a 11-76% prevalence of fragility fractures (Fx), potentially leading to impaired quality of life and increased mortality.

Bone and vascular diseases commonly coexist, sharing risk factors and pathogenic mechanisms, collectively referred as the "bone-vascular axis". Indeed, cellular, endocrine, and metabolic signals exhibit bidirectional communication between the vasculature and bone, essential for maintaining both bone and vascular health. Experimental evidence suggest that the excess of glucocorticoids may disrupt this axis, however effects in human remains uncertain.

Material and Methods

51 patients with overt CS were included in the study. Several biochemical parameters, as well as instrumental were analyzed, both related to bone and cardiovascular health.

Results

Fx were found in 62.7% of patients at diagnosis. Fractured patients had a longer disease duration ($p=0.025$), higher waist circumference ($p=0.006$), and were predominantly male ($p=0.008$), despite similar values of urinary free cortisol. Spine T-score was lower ($p=0.03$) in fractured than in non-fractured patients, whereas no differences were highlighted in terms of spine and femoral BMD. Increased serum uric acid levels ($p=0.001$), higher prevalence of venous thromboembolism events ($p=0.037$) and of atherosclerotic plaques ($p=0.002$) were described in the fracture group. Moreover, a greater intima-media thickness (IMT) ($p=0.017$) was observed in patients with Fx. Logistic regression identified atherosclerotic plaques, male gender, waist circumference, and osteoporosis as independent predictors of fractures. Following multiple regression, the presence of plaques and osteoporosis remained significant (OR 13.35, 95% CI 1.154-154.34, $p = 0.038$; OR 11.30, 95% CI 1.547-82.562, $p = 0.017$, respectively).

Trabecular Bone Score (TBS) values were comparable between patients with and without fractures, with TBS inversely correlated with BMI, fat and lean mass, and serum uric acid, and positively correlated with HDL cholesterol.

Conclusions

CS patients with higher overall burden of cardiovascular morbidity are more prone to experience Fx.

PhD COURSE "CLINICAL AND EXPERIMENTAL SCIENCES"

COORDINATOR: PROF. ROBERTA RAMONDA

Curriculum "HEMATOLOGICAL AND GERIATRIC SCIENCES"

OSTEOSARCOPENIA: EARLY DIAGNOSIS MODELS AND DISABILITY PREVENTION

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Ph.D. Course: Clinical and Experimental Sciences
Curriculum "Hematological and Geriatric Sciences"

Background

Osteosarcopenia is a condition characterized by the concurrent presence of sarcopenia and osteoporosis. This disorder represents a significant risk factor for disability and reduced quality of life in older adults, as it markedly increases the risk of falls and fractures. However, the underlying pathophysiology of osteosarcopenia includes malnutrition, low physical activity, sedentary behaviour, and chronic inflammatory states, making it a condition potentially present in adults and young adults as well. The objectives of this project are: 1) To investigate the prevalence and impact of early and late bone and muscle alterations on the functional performance of young and older individuals (COMET, SOS_TX studies). 2) To compare the efficacy of anti-resorptives and anabolic agents in maintaining muscle performance, balance, and fall prevention in older patients with osteoporosis under drug treatment (MITO study). 3) To explore the association between osteosarcopenia and inflammaging and its impact on the health of older individuals with chronic diseases (OPA study). 4) To examine the potential link between sarcopenia and neurodegenerative biomarkers.

Material and Methods

The project is divided into several studies, each involving different populations.
In the MITO and OPA studies, older patients over 65 years of age are recruited from the osteoporosis (MITO), obesity, neurology, and geriatrics clinics (OPA) of the Azienda Ospedale di Padova. The COMET study examines the effects of hormone therapy in a group of young adult transgender individuals, recruited from the Andrology and Reproductive Medicine clinics of the Azienda Ospedale Università di Padova, with subsequent evaluation at the Geriatrics clinics. The SOS_TX study evaluates patients over 20 years old, managed by the Thoracic Surgery Unit of the same Hospital for lung transplantation, who are then subjected to a comprehensive geriatric assessment. Finally, the study on neurodegeneration biomarkers is conducted using the SNAC-K database from the Karolinska Institutet, Aging Research Center.

Results

The ongoing studies have produced the following key findings:

- COMET: In a study involving 200 participants (100 transgender and 100 cisgender), it was observed that transgender individuals assigned male at birth have lower bone mineral density and bone geometry compared to their cisgender counterparts. Moreover, lower values of muscle parameters were observed in this population. These differences may be linked to lifestyle factors, including a tendency toward sedentary behaviour, frequent smoking, and a generally calcium-deficient diet.
- SOS_TX: Among patients with Cystic Fibrosis who underwent lung transplantation, low calf circumference—a proxy for muscle mass—was found to be associated with poorer lung function metrics (FVC, FEV1, TLC). This association remained significant even after accounting for vertebral fractures, steroid dosage, and the time elapsed since transplantation.
- A study involving 2,100 healthy elderly individuals in Sweden identified a link between elevated levels of neurofilaments and ptau181 and the development of sarcopenia.

Conclusions

Assessment of osteosarcopenia is crucial for improving patients' quality of life, regardless of age.

A DIAGNOSTIC APPROACH TO IMPROVE THE IDENTIFICATION OF INHERITED THROMBOCYTOPENIAS IN A COHORT OF ADULT PATIENTS

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Curriculum "Hematological and Geriatric Sciences"

Background and Aims

Inherited thrombocytopenias (ITs) represent a heterogeneous group of genetically determined disorders of platelet production or function. Currently, ITs include 45 known disorders caused by mutations in 40 genes, characterized by different degrees of severity related to a broad spectrum of hemorrhagic manifestations and additional comorbidities. Despite the advances in medical science, they often remain underdiagnosed or wrongly framed as immune forms, leading to inadequate clinical, diagnostic and therapeutic management. The introduction of multigenic sequencing techniques (NGS), may rapidly expand knowledge of these nosological entities and identify new gene loci responsible for their occurrence.

Methods

Since October 2021, all subjects, among those with thrombocytopenia of uncertain significance afferenting to First Medical Clinic of University Hospital of Padua were enrolled based on suspicious criteria such as thrombocytopenia with chronic course, family history, failure to respond to first-line therapy for ITP; patients with a previous normal platelet count, acute worsening of thrombocytopenia or acute hemorrhagic diathesis and all secondary thrombocytopenias were excluded. The diagnostic work-up first included the evaluation of platelet diameter and morphology on peripheral blood smear by May-Grunwald/Giemsa staining, the assessment of antigen expression on platelet surface through cytofluorimetric techniques, and platelet aggregation tests according to Born. A preliminary search for mutations in MYH9, in 5'UTR of ANKRD26 and genes coding for the GPIBa/GPIBb/GPIX/GPV complex was conducted by PCR amplification and sequencing using the Sanger's method. In cases in which first level genetic investigations did not lead to the identification of the causative mutation, the genetic study was expanded by NGS.

Results

152 patients belonging to 64 families are currently being enrolled. Sequencing by Sanger method was conducted in 133 and led to the identification of the causative mutation in 72 (47.3%) subjects. Among these, 36 (23.6%) had mutations responsible for Bernard Soulier Syndrome (BSS), and the most frequent variant was Ala156Val (6.5%). In 26 (17.1%) patients, variants associated with MYH9-related disease were detected. In 10 (6.5%) subjects a mutation in the 5'UTR region of the ANKRD26 was found.

NGS has been conducted so far in 13 patients resulting in wild type or VUS carriers at Sanger method. In one patient the variant NM_022437.3:c490C>T, p.(Arg164Ter) in homozygosity in the ABCG8 gene associated with Sitosterolemia 1 with autosomal recessive transmission was detected. In the other cases (23.6%) a variant of uncertain significance (VUS) was found.

Conclusions

Our proposed diagnostic work-up includes firstly the identification of clinical-anamnestic data, that should induce suspicion of a IT. The study of platelet morphology, immunophenotype and platelet aggregation, can reveal alterations that support the genetic hypothesis. The combination of these data could help to select subjects for in-depth genetic study, in which NGS could play a role in identification of additional unknown mutations.

According to literature, in our cohort the most common forms of ITs appear to be monoallelic BSS, MYH9-RD followed by ANKRD26. A mutation associated in the literature with Sitosterolemia 1 was found in one patient. Further investigations will be needed to clarify the pathogenic significance of VUS.

PHYSIOPATHOLOGY OF VENOUS THROMBOEMBOLISM IN PATIENTS WITH HYPERCOAGULABLE STATE RELATED TO INHERITED AND ACQUIRED THROMBOPHILIAS AND CLINICAL IMPLICATIONS

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Curriculum "Hematological and Geriatric Sciences"

Background
Thrombophilia, an inherited or acquired abnormality of the haemostasis and fibrinolysis, predisposes to hypercoagulability and, consequently, to venous and/or arterial thrombosis. Venous thromboembolism (VTE) is the third most common cardiovascular condition, affecting 1-2 individuals per 1000 annually. Despite its prevalence, around 50% of VTE cases are unprovoked, with patients potentially experiencing recurrent thrombotic events. This project aims to identify novel genetic defects, enhance the understanding of hypercoagulable states, and identify laboratory parameters that predict thrombotic risk.

Material and Methods
We are conducting a single-center, observational, non-interventional cohort study in adult patients with objectively confirmed VTE. We are gathering detailed data on demographics, clinical characteristics, etiology, predisposing risk factors, anticoagulant therapy, and long-term outcomes. For patients who test negative for known hereditary thrombophilia but have experienced early-onset thrombosis (ages 18-50) or have a strong family history of VTE, we plan further innovative tests. These include thrombin generation assays, thromboelastometry, extracellular vesicle analysis, and Next-Generation Sequencing (NGS) to identify underlying causes of thrombophilia.

Results
Up to October 2022, we gathered data from 3453 patients diagnosed with VTE since 1990. Among them, 821 patients (23.7%) were carriers of hereditary thrombophilia, and 74 (2.1%) were diagnosed with antiphospholipid antibody syndrome (APS). Unfortunately, 485 patients (14.0%) did not have access to thrombophilia testing. Prospective data collection from November 2022 to July 2024 includes 288 patients. Among this group, 37 patients (12.8%) are carriers of known inherited thrombophilia, 11 (3.8%) have APS, and 48 (16.6%) lack thrombophilia testing data. In a subgroup of 60 unrelated patients tested negative for thrombophilia who had experienced early-onset thrombosis, we conducted genetic analysis using NGS. We found 250 genetic variants, with an average of 64 variants per patient. Eleven patients shared multiple nucleotide variants (MNVs) in the SERPIN A5 gene, while other MNVs were found in the F5, F13B, and PROZ genes. The majority of these variants (94.4%) were Single-Nucleotide Variants (SNVs), with 126 being non-synonymous. Among these, 25 were previously unidentified. Out of the 37 patients with SNVs, the variants were categorized as pathogenic (1), likely pathogenic (10), or of unknown significance (VUS) (10).

Conclusions
Our current research aims to clarify the mechanisms underlying inherited and acquired thrombophilia and to identify novel genetic and laboratory markers for predicting thrombotic risk. Preliminary results suggest that a significant proportion of VTE cases may be related to previously undiscovered genetic variants with uncertain effects on prothrombotic and prohemorrhagic pathways, highlighting the need for advanced diagnostic approaches. We are currently conducting protein functionality assays to understand how these mutations affect procoagulant or anticoagulant protein function and to correlate these findings with clinical outcomes.

NEW PROTHROMBIN VARIANT ASSOCIATED WITH THROMBOPHILIA

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Background
Thrombophilia is defined as the tendency to develop venous thromboembolism (VTE) due to inherited or acquired conditions. Congenital thrombophilia derives from mutations which cause a deficiency and/or dysfunction in the coagulation proteins. We identified a novel prothrombin mutation in a 48-year-old male suffering from recurrent VTE episodes but with a pro-hemorrhagic laboratory phenotype. The aim of the study was to investigate the association of this novel prothrombin variant with VTE by in vitro functional studies.

Material and Methods
Genetic analysis was performed by NGS. Prothrombin activity and antigen levels were detected by the prothrombin-based clotting assay and ELISA using a matched pair of antibodies, respectively. Thrombin generation was measured in platelet poor plasma (PPP-TG) and whole blood (WB-TG) of the patient and normal control. PPP-TG was triggered with 5 pM tissue factor (TF), synthetic phospholipids and CaCl2. WB-TG was triggered using 1 pM TF and CaCl2 without synthetic phospholipids. TG was monitored for 60 min after addition of a fluorogenic thrombin substrate by a microplate fluorometer. TG curves were described in terms of lag time, peak thrombin (peak), time to peak (TTP) and endogenous thrombin potential or area under the curve (ETP). TG assays were also performed after addition of thrombomodulin (TM) to evaluate the functional status of the anticoagulant protein C (PC) pathway. Ratios of ETP and peak were calculated from dividing the results in the presence of TM by the results in the absence of TM. Increased ratios of ETP and peak compared to normal control indicated PC anticoagulant function was impaired.

Results
Genetic analysis revealed a novel heterozygous missense mutation, c.1303G>A (p.Glu435Lys), in exon 11 and a 3000 bp deletion including exon 13 of *F2* gene. Prothrombin activity and antigen levels were reduced to 1% and 20%, respectively (normal range, 80-120%). In PPP-TG assay performed in the absence of TM, lag time and TTP were significantly prolonged in the patient (6.3 min and 8.7 min, respectively) compared to normal control (1.7 min and 4.6 min, respectively). Peak and ETP values were greatly reduced in the patient (42.6 nM and 226.6 nM*min, respectively) compared to normal control (215.4 nM and 1274.3 nM*min, respectively). In WB-TG measured without TM, the patient had lag time and TTP (12.3 min and 16.7 min, respectively) markedly prolonged compared to normal control (3.2 min and 9.4 min, respectively). The patient presented peak and ETP values (65.7 nM and 351.8 nM*min, respectively) significantly decreased compared to normal control (492.0 nM and 4220.1 nM*min, respectively). Peak and ETP ratios were significantly higher (~ 0.97) in the patient than in normal control in both TG assays.

Conclusions
The PC pathway is one of the most important anticoagulant systems. Defective PC pathway leads to blood hypercoagulability and predispose to thrombosis. Reduced prothrombin activity and antigen levels as well as prolonged lag time and TTP and decreased values of peak and ETP, detected in our patient by TG assays in the absence of TM, were consistent with a hypocoagulative state that could be explained by the presence of a 3000 bp deletion in *F2* gene. However, TM-modified TG assay performed on both plasma and whole blood of the patient showed elevated peak and ETP ratios (close to 1.0), indicating functional impairment of PC anticoagulant system. In conclusion, the prothrombin p.Glu435Lys variant identified in our patient could represent a new genetic risk factor for VTE, shifting the hemostatic balance towards hypercoagulability. Further studies with recombinant proteins are underway to better clarify the underlying thrombotic mechanisms.

PhD COURSE "CLINICAL AND EXPERIMENTAL SCIENCES"

COORDINATOR: PROF. ROBERTA RAMONDA

Curriculum "HEPATOLOGY AND TRANSPLANTATION SCIENCES"

ARE THERE ANY BENEFITS OF PROLONGED HYPOTHERMIC OXYGENATED PERFUSION OF THE LIVER GRAFTS FOR TRANSPLANTATION? RESULTS FROM A MULTICENTRIC STUDY

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Background
Machine perfusion is increasingly used to preserve liver grafts before transplantation. Dual hypothermic oxygenated perfusion (DHOPE) enables high-quality preservation and metabolic reconditioning, while normothermic machine perfusion (NMP) permits graft evaluation and selection before implantation. Different advantages of these technologies have been confirmed in the field of donation after cardiac death (DCD), but their use in some specific categories (e.g., elderly donors, donors with extended criteria, steatotic livers, and prolonged preservation times) needs to be further explored. We have retrospectively and prospectively collected data on the use of machine perfusion in liver transplantation from 12 Italian centres. We present here a subgroup analysis of the livers treated with DHOPE, aiming to analyse the impact of the duration of the perfusion on the transplant outcome.

Material and Methods
We performed a cohort study of liver transplants preserved with DHOPE between 2015 (i.e., the start of the use of this technology in Italy) and 2023. Every liver was connected to DHOPE after a certain period of static cold storage for transport and back-table preparation and continued until implantation in the recipient. We have adopted a cutoff of ≥4h to define prolonged DHOPE, in contrast to the current practice of 1–2h of perfusion (short DHOPE). The impact of risk profiles and preservation times (short vs. prolonged DHOPE) on the transplant outcomes was assessed using univariate and multivariate regression models. Primary outcomes were 90-day graft survival and graft-related complications.

Results
A total of 677 liver transplants with DHOPE were initially considered (177 with prolonged DHOPE and the remaining 500 with prolonged DHOPE). A 1:1 propensity score matching was carried out to establish a comparable control group for short DHOPEs. No significant differences in post-transplant outcomes were found between prolonged and short DHOPEs. However, the prolonged group had a significantly lower incidence of post-transplant AKI of stages 2 and 3 compared to the short DHOPE group (30.5% vs. 44.6%, p=0.008). Prolonged HOPE confirmed its protective effect against AKI in a multivariable model adjusted for donor and recipient predictors of AKI [OR: 0.412, 95%CI: 0.200–0.850, p=0.016]. Among prolonged DHOPEs, no differences in transplant outcomes were observed according to donor risk index (DRI≤2 vs. DRI>2), Eurotransplant definition for marginal grafts (standard vs. marginal), and balance of risk score (BAR≤9 vs. BAR>9).

Conclusions
Prolonged DHOPE to improve transplant logistics provides good results with high-risk grafts and appears to be associated with a lower risk of post-transplant AKI. These results provide further insight into the important role of DHOPE in preventing post-transplant complications. Further research should clarify what metabolic differences exist between short and prolonged DHOPE.

URINARY BIOMARKERS OF TUBULAR AND GLOMERULAR DAMAGE PREDICT RENAL RECOVERY IN PATIENTS WITH HEPATORENAL SYNDROME-ACUTE KIDNEY INJURY

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Background
Hepatorenal syndrome-acute kidney injury (HRS-AKI) is the most severe phenotype of AKI in patients with cirrhosis. The combination of vasoconstrictors, particularly terlipressin, and albumin is the first line medical treatment of HRS-AKI. However, less than 50% of patients with HRS-AKI show renal function recovery with vasoconstrictors and albumin. The lack of renal function recovery is still poorly understood and currently there are no biomarkers able of predicting it. The aim of this study was to identify biomarkers of renal function recovery and 90-day mortality in patients with HRS-AKI. We evaluated urinary biomarkers of tubular and glomerular damage, cell cycle arrest and metabolites of *de novo* NAD+ biosynthetic pathway.

Material and Methods
We identified patients with a clinical diagnosis of HRS-AKI, serum creatinine > 1.5 mg/dL and available urine samples among those enrolled in the PREDICT and ACLARA studies (two prospective multicenter observational studies promoted by the European Foundation for the Study of Chronic Liver Failure and performed in Europe and Latin America, respectively, which included more than 2500 patients hospitalized for Acute Decompensation of cisshosis). Demographics, clinical and laboratory characteristics were collected. Urinary biomarkers of glomerular and tubular damage (neutrophil gelatinase-associated lipocalin [NGAL], Cystatin C, Albumin, Epidermal growth factor, Osteopontin) and cell cycle arrest (insulin growth factor binding protein 7 [IGFBP-7]) were measured by multiplex ELISA. Urinary metabolites of *de novo* NAD+ biosynthetic pathway were measured by HPLC-MS. Serum inflammatory cytokines (IL-1beta, IL-1ra, IL-6, IL-8, TNF-alpha, MCP-1, VEGF-A) were measured by a multiplexed immunoassay. Primary outcome was renal recovery (defined as serum creatinine < 1.5 mg/dL) and all the analysis were adjusted for false discovery rate. Predictors of 90-day mortality were explored using renal recovery as a time-varying covariate.

Results
Overall, 171 patients with HRS-AKI were identified. Most frequent etiology of liver cirrhosis was alcohol-related liver disease (61%) and 138 patients (81%) had acute on chronic liver failure (ACLF). Renal function recovery was achieved in 69 patients (40%). Patients with renal recovery were younger (57 vs 62 years; p=0.021), had lower serum creatinine (2.1 vs 2.7 mg/dl; p<0.001) and lower prevalence of ACLF (69 vs 88 %; p=0.033). Patients with renal recovery had significantly lower levels of urinary NGAL (184 vs 533 ng/ml; p=0.007), urinary albumin (4.3 vs 8.2 mg/dl; p=0.025) and serum MCP-1 (235 vs 329 pg/ml; p=0.033) than those without. Metabolites of de novo NAD+ biosynthetic pathway were not significantly different between the two groups. During 90-day follow up 88 patients died. In multivariable analysis, white blood cell count (hazard ratio [HR]=1.05; p=0.001], bilirubin (HR=1.04; p<0.001) and IGFBP-7 (HR=1.27; p=0.002) were identified as independent predictors of 90-day mortality, while renal recovery was associated with a lower risk of 90-day mortality (HR=0.31; p<0.001).

Conclusions
Urinary biomarkers of tubular (NGAL) and glomerular damage (albumin) are strong predictors of renal recovery in patients with HRS-AKI. These data suggest that patients with HRS-AKI have some degree of parenchymal kidney damage that can affect renal recovery. Urinary IGFBP-7, a biomarker of cell cycle arrest, predicts survival independently from renal recovery in patients with HRS-AKI.

PhD COURSE "DEVELOPMENTAL MEDICINE AND HEALTH PLANNING SCIENCES"

COORDINATOR: PROF. GIANNI BISOGNO

Curriculum "ONCOHEMATOLOGY, MEDICAL GENETICS, RARE DISEASES AND PREDICTIVE MEDICINE"

PROTOCOLS FOR "ONE DIGITAL HEALTH" SURVEILLANCE SYSTEMS

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Background
"One Digital Health" (ODH) is an integrated approach that extends the "One Health" concept, which recognises the close interconnection between human, animal, and environmental health, combining it with medical informatics and data science. ODH promotes a holistic and interconnected approach to healthcare by leveraging technologies such as telemedicine, wearable devices, health information systems, and Artificial Intelligence (AI). Despite its promising potential, several challenges hinder the widespread adoption of ODH surveillance systems (SSs). Key obstacles include privacy, lack of interoperability between sectors and stakeholders, and the complexity of integrating diverse data sources. Additionally, evaluating the effectiveness and impact of ODH SSs remains not straightforward.
Materials and Methods
Multiple literature reviews of the Scopus and Embase databases were conducted to assess the implementation details, data sources, and modelling of an infection SS from an ODH perspective across various contexts and applications. A first systematic review with meta-analysis aimed to evaluate the diagnostic performance and impact of AI in healthcare-associated infections (HAIs) surveillance. A second literature review focused on understanding the operational aspects and challenges for implementing an ODH SS for zoonotic pathogens and Antimicrobial Resistance (AMR). Studies were performed in accordance with PRISMA guidelines.
Results
More than 4,000 publications were screened, with 8% deemed eligible. In the case of HAI surveillance, although the performance of AI models was generally high (pooled sensitivity and specificity > 83.5%), adoption in clinical practice remains uncommon (3.6%). Among the approximately 36.7% of studies that compared AI system performance with other methods, most achieved better or comparable results than traditional clinical scores or manual surveillance. The review of ODH surveillance systems for zoonotic pathogens identifies bacteria as the primary target, particularly AMR strains (notably *E. coli*, *S. aureus*, *Enterococcus*, and *Campylobacter*), as well as anthrax, and foodborne infections like *Salmonella*. Other high-priority pathogens include arboviruses (especially West Nile), rabies, and parasites like *Echinococcus*. Studies highlight the importance of engaging local stakeholders. Many cases reveal a lack of systematic data-sharing practices, limited diagnostic capacity, outdated and unclear surveillance guidelines, and inadequate operational research. Overall, integrated surveillance improved risk assessment, better defined risk factors, and promoted coordinated prevention and control measures for infections and antimicrobial stewardship. However, evidence remains scant, particularly for AI-based systems, with less than 7.6% of studies measuring real-word impact.
Conclusions
Common definitions and protocols for "One Digital Health" surveillance systems are necessary to promote their adoption. Public and government collaboration, sharing and timely availability of information flows are crucial for comprehensive monitoring. Further research is needed to assess ODH SSs real-world impact in reducing burden, costs, and resource use.

ENGINEERED EXTRACELLULAR VESICLES LOADED WITH MICRORNA-31 AS THERAPY FOR INFLAMMATORY BOWEL DISEASES

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Background and Aim
Inflammatory Bowel Diseases (IBD) are chronic inflammatory diseases of unknown aetiology and for which therapy should be improved. The extracellular Vesicles derived from Mesenchymal stromal cells (MSC-EVs) have shown the potential to reproduce many of the therapeutic properties of their cells of origin, like the potential to repair damaged tissues. Notably, microRNA-31 (miR-31) has been related to regulating the WNT and anti-inflammatory pathways relevant to IBD. A therapy focused on delivering MSC-EVs loaded with microRNA-31 could bring more knowledge of the regeneration mechanism during IBD.
Aim
This project proposal aims to expand the therapeutic approaches in regenerative medicine in IBD.
Material and Methods
Colon-derived organoids were used to recapitulate in vivo IBD. The IBD damage on organoids was assessed using a pro-inflammatory cytokines cocktail (TNF-alpha, Interleukin-6, and Interleukin-1beta) for 72 hours. Mimic miR-31 was synthesised with a fluorescent dye. Electroporated EVs were loaded with miR31, and the efficiency was evaluated by flow cytometry. Surface area size was monitored during the damage, immunofluorescence, and gene expression for miR-31 targets, and pro- and anti-inflammatory pathways were analysed by qRT-PCR targeting Il6r, Il17ra, Axin2, Lats2, Il10, Occludin, TGF-beta receptor, TNF-alpha receptor, before and after IBD induction and after treatment with naïve-EVs and EVs-loaded miR31.
Results
Mimic miR-31 was efficiently loaded into the MSC-EVs. IBD-damage organoids decreased their surface area after the pro-inflammatory cocktail and recovered their sizes significantly after naïve-MSC-EVs and MSC-EVs-loaded miR-31 treatment. miR-31 targets genes (Il6r and Il17ra), tending to decrease their expression after MSC-EVs-loaded miR-31 and näive MSC-EVs treatment, reducing the inflammatory process. WNT target genes intend to increase, and EVs-loaded miR-31 treatment IBD-damage organoids and HIPPO targets decreased.
Conclusions
These findings highlight the potential of targeted delivery of miR-31 via MSC-EVs as a promising avenue for IBD therapy, offering insights into specific mechanisms for anti-inflammatory mechanisms and regeneration.

INFLAMMATION AND IMPAIRED AUTOPHAGY IN PRESYMPTOMATIC GAUCHER AND FABRY PATIENTS IDENTIFIED BY NEWBORN SCREENING

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Background
Gaucher (GD) and Fabry (FD) diseases are lysosomal storage disorders, characterized by accumulation of sphingolipids, glucocerebroside and globotriasylceramide, respectively. The storage of these macromolecules leads to impairment of autophagic flux, inflammation and oxidative stress. It is known that the accumulation begins before the birth, but it is not known if the secondary abnormalities are already present in the first years of life, especially in presymptomatic patients with late onset phenotype. The aim of our study is to investigate secondary anomalies in presymptomatic pediatric patients with FD and GD, diagnosed by newborn screening.

Material and Methods
Our population consists of 11 pre-symptomatic patients affected by GD (n=5, age 3-6 yrs) or FD (n=6, males, age 2-7 yrs) and 4 healthy controls. We measured the levels of inflammatory cytokines (IL1β, IL6, TNFα) and studied the MAPK pathway, in particular P-P38 expression, and LC3 II, as marker of autophagic flux, by western blotting on peripheral mononuclear cell lysate.

Results
All GD patients showed increased TNFα values (mean 16.2 ng/l, range 10.4-28.6, nv < 8.1) which was correlated with the lysoGb1 values (mean 21.74 mmol/l, range 2.37-88.72, nv <1.93, r2=0.95, P=0.004). Only the patient with marked increase in LysoGb1 level (88.72 mmol/L) had an increase in P-P38 activation. All patients showed reduced levels of LC3 II (mean 48%, SD 27%), indicating impaired autophagic flux. Patients with Fabry disease showed increased P-P38 values (mean 1,31 times greater that controls, SD 1) and decreased LC3 II levels (mean 47%, SD 54%). The also showed increased IL1β (mean 9.52 ng/l, SD 5.96, nv <7) and TNFα (mean 10.15 ng/l, SD 6.60, nv <8.1) levels, while in all IL6 levels were reduced, below the minimum cutoff (3 ng/l).

Conclusions
Our data indicate that in patients with late-onset GD and FD there is an early activation of inflammatory pathways, and impairment of autophagic flux. This increases our knowledge on the natural history of the diseases and opens the way to the possibility of specific therapies that could act on these specific pathways, preventing or delaying the onset of the clinical disease.

TP53 AND TPM2: NEW POTENTIAL BIOMARKERS IN PEDIATRIC BURKITT LYMPHOMA

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Background
Burkitt lymphoma (BL) is the most common subtype of non-Hodgkin lymphoma (NHL) in pediatric patients. Despite current chemotherapy regimens are extremely effective, the outcome for patients with primary refractory or relapsed disease still remains very poor, with a survival rate of less than 30%. Aiming to implement new biomarkers for risk assessment, we conducted a mutational study of the *DNA binding domain* (DBD) of *TP53* in a cohort of 214 BL patients treated according to the LNH-97 protocol. Furthermore, we performed single-cell transcriptomic analysis on a small cohort of tumor samples to investigate BL heterogeneity and the features distinguishing therapy responders from non-responders.

Material and Methods
BL diagnostic samples, including tumor tissue and pleural effusions, were collected at diagnosis. *TP53* gene hot-spot exons 5, 6, 7 and 8 of the DBD were amplified according to the IARC protocol; PCR amplicons were purified and then sequenced by Sanger method. Specimens used for sc-transcriptomic analysis were obtained from 11 patients, three of whom experienced relapse. The validation cohort, analyzed by qRT-PCR, included 61 pediatric BL patients.

Results
In line with previous studies conducted by UK and German groups, *TP53* mutations were detected in 87/214 (40.7%) of our cases. The presence of *TP53* mutations was associated with a significantly inferior outcome (3-year PFS of 91%±3% for *TP53* wild-type vs. 76%±5% for patients bearing *TP53* mutations, p-value 0.005). The multivariate analysis, which also considered the main clinical parameters, showed the prognostic impact of *TP53* mutations for the early identification of "high-risk" BL patients.
Single-cell transcriptomic analysis revealed an unexpected and high inter- and intra-tumoral heterogeneity. Additionally, numerous transcripts were found to be differentially expressed among patients with different prognoses, including Tropomyosin 2 (TPM2), a member of the actin filament-binding protein family, which was confirmed to be significantly higher in the majority of relapsed cases at both RNA and protein levels in an independent cohort of 61 BL cases (p-value <0.05). Furthermore, TPM2 expression can also discriminate high-risk patients with *TP53* mutations (3-year PFS 85% TPM2 low and *TP53* mutated vs. 42% TPM2 high and *TP53* mutated, p-value 0.008).

Conclusions
We demonstrated the prognostic impact of *TP53* mutations for the identification of pediatric patients with BL at higher risk of treatment failure. Moreover, to further explore the pathogenic role of identified mutations, we are conducting *in-vitro* evaluations using the H1299 lung cancer cell line that does not express *TP53*, introducing hotspot mutations and wild-type variants by lentiviral vectors. For this reason, I spent the last two months at the University Hospital of Münster, in Germany, to learn about specific techniques crucial to achieve our purpose.
The aberrant expression in BL non-responders of TPM2 strongly suggests its contribution in therapy resistance of BL. This novel observation not only provides a new potential biomarker for early detection of patients at high risk of refractory disease, but also instructs on the need to investigate the role of cytoskeleton features in BL pathogenesis.

TARGETING CD84 IN PEDIATRIC ACUTE MYELOID LEUKEMIA: A NOVEL APPROACH FOR CAR T CELL THERAPY

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Curriculum "Oncohematology, Medical Genetics, Rare Diseases and Predictive Medicine"

Background
Over the past decade, significant advances in immunotherapy have revolutionized the approach to cancer treatment, especially for patients with relapsed/refractory disease. In pediatric oncology, where treatment success is often hampered by long-term side effects, immunotherapy represents a concrete avenue. Currently, numerous clinical trials are evaluating new immunotherapy-based drugs for patients with hematological malignancies, with the most promising results seen in the application of chimeric antigen receptor (CAR) T cell technology in lymphoblastic malignancies and solid tumors.
Aiming to create a highly specific CAR-T cell therapy for pediatric acute myeloid leukemia (AML), we conducted an extensive analysis of a gene expression dataset from AML patients and discovered, for the first time, that CD84 is a novel AML-specific marker. We aim to test the expression of the CD-84 in the bone marrow of AML patients at diagnosis, relapse, and follow-up to test its specificity, and in bone marrow healthy hematopoietic stem cells (CD34+) samples for its selectivity and safety.
Material and Methods
We analyzed 145 paediatric AML BM samples including 102 AML at diagnosis, 32 AML at relapse and 11 AML samples collected during therapy. CD84 expression was performed by flow cytometry in accordance with the World Health Organization (WHO)-style tripartite consensus rating.
Results
Patients were 75(52%) male and 70(48%) female, the average age was 10,4 years. By cytogenetic and molecular aberrations, main factors currently dictating risk stratification and adopted therapy, patients were distributed in twenty-five patients out of 145 (17%) harboured rearrangements of the core binding factor (CBF), 12 cases (8,5%) were NPM1 mutated, 38 (26%) harboured a KMT2A-rearrangement, 15(10,5%) had the FLT3-ITD mutation and 18 (12,5%) had other rare aberrancies. 37/145 (25,5%) cases were negative for the markers routinely screened. CD-84 was rated as positive in 131/145 (90,3%) patients including 91/102 (89%) at diagnosis, 29/32 (91%) at relapse. Interestingly, during the follow-up we monitored 11 patients having positive minimal residual disease (MRD) by flow cytometry (1-5% blasts) and 11/11(100%) expressed CD84. Notably CD-84 was found positive in all KMT2A-r AML(38/38), which is a highrisk subgroup of AML with increased relapse occurrence. We deep into antigen density since insufficient reactivity against cells with low antigen density has emerged as an important cause of CAR T-cell resistance. Flow cytometric Median Fluorescence Index (MFI), analysis of primary AML cells showed that CD84 was highly expressed on AML blasts (MFI= 4780) but not on normal lymphocytes (MFI 310), and in other healthy myeloid cells (MFI=470). To support that targeting CD84 is safe and would not affect hematopoietic stem cell compartment, CD34+ were tested and documented to expressed very low levels of CD84 (MFI=517).
Conclusions
These results address a fundamental challenge in immunotherapy for AML and identify that CD84 is an AML-restricted target. Its expression in >90% of the AML samples provide the foundation for further pre-clinical studies aimed at testing the potency of CD84-directed CAR T cells as a potential therapeutic approach for all AML regardless of the genetic lesion.

PREDICTIVE VALUE OF PLASMA AND URINARY METABOLOMIC PROFILE IN NEWBORNS WITH CONGENITAL HEART DISEASE

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Ph.D. Course: Developmental Medicine and Health Planning Sciences
Curriculum "Oncohematology, Medical Genetics, Rare Diseases and Predictive Medicine"

Background
The incidence of adverse outcomes in infants undergoing surgery for complex congenital heart disease (CHD) remains high, with early identification and treatment of high-risk patients being particularly challenging. Traditional clinical risk factors often fail to fully explain the variability in outcomes, highlighting the need for new approaches. Metabolomics offers a promising method for identifying physiological disruptions and potential therapeutic targets in neonates undergoing CHD surgery. Previous studies have shown that metabolic profiling can effectively detect CHD during fetal life. Additionally, it may be useful in understanding the postoperative metabolic changes in this population. This study aims to explore the association between metabolic profiles and major clinical outcomes in newborns undergoing elective surgery/catheterization for CHD, potentially identifying early metabolic biomarkers of postoperative morbidity and mortality.

Material and Methods
This prospective, observational, single-center study is conducted at the Neonatal Intensive Care Unit (NICU) of Padua University Hospital. It includes full-term newborns (gestational age >37 weeks) with complex CHD who require either corrective or palliative cardiac surgery or hemodynamic catheterization. The study aims to enroll approximately 30 newborns over 18 months. Data collection includes anthropometric, demographic, clinical, laboratory, and instrumental information at birth and during hospitalization. Plasma and urine samples are collected at three time points: at birth (within 72 hours), pre-cardiac surgery (within 24 hours), and post-cardiac surgery (within 3 days). These samples will be analyzed using Untargeted metabolomic profiling through Ultra Performance Liquid Chromatography (UPLC)-Mass Spectrometry (MS) at the Mass Spectrometry and Metabolomics Laboratory of the University of Padua. Additionally, cerebral ultrasound and NIRS monitoring (cerebral and renal) will be performed at birth and post-surgery.

Objectives
The primary objectives of the study are to evaluate the changes in plasma and urinary metabolomic profiles before and after surgery and to determine whether early metabolomic profiles (within 72 hours of life and within 24 hours pre-surgery) can predict the need for ECMO and post-surgical mortality. Secondary objectives include assessing whether early and pre-surgery metabolomic profiles can predict the risk of intraventricular haemorrhage (IVH), acute kidney injury (AKI), necrotizing enterocolitis (NEC), and neurodevelopmental outcomes at discharge, measured by the Hammersmith Functional Motor Scale Expanded (HFMSE).

Results
To date, 12 newborns (4 females and 8 males: median gestational age at birth 38 gestational weeks; median birth weight 2378g)) have been enrolled in the study, with diagnoses including TOF (Tetralogy of Fallot); DORV (Double Outlet Right Ventricle)-TOF with pulmonary valve atresia; D-TGA (Transposition Great Vessels); Aortic Coarctation; Aortopulmonary Window; Unbalanced complete AVSD (Atrioventricular Septal Defect); Double aortic arch and others. Patient recruitment, data and samples collection and metabolomic analysis with interpretation of results is still ongoing.

Conclusions
Metabolomic profiling has the potential for enhancing the care of infants undergoing cardiac surgery for CHD. By offering insights into the metabolic alterations caused by surgical stress and recovery, this approach could help in identifying high-risk patients, guiding perioperative management, and ultimately improving clinical outcomes.

LEVERAGING PHENOTYPIC PLASTICITY TO OVERCOME DRUG RESISTANCE IN PEDIATRIC NEUROBLASTOMA

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Background
Neuroblastoma (NB) is a pediatric tumor of the developing sympathetic nervous system that accounts for 15% of pediatric cancer-related deaths. High-risk NB patients are subjected to an intensive multimodal treatment regimen. However, many high-risk patients with NB experience relapse due to the development of therapy-resistant tumors, a phenomenon frequently associated with phenotypic cell plasticity.
Recent studies employing gene expression and epigenetic profiling of NB cell lines have identified two predominant cell types: adrenergic (ADRN) and mesenchymal (MES), which can spontaneously switch states through epigenetic regulation. ADRN cells exhibit markers of sympathoadrenergic differentiation, whereas MES cells resemble undifferentiated neural crest progenitors and are dominant among drug-resistant clones. This phenotypic cell plasticity may confer adaptive advantages under selective pressure, contributing to drug resistance and cancer progression. Here, we aimed to investigate the mechanism by which neuroblastoma cells rewire their phenotypic state to acquire drug-resistant features.
Material and Methods
In this study, we utilized two isogenic tumor-derived cell lines deriving from primary tumor mass (T) or bone marrow metastasis (B), namely 691B/691T and 700B/700T, to examine the epigenetic mechanisms associated with drug resistance. These cell lines were characterized using qPCR and WB to classify them as either ADRN or MES. The cells were treated with the Axl inhibitor TP-0903, proposed for mesenchymal cell treatment, and cell viability and apoptosis were analyzed using resazurin and flow cytometry, respectively. The effects of different drug concentrations on epigenetic marker expression were also investigated. In addition, a combinatorial approach with BIX-01294, a selective inhibitor of H3K9me3, was adopted to evaluate the effects on cell viability, apoptosis, and proliferation using a colony formation assay. Western blotting was adopted to follow the changes in the expression levels of different markers related to ADRN and MES state.
Results
Our investigation paves the base for a rapid and univocal validation of NB cells as ADRN or MES. As well, it revealed that treatment with TP-0903 resulted in an increase in H3K9me3 expression in MES cells. Furthermore, we observed that chemical inhibition of H3K9me3 using BIX-01294 potentiated the cytotoxic effects of TP-0903. Additionally, our data showed that H3K9me3 expression was differentially regulated in MES and ADRN cells. Importantly, in MES cells, inhibition of H3K9me3 partially reversed the cell phenotype, leading to an increase in the expression of ADRN markers and a decrease in the expression of MES markers.
Conclusions
Based on our findings, we propose that H3K9me3 plays a crucial role in facilitating phenotypic plasticity and promoting MES phenotype and drug resistance in NB cells. Our results suggest a strong dependence of aggressive NB phenotype on epigenetic regulation suggesting it as a therapeutic vulnerability in this tumor. This opens a window for a novel therapeutic strategy that combines conventional therapies with epigenetic drugs to target the intra-tumor cell heterogeneity and hence clone selection during treatment.

A NOVEL AND EMPOWERED TARGETED GENE ADDITION APPROACH AT A RELEVANT MICROGLIA LOCUS FOR THE TREATMENT OF X-LINKED ADRENOLEUKODYSTROPHY

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Background

Adrenoleukodystrophy is an X-linked disorder (X-ALD, OMIM: 300100) resulting from a mutation in the ABCD1 gene, which causes a defect in peroxisomal beta-oxidation and leads to the accumulation of saturated very long chain fatty acids in body tissues primarily impacting the Central Nervous System (CNS). Its pediatric manifestation, named Childhood Cerebral ALD, still represents a frequent cause of unrelenting neuropathology and death early in infancy. Although allogeneic Hematopoietic Stem and Progenitors cells (HSPCs) transplantation and lentiviral gene therapy (GT) for X-ALD are considered valuable treatment options for childhood cerebral X-ALD patients, their use is limited by the slow engraftment of transplanted cell progeny in the CNS as compared to the rapid progression of neurodegeneration. Moreover, the allogeneic transplant is affected by donor availability and graft versus host disease, whereas lentiviral gene therapy by the risk of clonal expansion events consequent to vector integration.

We have identified CX3CR1, a chemokine receptor expressed on microglia that binds fractalkine (CX3CL1) and regulates recruitment to sites of neuroinflammation and microglia ontogeny, as a key locus to be targeted to enhance the ability of HSPCs to generate microglia-like cells (MLC) upon transplantation. Indeed, we demonstrated that transplantation of CX haploinsufficient HSPCs results in a faster and improved engraftment and generation of MLCs as compared to wild type cells, as well as a more efficient acquisition of mature microglia-like phenotype by the transplanted cell progeny. Based on this evidence, we have developed a CRISPR/Cas9 gene editing and targeted gene addition strategy to insert the human ABCD1 cDNA at the CX3CR1 locus to simultaneously generate a haploinsufficiency condition at the locus and drive the expression of the therapeutic cassettes under the control of the CX3CR1 locus. This could allow coupling an anticipated microglia replacement effect by the gene corrected cells with a regulated microglia-specific expression of the therapeutic gene, also reducing the safety concerns related to the use of integrating vectors.

Material and Methods

We targeted a promoter less, splice trapping cassette encoding hABCD1 cDNA to a CX3CR1 intron achieving highly efficient targeted insertion and regulated transgene expression in microglia cell lines and human HSPCs.

Results

Our data, demonstrate a successful and efficient (up to 60% of targeted alleles) transgene integration in hHSPCs, a robust and CX3CR1_promoter regulated hABCD1expression in HSPCs progeny cells upon myeloid differentiation and the establishment of a CX3CR1 haploinsufficiency maintaining a functional copy of the gene.

Conclusions

We are now reproducing these data employing engineered Lipid Nanoparticles (LNPs) for the delivery of gene editing reagents to CD34+ HSPCs to alleviate the well-known effects of the editing procedure and establish a new harmless ex vivo gene editing protocol with improved tolerance to cell manipulation, as well as testing protocols compatible with large scale manipulation. Simultaneously, we are testing the CX3CR1 edited human HSPCs into relevant immunodeficient models allowing to either enhance microglia engraftment (IL34 and CSF1 NSG models) or to establish proof of concept data in correcting the disease associated biochemical abnormalities (in the NSG SGM3 ABCD1-KO (Δ897) disease model). Early in vivo data will be presented.

PhD COURSE "DEVELOPMENTAL MEDICINE AND HEALTH PLANNING SCIENCES"

COORDINATOR: PROF. GIANNI BISOGNO

Curriculum "HEALTH PLANNING SCIENCES"

CHILDREN EXPOSURE TO DRUGS OF ABUSE: STUDY OF HAIR ANALYSIS REPORTS FROM 2018 TO 2022 AND PRELIMINARY STUDY OF 2023 REPORTS

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Background
Children exposure to drugs of abuse is an emergent problem which affects children health. The exposure may occur via different routes, whose likelihood changes depending on the age of the child, and includes in utero, breastfeeding, passive inhalation, intentional administration, and accidental intake. The suspicion of exposure may raise from a maternal history of substances use during pregnancy or lactation, from the presence of withdrawal symptoms at birth, or signs of acute intoxication, or it may also raise when assessing a case of abuse or neglect. In addition to blood and urine analysis, hair analysis is increasingly used in our clinical context to investigate such exposure, but its interpretation in children is still difficult and does not allow the specific mode of exposure to be traced.
Material and Methods
To investigate the extent of this phenomenon in our setting and to pick up any suggestions to guide the clinical practice, the reports of hair analysis of children aged 0-16 years, performed at the Legal Medicine and Toxicology Unit of the University Hospital of Padova from 2018 to 2023, were collected and then analyzed focusing on infants of 0-1 year. Reports of parents' hair analysis were also collected, if present, and results of children hair analysis were compared with those of their mothers, when available.
Results
375 reports of hair analysis of children aged 0-16 years were included, 240 referred to the period 2018-2022 and 135 referred to 2023. Of these, respectively 101 and 57 referred to infants of 0-1 year. Maternal hair analysis was available for 37 infants of 0-1 year (including 3 twin sibling pairs) in period 2018-2022, and for 25 infants of 0-1 year in 2023. Cocaine represented the most frequently detected substance in both periods, 2018-2022 and 2023, being respectively 94 (39.2%) and 44 (32.6%) children of 0-16 years positive for this substance, and particularly 42 (41.5%) and 24 (42.1%) infants of 0-1 year. This emphasizes the need to implement information on the risks of this substance and to spread it to parents and people of child-bearing age. In most of requests for neonatal hair analysis, no clinical symptoms of the child were described, suggesting the importance of a comprehensive assessment not limited only to the clinic and suggesting the need to pay attention to the interview and the relationship of trust with pregnant women. The analysis of period 2018-2022 showed the highest number and rates of positives in young children within 3 years of age, and highest median concentrations of cocaine, methadone, tetrahydrocannabinol in infants within the first year, suggesting that exposure to drugs of abuse represents a non-negligible problem particularly in infants and toddlers, which requires special attention in clinical and social setting.
Conclusions
Notwithstanding the difficulties inherent in the subject, all these elements could be used to build shared prevention and management protocols for this phenomenon.

PhD COURSE "MOLECULAR MEDICINE"

COORDINATOR: PROF. ARIANNA LOREGIAN

Curriculum "BIOMEDICINE"

DEVELOPMENT AND CHARACTERIZATION OF NOVEL ANTIVIRALS AGAINST CORONAVIRUSES

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Curriculum "Biomedicine"

Background

The main protease MPro of coronaviruses, including SARS-CoV-2, is considered a good drug candidate and research has been intensely pursued in screening molecules that bind its catalytic pocket. We focused our efforts on the characterization of novel inhibitors of this protein. Furthermore, we recently proposed the first indomethacin (INM)-based PROTAC degraders endowed with SARS-CoV-2 inhibitory activity and we characterized the mechanism of action of VHL-recruiting PROTACs, that we previously reported, both in transfected and infected cells. In addition, we explored the use of cereblon (CRBN) as another E3 ligase (PROTACs 12–14 and 18-21) for INM-based PROTACs.

Material and Methods

The antiviral activity of the MPro inhibitors and degraders was first evaluated using plaque reduction assays (PRAs) in Vero E6 cells infected with SARS-CoV-2/NL/2020. Moreover, their antiviral activity was assessed by PRAs against the endemic β-coronavirus HCoV-OC43 and α-coronavirus HCoV-229E. In parallel, the cytotoxicity of the compounds was evaluated by MTT assays.

Several stereoisomers and analogues of the MPro inhibitor Cov07 were characterized by testing their anti-MPro activity both in transfected and infected cells. In parallel, to investigate the PROTACs' mode of action, we compared the degradation effects of the best PROTACs with their respective negative controls, *i.e.*, compounds that were unable to bind the E3 ligase VHL or the target MPro, or co-treatment with the VHL ligand VH298. The activity of the most promising compound, PRO-INM-09, was confirmed through Western blot analysis of MPro levels in human Calu-3 cells.

Results

We demonstrated that compounds Cov07 and Cov37 are able to inhibit the activity of MPro in a cellular context, while the exploration of the activity of their stereoisomers is still ongoing. The biological evaluation of INM-based PROTACs recruiting the E3 ligase VHL demonstrated that PRO-INM-04 and -09 can induce the concentration-dependent degradation of SARS-CoV-2 MPro both in transfected and in SARS-CoV-2-infected cells and that this degradation is dependent on VHL. PRO-INM-09 also exhibited MPro degradation activity in human lung Calu-3 cells. Furthermore, the antiviral activity of INM-based PROTACs was susceptible to the modification of the E3 ligase ligand, since CRBN-recruiting PROTACs were inactive.

Conclusions

The reported studies allowed us to better characterize the activity of the candidate MPro inhibitor Cov07 and its analogue Cov37; furthermore, we identified the target of the INM-based PROTACs in the SARS-CoV-2 MPro. These results pave the way for possible strategies that can be adopted to counteract the coronavirus infection in cells.

CHARACTERIZATION OF MICROBIOME, VIROME AND HOST IMMUNE RESPONSE IN INFLAMMATORY DISEASES OF THE GASTROINTESTINAL TRACT

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Curriculum "Biomedicine"

Background
The microbiota has been already proven to modulate inflammation in the gastrointestinal tract and airways by stimulating the host epithelial cells to produce pro-inflammatory cytokines. Eosinophilic esophagitis, EoE, is a Th-2 antigen-mediated chronic inflammatory disease which is triggered by food and airborne allergens. The chronic inflammation reshapes the oesophageal epithelium, leading to difficulties in swallowing. To date, the diagnosis requires an endoscopy with the collection of at least 6 biopsies along the oesophageal tract, followed by the counting of eosinophils and the exclusion of other morbidities. The aim of this project is to better characterise the microbiome in saliva, stool and tissue samples of EoE patients compared with healthy controls. Moreover, it aims to identify potential biomarkers for a non-invasive diagnostic method relying on features detectable in saliva or stool samples.
Material and Methods
Self-collection kits for saliva and stool samples, together with surveys about clinical and lifestyle metadata, were delivered to subjects with a confirmed EoE diagnosis and to matched healthy controls. Oesophageal biopsies were also collected from EoE patients. Metagenomics and metatranscriptomics were tested on a saliva and on a stool sample extracted multiple times with different experimental conditions, on a bioptic sample, and on a standard mock community. A bioinformatic pipeline was then designed to process the raw sequencing data. Finally, the number of informative features detected at different sequencing depths was assessed.
Results
Saliva, stool and oesophageal biopsies, together with clinical and lifestyle metadata, were collected from 52 subjects with a confirmed EoE diagnosis. So far, biological samples and metadata have been collected from seventeen healthy volunteers matched for age, sex BMI and diet. Kneaddata was selected as the bioinformatic tool to clean and decontaminate raw reads. The taxonomic profile was obtained running metaphlan, whereas gene families and pathways were reconstructed with humann. Overall, the taxonomic profile of the mock community highlighted a suboptimal lysis in favour of gram-negative bacteria, whose relative abundance resulted to be overestimated. Unfortunately, no biological information could be retrieved from the oesophageal biopsy due to insufficient microbial genetic material. A sequencing depth of 40M reads per strand per sample was identified as the most adequate sequencing depth.
Conclusions
While a sufficient number of EoE patients was recruited, the collection of samples from matched healthy controls is still ongoing. In order to improve the lysis protocol and to even the lysis of gram-negative and gram-positive bacteria, we are optimizing the lysis protocol by substituting the vortex with the omni bead ruptor. The oesophageal biopsies will probably undergo RNA sequencing instead of shotgun metagenomics and metatranscriptomics to retrieve interesting biological information. To end with, the sequencing depth and the pipeline to perform the initial analysis have been defined, but more pipelines have to be designed and tested to perform more extensive analyses.

VIRNA: INVESTIGATING VIRAL CONTAGION PATTERNS VIA DIRECTED MINIMUM SPANNING NETWORKS

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Background

Next Generation Sequencing technologies are nowadays essential in public health surveillance for tracking pathogen evolution, spread, and the emergence of new variants. The sequencing of millions of viral genomes during recent pandemics has highlighted limitations of the existing molecular phylogenetic algorithms in analyzing rapidly evolving pathogens. Traditionally, phylogeny has been the milestone method for studying pathogen evolution. However, its statistical accuracy diminishes when analyzing highly similar sequences, a scenario that has become increasingly common during recent pandemics and is expected to become the norm in pathogen surveillance, with thousands of genomic sequences produced for an efficient monitoring of pathogen spread. This situation has highlighted the urgent need for advanced analytical approaches to manage and interpret such data. To address this need, we have developed VirNA (Viral Network Analyzer), a tool designed to reconstruct detailed patterns of viral transmission and spread within specific regions and time periods.

Material and Methods

The algorithm of VirNA implements Minimum Spanning Networks as defined by Bandelt et al., introducing novel elements to enhance the interpretation of viral evolution. The algorithm takes a set of viral genomic sequences as input and generates a topological structure, consisting in nodes connected by edges, where nodes represent groups of identical sequences and directed edges represent the putative evolutionary paths of the evolving virus. Two nodes are connected only if the mutations characterizing one node are entirely contained in the mutation set of the other node or vice versa. The direction of the connection always goes from sequences with fewer mutations to sequences with more mutations, following a mutation accumulation rationale, that is the most probable scenario in the context of rapidly evolving pathogens. VirNA also extracts information regarding the putative introduction (founder effect) or exit (spread) of the analyzed pathogen into and out of specific geographic areas. The efficient implementation of VirNA allows for the rapid analysis of thousands of sequences.

Results

The topological structures generated by VirNA enable the user to understand the putative evolutionary pathways taken by the analyzed pathogen both genomically and geographically. The directed connections between nodes effectively identify connections between highly similar sequences, typical of rapidly evolving pathogens, and datasets aligned with new trends in pathogen surveillance, where thousands of sequences are daily generated from around the world.

VirNA does not estimate any node of the network, making it suitable for analyzing simulated data or highly granular real datasets. However, this approach may be a limitation when dealing with less homogeneous datasets, where alternative methods might be more appropriate. VirNA is optimized to handle large datasets, such as those of SARS-CoV-2 or arboviruses sequences, making it suitable for big data analyses.

Conclusions

VirNA is a powerful tool for understanding and tracking rapidly evolving pathogens within extensive and fine-grained datasets, making it a useful resource in pathogen surveillance effort.

IDENTIFICATION OF A NEW NON-CANONICAL STRUCTURE IN THE GENOME OF *MYCOBACTERIUM TUBERCULOSIS*

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Curriculum "Biomedicine"

Background

Tuberculosis (TB) is caused by the bacillus *Mycobacterium tuberculosis* (Mtb) and is one of the leading causes of death worldwide, as reported in the "Global tuberculosis report 2023".

The lack of an effective vaccine and the ever-increasing resistance to antibiotics makes the search for new anti-TB drugs a significant challenge for biomedical research.

Material and Methods

Pull-down of mycobacterium proteins: proteins were extracted from Mtb H37Rv, pulled down with streptavidin coated paramagnetic beads derivatized with biotinylated oligonucleotides. The bounded proteins were visualized by SDS-PAGE and identified by mass spectrometry.

SDS-PAGE: precasted gels were used, NuPAGE™ 4 to 12%, Bis-Tris protein gel.

Expression and purification of proteins: the Rv1488 and SPFH sequences were cloned into pEt23b, pGex6P-1 and pRESTb plasmids and the expression was induced with BL21 competent cells. The interest proteins were purified in batch by a GST-tag or Ni-NTA affinity resins.

Fluorescence anisotropy: samples were read by the Tecan plate reader. The reading mode is fluorescence polarization with excitation wavelength: 485 nm and emission wavelength: 535 nm. Anisotropy calculation has been done: $(I_{parallel} - I_{perpendicular})/(I_{parallel} + 2*I_{perpendicular})$.

Results

G-rich regions of the genome of Mtb were investigated in order to find a sequence pattern potentially capable of folding into a nucleic acid secondary structure. The aim was to study the epigenetical regulation of cellular functions, as a potential pharmacological target.

We identified CORE-1, a highly repeated sequence within the genome of Mtb but very rarely repeated in the human genome, that is capable of folding in vitro into a G-hairpin.

The G-hairpin is a secondary structure that has recently attracted interest. It consists in a filament folded back on itself forming a loop and stabilized by both Watson-Crick and Hoogsteen bonds.

Since we had no information about the physiological role of CORE-1 G-hairpin, we performed a pull-down assay with mycobacterium proteins that allowed us to identify a protein (Rv1488) interactor of CORE-1, potentially involved in the physiological activity of the structure.

The interaction CORE-1 – Rv1488 to be confirmed should be demonstrated through different techniques: it is still today object of our study. In particular they are: EMSA and fluorescence anisotropy.

Additionally, as Rv1488 is an uncharacterized protein, we are carrying out some functional assays. And in order to perform them the protein has been produced, through the recombinant DNA technique, exploiting different tags and plasmids.

Conclusions

Although the results are still preliminary, our attention is now focused on both CORE-1 and the protein since, directly or indirectly, they could be interesting candidates as therapeutic targets.

INVESTIGATING THE ROLE OF THE PROTEIN LYSX2 OF *MYCOBACTERIUM TUBERCULOSIS*

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Background

LysX2 is a protein of *Mycobacterium tuberculosis (Mtb*), characterized by a domain with high similarity to aminoacyl-phosphatidylglycerol synthases, that belongs to the family of MprF-like proteins. However, unlike homologous domains of MprF of *S. aureus* and LysX of *Mtb*, positioned in the cytoplasm, the MprF-like domain in LysX2 is located in the extracytoplasmic region. LysX2 orthologues can be found in major human pathogens and in rapid-growing mycobacteria frequently associated with human infections, but not in environmental and non-pathogenic mycobacteria. To understand its role, LysX2 was introduced in the non-pathogenic *M. smegmatis*, which naturally lacks this protein. Bacteria were then exposed to several stressful conditions and it was observed an increased resistance to acidic pH, nitrosative stress, Cationic Antimicrobial Peptides (CAMPS), a delay in biofilm formation and a reduction on the negative net charge of the bacterial surface.

Material and Methods

To investigate the role of LysX2, a knockout strain was generated in *Mtb* using a genetic engineering tool for mycobacterial chromosomes known as ORBIT. During the various stages of mutant generation, a lipid profile analysis was performed, specifically focusing on PDIM, to rule out the emergence of mutations affecting the genes involved in their biosynthesis. Considering that the phenotypes observed in *M. smegmatis* were associated with acidic pH, the ability of the knockout strain to grow in this stressful environment was tested. Additionally, the role of LysX2 in response to nitrosative stress was assessed using a REMA assay on bacteria grown in the presence of different concentrations of sodium nitrite.

Results

The lipid profile analysis demonstrated that during the generation of the *lysX2* knockout mutant, no mutations occurred that could impair the biosynthesis of PDIM, as the lipid pool of bacteria remained unaltered. The growth of *Mtb* at acidic pH is slowed due to metabolic changes, but while the wild-type strain, after an extended latency phase, enters an exponential growth phase, the *lysX2*-deficient strain maintains significantly reduced growth. Additionally, when the same strains were incubated with sodium nitrite, which generates nitrogen radicals and thus nitrosative stress in an acidic solution, it was observed that the MIC90 of the wild-type strain was two-fold higher compared to that of the KO strain.

Conclusions

LysX2 could be a prototype of a new class within the MprF-like protein family exerting its function through a novel still unknown mechanism that is important for modulating cell surface and consequently bacterial fitness and virulence, in particular in response to phagosome-related stresses, such as acidic pH and nitrosative stress.

IDENTIFICATION OF ANTI-FLAVIVIRUS ACTIVITY OF APPROVED ANTIFUNGAL DRUGS BY A DRUG REPURPOSING APPROACH

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Curriculum "Biomedicine"

Background
Mosquito-borne flaviviruses, which include West Nile virus (WNV), dengue virus (DENV), and Zika virus (ZIKV), are single-stranded positive-sense RNA viruses that can cause from asymptomatic to severe human infections. The increasing risk of potential flaviviral infection outbreaks and the lack of specific antiviral agents have seriously threatened human health in recent years. For this reason, there is an urgent need for novel antivirals against these viruses. Numerous strategies have been adopted and among them, drug repositioning is an approach relatively faster and cheaper than *de novo* drug discovery. Interestingly, some antifungal azoles have been recently identified as possessing anti-flaviviral activity. Therefore, this study aims to exploit a drug-repurposing approach to identify clinically approved antifungal azoles able to inhibit flaviviral replication and characterize the mechanism of action of the active compounds.
Material and Methods
The cytotoxicity and antiviral activity against different flaviviruses (ZIKV, WNV, and DENV) of selected antifungals were determined by MTT and plaque reduction assays (PRAs), respectively, first in Vero cells. Then, given the wide tropism of flaviviruses, both the anti-ZIKV activity and cytotoxicity of the two most active antifungals, posaconazole (PCZ) and isavuconazole (ICZ), were assessed in two human cell lines, i.e., JEG-3 and SH-SY5Y cells. Finally, the mechanism of action of isavuconazole against ZIKV has begun to be elucidated through time-of-addition (TOA) and virucidal assays in ZIKV-infected Vero cells.
Results
Among the antifungals assayed against different strains of ZIKV, DENV, and WNV, some inhibited the flaviviral replication in Vero cells at low micromolar concentrations, without being cytotoxic. In contrast, some others did not show antiviral activity in the range of tested concentrations. Posaconazole and isavuconazole were the two most active antifungal compounds against ZIKV, WNV, and DENV. Moreover, these azoles also exerted their antiviral activity against ZIKV in the disease-relevant JEG-3 and SH-SY5Y cells. Regarding the mechanism of action of isavuconazole against ZIKV, direct activity on the virus was excluded by performing a virucidal assay. However, from the time-of-addition assay preliminary results, multiple mechanisms of antiviral activity could be hypothesised.
Conclusions
Repurposing clinically licensed antifungals with well-known safety profiles could be a faster and cheaper approach to fight against the lack of anti-flaviviral drugs, as well as a promising starting point for the development of novel and specific antiviral strategies. Isavuconazole showed a promising anti-ZIKV activity. However, further studies are needed to identify its specific mechanism of action. This compound may target both the viral replication cycle and a cellular pathway, possessing multiple mechanisms of antiviral activity. Therefore, both hypotheses will be considered. Particularly since the characterization of the anti-flavivirus activity of this antifungal azole could pave the way to the development of new antiviral compounds against flaviviral infections.

PhD COURSE "MOLECULAR MEDICINE"

COORDINATOR: PROF. ARIANNA LOREGIAN

Curriculum "REGENERATIVE MEDICINE"

APPLYING SYNTHETIC BIOLOGY TOOLS TO FIGHT ANTIMICROBIAL RESISTANCE

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Curriculum "Regenerative Medicine"

Background

Antimicrobial resistance (AMR) is a health emergency spreading worldwide. The WHO identified ESKAPE bacteria (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp.,* sometimes extended to ESKAPEE to include *Escherichia coli)* among frightens pathogens to be monitored. The request for developing and evaluating new therapies and treatment against AMR is massive and novel approaches must be explored to overcome the onset of new AMR strains. Synthetic biology, combining biological and engineering knowledge, and phage engineering can play a relevant role in this run.

Material and Methods

To restore sensitivity to antibiotics in resistant bacteria, we are developing a delivery platform using engineered M13 phage particles that carry a phagemid designed to encode a CRISPR interference (CRISPRi) system, specifically targeting AMR genes. Upon a preliminary characterization of the delivery efficacy through a phagemid bearing the Red Fluorescent Protein (RFP) expression cassette, we produced phage particles encapsulating a phagemid with a CRISPRi circuit containing dCas9 and sgRNA targeting a RFP. This was achieved through the interaction between M13KO7 (a helper phage) and the CRISPRi-carrying phagemid. The engineered phage particles were purified using 5% polyethylene glycol precipitation and assessed via transduction assay. Phage preparations incorporating this circuit were evaluated for CRISPRi-mediated repression of RFP expression using a microplate reader assay to detect fluorescence signal (excitation 535 nm and emission 610 nm).

To investigate CRISPRi effects in target bacteria such as *Acinetobacter baumannii*, we optimized an electroporation protocol and tested an *E. coli-A. baumannii* shuttle vector carrying RFP. Initially, we characterized a library of parts for reliable expression of recombinant genes in those strains and; then, a CRISPRi circuit consisting of a sgRNA targeting RFP designed for this purpose. The efficacy of target silencing was evaluated assessing the expression of RFP using a microplate reader assay (excitation 535 nm and emission 610 nm) and confocal microscopy (using a 594 nm laser).

Results

The phage preparation harbouring the CRISPRi system targeting RFP effectively delivered the CRISPRi machinery to silence RFP; however, repression was not completely effective. Optimization of the electroporation protocol enabled successful electroporation of *A. baumannii*, as confirmed by the appearance of red colonies on selective plates. Subsequent characterization of CRISPRi targeting RFP in *A. baumannii* revealed that while dCas9 expression alone can be toxic, the presence of sgRNA may reduce some of these adverse effects. Additionally, both confocal microscopy and microplate reader results supported the effectiveness of CRISPRi technology in suppressing target gene expression in *Acinetobacter baumannii*.

Conclusions

According to our preliminary results with the delivery platform, we created an effective CRISPRi-based phage systems in *E.coli*; on the other hand, to achieve a therapeutically-relevant effect on target AMR genes, further optimizations are necessary. Analogously, the incomplete silencing observed in *A. baumannii* requires further investigation of the CRISPRi along with the exploration of AMR targets in this ESKAPEE bacteria. Finally, to expand the delivery capabilities of the conceived phage-based CRISPRi platform to other AMR bacteria, the phage tropism must be broadened.

PhD COURSE "PHARMACOLOGICAL SCIENCES"

COORDINATOR: PROF. NICOLA FERRI

Curriculum "PHARMACOLOGY, TOXICOLOGY AND THERAPY"

Polymedication and Multimorbidity Patterns of Atrial Fibrillation Patients Using Latent Class Analysis and Their Association with Thromboembolic Events, Bleeding, and Falls

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Ph.D. Course: Pharmacological Science
Curriculum "Pharmacology, Toxicology and Therapeutics"

Background
The combination of age, frailty, multimorbidity, and concurrent drug use leads a challenge for the management of atrial fibrillation (AF) patients. This study aimed to determine the attributes of AF patients using latent class analysis based on their comorbid conditions and concomitant drug use and explore the associations of comorbidity and polymedication profiles with the risk of adverse outcomes.
Material and Methods
This monocentric, retrospective cohort study included 633 non-valvular AF patients who accessed the outpatient clinic for anticoagulant therapy at the Geriatric Unit of Padua University Hospital. Sociodemographic and medical records were collected from each patient, with 6-month follow-up assessments up to 36 months from the first visit. The combination of multimorbidity and polymedication patterns were identified using latent class analysis on 18 drug and 17 disease groups. Observed/expected (O/E) ratio (≥2) and exclusivity (≥25%) of the combined comorbidity and polymedication were calculated for each cluster. Then, the association of latent classes with clinical outcomes (thromboembolic events, haemorrhages, and falls) was tested using Cox regressions.
Results
633 patients with AF aged 65 years and above (mean age 80.5±6.9 years, 52.5% women) were included to the analysis. The median (IQR) follow-up was 24.2 (12.1–35.5) months. We identified four latent classes considering the disease and concurrent drug use of patients: Unspecific Class (39.0%), Diabetes and Liver Diseases Class (14.8%), Neurocognitive and Psychiatric Diseases Class (14.1%), and Musculoskeletal, Immunologic and Dermatologic Diseases Class (32.1%). The incidence rates were higher in Neurocognitive and Psychiatric Diseases Class for all clinical outcomes among latent classes. Neurocognitive and Psychiatric Diseases Class was associated with an increased risk of composite (adjusted hazard ratio [aHR] [95% CI]: 1.75 [1.56-3.82]), and thromboembolic (aHR [95% CI]: 3.04 [1.28-7.22]) outcomes.
Conclusions
Patients assigned Neurocognitive and Psychiatric Diseases Class had a higher incidence rate of composite (thromboembolic, haemorrhage, all cause death), bleeding, thromboembolic, and fall events than other latent classes. These patients also had a significantly increased risk of composite and thromboembolic events. Concomitant drug use and multimorbidity resulted in complex clinical characteristics and adverse outcomes in AF patients classified into Neurocognitive and Psychiatric Diseases Class. The study findings may help with clinical decision-making, research, and policy.

PAIN ASSESSMENT IN PEDIATRIC PATIENTS UNDERGOING ORTHODONTIC THERAPY WITH RAPID PALATAL EXPANDER

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Background
Rapid Palatal Expansion (RPE) therapy is an orthodontic practice used to correct transverse defects of the maxilla, improving the harmony and functionality of the dental arches. By using a rapid expander, the treatment is particularly effective in young patients, as it facilitates the widening of the upper jaw while positively influencing skeletal development. This research aims to evaluate the pain and discomfort experienced during RPE therapy, collecting real-time data from patients to examine the variables that influence pain perception, such as demographic characteristics, type of expander, activation protocol, and medication use.

Materials and Methods
The study collected data through questionnaires administered to patients and parents, recording information on age, sex, type of expander, activation protocol, discomfort, and the use of pain control medication. The sample includes 30 patients, divided between those treated with Hyrax (17 patients) and Haas (13 patients), with an average age of 8.33 years.
The study examined two types of expanders, Hyrax and Haas, and considered variables such as the positioning of the bands on the teeth and different activation protocols to determine the effectiveness and associated discomfort of the treatments.

Results
Pain associated with the use of expanders was measured over a 30-day period. Patients treated with Hyrax reported higher initial pain, which, however, decreased rapidly, stabilizing at nearly negligible levels by the twelfth day. In contrast, patients treated with Haas reported lower initial pain but more persistent discomfort, stabilizing only around the twenty-second day. Error bars indicated a reduction in pain variability among patients over time for both devices.

Conclusions
The analysis showed that the Hyrax expander causes sharper but shorter-lasting pain, while the Haas expander causes less intense but more prolonged pain. These findings suggest that Hyrax may offer quicker pain relief compared to Haas. The study provides valuable insights for optimizing pain management and improving patient experience. The study provides a comprehensive overview of the pain dynamics during RPE therapy, with potential implications for improving orthodontic treatment strategies and ensuring greater patient comfort.

HIGH-FAT DIET INDUCED OBESITY AS A PREDISPOSAL FACTOR TO PARKINSON DISEASE VIA INTERPLAY BETWEEN INFLAMMATION AND AUTOPHAGY

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Background
The underlying mechanisms of the neuronal degeneration in Parkinson's Disease (PD) are not well understood, but mitochondrial dysfunction, chronic inflammation, oxidative stress, and defect in autophagy have been implicated in different animal models of PD. Several studies indicated that among factors associated with risk of developing PD, dietary habits have an important role. In addition, high-fat diet (HFD) is associated with an increased risk of developing neurodisorders and this effect seems to be related to obesity-induced inflammation. For this reason, our aim is investigating if the peripheral inflammation driven by HFD could contribute to PD-associate alterations at central level, through the gut-brain axis.

Material and Methods
C57BL/6J male mice were divided in three groups: control (1), HFD (2) and rotenone (3). The control group were fed with standard diet (SD,10% of energy from fat) until all the protocol. HFD mice were fed with high-fat diet (HFD, 60% of energy from fat) from 4 weeks age old until complete 13 weeks age old. The third group received rotenone (10mg/Kg) by oral gavage, from 8 until completed 13 weeks age old. Body weight was measured weekly, rotarod test was performed in the last week of treatment, and then mice were sacrificed, and feces, blood, and tissues (colon and midbrain) were collected for analyses. Inflammatory parameters (ASC, IL-1β, Iba-1), PD biomarkers (TH, α-synuclein), autophagic pathway proteins (mTOR and LC3BII) were analyzed by ELISA and western blot.

Results
As expected, rotenone administration reproduces PD features in mice, such as motor deficit, overexpression of α-synuclein at peripheral and central level, as well as loss of dopaminergic neurons and neuroinflammation. Interestingly, rotenone administration also reproduces non-motor symptoms related to gut, increasing intestinal permeability and inflammation. HFD-induced obesity protocol determines alterations in body weight, metabolic parameters, intestinal transit and permeability. Furthermore, we observed that obese mice developed peripherical and central inflammatory process and were found to have the similar alteration as PD mice, but in lesser extension. Furthermore, mice fed with HFD presented an increase mTOR phosphorylation and a reduction of LC3B-II expression, resulting in an impairment of autophagic pathway. These autophagic parameters were found similarly altered but in more significant extent in rotenone-treated mice.

Conclusions
Our data emphasize that gut-brain axis plays an important role in PD, since accumulation of α-synuclein and consequent inflammatory process was also detected on gut tissue. In addition, HFD driving-inflammation promotes defective autophagy and predispose to toxics proteins accumulation contributing to develop neurovegetative diseases such as PD.

PhD COURSE "PHARMACOLOGICAL SCIENCES"

COORDINATOR: PROF. NICOLA FERRI

Curriculum "MOLECULAR AND CELLULAR PHARMACOLOGY"

PD-L1 TARGETED LIPOSOMAL DOXORUBICIN EXERTS AN IMMUNOMODULATORY ROLE AND REDUCES INVASIVENESS OF HEPATOCELLULAR CARCINOMA

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Curriculum "Molecular and Cellular Pharmacology"

Background
Hepatocellular carcinoma (HCC) is a highly malignant cancer, characterized by elevated morbidity and mortality. Many tumors, including HCC, exploit the upregulation of PD-L1 as a mechanism to modulate T-cell–mediated antitumor activity. Therefore, PD-L1 is a commonly exploited target for tumor immunotherapy. The immune checkpoint inhibitor atezolizumab, a monoclonal antibody targeting PD-L1, in association with bevacizumab, has become the new standard of care in patients with advanced HCC. A critical process in the progression of HCC is the epithelial-to-mesenchymal transition (EMT), that is linked to PD-L1 expression in HCC patients and drug resistance in multiple targeted therapies.
The aim of this study is to investigate the immunomodulatory role of atezolizumab-targeted liposomal DXR (Stealth Immunoliposomes, SIL) by evaluating its effects on PD-L1 expression and on the phenotype of tumor associated macrophages, and on the EMT, by using as control its untargeted counterpart (Stealth Liposomes, SL).
Material and Methods
The effect of SIL and SL treatments on PD-L1 expression was evaluated in human hepatocellular carcinoma cells HepG2 treated with INF- γ to induce its overexpression. The effect of the two formulations on PD-L1 and p65 expression were determined by immunocytochemistry and qPCR, which was used also for measuring the mRNA expression of JAK, STAT, p50, IL-6 on INF- γ -treated HepG2 cells. Spheroids with HepG2 and the monocytic cells THP-1 were set up to assess the effect of SIL on PD-L1 expression, macrophage polarization and clonogenicity and invasiveness of HepG2. The effect of SL and SIL was evaluated also on HepG2 cells treated with TNF-α to increase their migration features, by the expression of PD-L1 and p65, and of the epithelial markers E-cadherin and β-catenin and the mesenchymal marker Vimentin by ICC and qPCR.
Results
Only SIL was able to decrease PD-L1 and p65 protein and mRNA expressions ($p<0.05$ for both) on INF- γ -treated HepG2 cells. Atezolizumab targeting caused a reduction of the mRNA expression of JAK2 ($p<0.05$), STAT3 ($p< 0.01$), p50 ($p<0.05$) and IL-6 ($p<0.05$), indicating that the PD-L1-decreasing effect occurs via the JAK/STAT and NF-kB pathways. In THP-1/HepG2 spheroids, both SL and SIL decreased clonogenicity ($p<0.0001$) and invasiveness of HepG2 cells and reduced pro-tumoral CD-163-expressing macrophages ($p<0.0001$), but only SIL decreased PD-L1 expression ($p<0.01$), confirming its peculiar immunomodulatory activity. Furthermore, SIL decreased PD-L1 protein and gene expression ($p<0.001$ and $p<0.05$ respectively) and p65 protein and gene expression ($p<0.05$) also in the TNF-α migration model. Accordingly, SIL significantly increased E-cadherin ($p<0.05$), upregulated by TNF-α, and decreased Vimentin ($p<0.001$) and β-catenin, downregulated by TNF-α, indicating their role in decreasing EMT.
Conclusions
PD-L1-targeted liposomal DXR, beside exerting an immunomodulatory role in the tumor microenvironment, reduces tumor cell invasiveness by acting on the EMT. The active targeting of doxorubicin to tumor cells could be helpful in developing new therapeutic strategies for HCC treatment.

PhD COURSE "TRANSLATIONAL SPECIALISTIC MEDICINE «G.B. MORGAGNI»"

COORDINATOR: PROF. DARIO GREGORI

Curriculum "BIOSTATISTICS AND CLINIC EPIDEMIOLOGY"

WIN STATISTICS IN OBSERVATIONAL CANCER RESEARCH: INTEGRATING CLINICAL AND QUALITY-OF-LIFE OUTCOMES

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Curriculum "Biostatistics and Clinical Epidemiology"

Background
Quality-of-life metrics are increasingly important for oncological patients alongside traditional endpoints like mortality and disease progression. Statistical tools such as Win Ratio, Win Odds, and Net Benefit prioritize clinically significant outcomes using composite endpoints. In randomized trials, Win Statistics provide fair comparisons between treatment and control groups. However, their use in observational studies is complicated by confounding variables. Propensity score (PS) matching mitigates confounding variables but may reduce the sample size, affecting the power of win statistics analyses. Alternatively, PS matching can stratify samples, preserving the sample size. This study aims to assess the long-term impact of these methods on decision making, particularly in colorectal cancer patients.
Methods
A motivating example involves a cohort of patients from the ReSARCh observational study (2016–2021) with locally advanced adenocarcinoma of the rectum, situated up to 12 cm from the anal verge. These patients underwent either a watch-and-wait approach (WW) or trans-anal local excision (LE). Win statistics compared the effects of WW and LE on a composite outcome (overall survival, recurrence, presence of ostomy, and rectum excision). For matched win statistics, we used robust inference techniques proposed by Matsouaka et al. (2022), and for stratified win statistics, we applied the method proposed by Dong et al. (2018). A simulation study assessed the coverage probability of matched and stratified win statistics in balanced and unbalanced groups, calculating how often the confidence intervals included the true values of WR, NB, and WO across 1000 simulations.
Results
The results suggest a better efficacy of the LE approach when considering efficacy outcomes alone (WR: 0.47 (0.01 to 1.14); NB: −0.16 (−0.34 to 0.02); and WO: 0.73 (0.5 to 1.05)). However, when QoL outcomes are included in the analyses, the estimates are closer to 1 (WR: 0.87 (0.06 to 2.06); WO: 0.93 (0.61 to 1.4)) and to 0 (NB: −0.04 (−0.25 to 0.17)), indicating a negative impact of the treatment effect of LE regarding the presence of ostomy and the excision of the rectum. Moreover, based on the simulation study, our findings underscore the superior performance of matched compared to stratified win statistics in terms of coverage probability (matched WR: 97% vs. stratified WR: 33.3% in a high-imbalance setting; matched WR: 98% vs. stratified WR: 34.4% in a medium-imbalance setting; and matched WR: 99.2% vs. stratified WR: 37.4% in a low-imbalance setting).
Conclusions
In conclusion, our study sheds light on the interpretation of the results of win statistics in terms of statistical significance, providing insights into the application of pairwise comparison in observational settings, promoting its use to improve outcomes for cancer patients.

MONITORING PATIENT REPORTED OUTCOMES AND LIFESTYLES BY INTEGRATING CLINICAL RECORDS WITH DIGITAL AND SOCIAL MEDIA DATA

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Curriculum "Biostatistics and Clinical Epidemiology"

Background
The Patient State Index (PSI) is a widely used tool for monitoring sedation levels in pediatric anesthesia, providing an indication of the patient's level of consciousness and depth of anesthesia. The stability of sedation during anesthesia is equivalent in statistical jargon to the stationarity of the process. However, the detection of time points when the PSI level changes from a stable sedation level remains a challenge. Since the distribution of PSI is usually non normal and is expected to have outliers, a robust method is required to evaluate the phases. The anesthesia period is clinically divided into well-defined five phases; however, recent studies have unexpectedly detected large variations of PSI have even within same phase during pediatric anesthesia.

Material and Methods
This study proposes the Variability Ratio Index (VARI), a simple statistical tool based on the deviation of PSI from its stationary process, to evaluate sedation phases. We considered the probability of a change point, where PSI deviates, can vary across different phases. We used quasi-binomial distribution that describes additional variation of PSI from its stationary process, to develop VARI. Change points are detected using the pruned exact linear time algorithm. We also checked the robustness behavior of VARI in both parametric bootstrapping in Bayesian paradigm and Monte Carlo simulation.

Results
To demonstrate the practical application of VARI, a single-center retrospective study was conducted using PSI data from pediatric patients undergoing cardiac surgery with extracorporeal circulation. The study included twenty patients monitored using the Sedline monitor at 124,699 time points. We observed large variation of PSI within each phase and VARI evaluated all phases with satisfactory results. VARI successfully identified the hypothermic phase with the lowest value and the awakening phase with the highest value of it, highlighting its potential in assessing sedation depth during anesthesia. VARI showed robust behavior in both parametric bootstrapping under Bayesian paradigm and Monte Carlo simulation. VARI converged into Beta distribution with parameters Shape 1 < Shape 2 in 10,000 iterative schemes. Finally, we applied Generalized Estimating Equation to capture the correlation structure of each patient to make patient wise predictions on the deviations of PSI.

Conclusions
We developed R package "varifinder" to facilitate the use of VARI. Further research and data are necessary to fully explore the utility of VARI in different clinical settings.

PhD COURSE "TRANSLATIONAL SPECIALISTIC MEDICINE «G.B. MORGAGNI»"

COORDINATOR: PROF. DARIO GREGORI

Curriculum "CARDIOVASCULAR SCIENCES"

BIODEGRADABLE EXTRA-VASCULAR SUPPORT FOR CARDIOVASCULAR SURGERY INVOLVING VALVAR GRAFTS IN PEDIATRIC PATIENTS

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Curriculum "Cardiovascular Sciences"

Background
Progressive vascular dilatation following cardiovascular surgery involving valvar grafts may lead to valvular incompetence and potential regurgitation. Nevertheless, the use of valvar grafts, such as in pulmonary autotransplantation (Ross operation), remains one of the best options for young patients needing aortic valve replacement. While external vascular reinforcement with synthetic material can help address this issue, it also restricts the growth of the graft, which is critical for pediatric patients. Therefore, we aimed to develop a biodegradable extra-vascular support specifically for cardiovascular surgical procedures in pediatric patients.

Material and Methods
Thermoplastic polymers such as polycaprolactone (PCL) are biodegradable, easy to handle, and FDA/EMA-approved polyesters. We propose a PCL-based 3D-printed biodegradable extravascular support created using melt electrowriting (MEW). This patch replicates the mechanical properties of the aorta, with thin fibres and a structured scaffold that accommodates physiological deformation during the cardiac cycle. PCL and similar polymers are extensively used in regenerative medicine due to their thermal stability and compatibility with 3D printing techniques like MEW. MEW allows for the fabrication of intricate structures, such as highly porous scaffolds, by depositing micrometric fibres. By fine-tuning the fibre dimensions, we can enhance cellular infiltration and tissue remodelling. Current MEW technology enables the printing of well-defined scaffolds with interfibre distances as small as 25 μm, fibre diameters below 1 μm, and scaffold thicknesses up to 1 cm. The size of extrusion-based filaments is limited by the die, but MEW filament size is determined by the pulling force acting on the filament, allowing for precise adjustments.

Results
Triangular scaffolds successfully replicated the properties of soft biological materials, highlighting the significance of mimicking fibrous protein patterns in blood vessels to ensure proper function. Challenges in printing curved structures using MEW were explored through various samples, underscoring the need for optimized designs and printing processes. The study's results centered on optimizing electrospinning parameters, testing scaffold designs, and addressing challenges to improve functionality in biomaterial applications. Mechanical characterization of the patches and native rodent aorta was conducted using tensile tests.

Conclusions
In summary, our study has demonstrated promising early results, showcasing the favorable mechanical properties of a biodegradable extravascular PCL-based MEW patch. The next phase of our research will involve animal implantation to assess in vivo performance and validate the potential effectiveness of our innovative biodegradable support system.

AUGPRED AND COMPLEX NETWORK ANALYSIS APPROACH FOR ENHANCING HEART TRANSPLANT FOLLOW-UP

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Curriculum "Cardiovascular Sciences"

Background
Nowadays, machine learning (ML) has emerged as a powerful tool in clinical applications, offering the potential to analyze complex data and predict clinical outcomes with remarkable precision. In parallel, complex network theory, which is a physical approach to studying relationships between nodes, is a growing field in understanding the intricate web of relationships within biological systems. My work aims to introduce: a) *AugPred*, an explainable ML-based pipeline for classification tasks, and b) Network-based analysis to investigate inter and intra hub and node connection within the biological system to enhance post-transplant surveillance.

Material and Methods
Data examined include microRNA and mRNA expression profiles of 16 post-heart transplant patients: 5 in the control group (R0), 6 with infections, and 5 with rejection. a) AugPred was used to augment the data, generating synthetic datasets for each condition to enhance classifier training. This augmented data trained a logistic regression model with L1 regularization to select influential microarray expression features, which were then used to train a Kernel-SVM (KSVM) model for phenotype classification. b) Network analysis involves enriching data from multiple databases, constructing multilayer networks of biological interactions, and measuring node centrality using a multiplex PageRank algorithm. Pathway enrichment analysis then highlights information propagation through networks of different phenotypes to determine relevant targets.

Results
a) The AugPred pipeline has been developed in multiple sections. First, a random sampling model based on the available data distribution has been created and used to generate 100 synthetic samples for each condition. Subsequently, the logistic regression model with L1 lasso regularization automatically selected relevant features. For each k-fold cross-validation, the model determined multiple non-zero coefficients. By focusing on miRNAs with non-zero coefficients in over 50% of the 100 iterations, we identified consistently maintained features. The Kernel-SVM (KSVM) model then classified rejection and infection cases, achieving a 91% classification accuracy in cross-validation, significantly outperforming a baseline logistic regression model, which reached 75% and misclassified only one case. b) For network analysis, microRNA expression profiles were enriched by extracting target genes from miRDB and TargetScan databases, along with their Gene Ontology (GO) functional information. A multilayer network for each phenotype (control, infection, and rejection) to represent biological interactions at different levels, has been created. Moreover, an adapted PageRank algorithm for the exploration of multilayer networks has been created. It has been used to detect highly connected hubs and rank them based on centrality measures. Enrichment analysis was performed to identify the most affected pathways across conditions.

Conclusions
The AugPred approach is a valid and innovative approach for data augmentation microarray expression profiles showing excellent accuracy in the classification of different outcomes. Furthermore, the automated selection and extraction of relevant features leverage the explainability of the model, making it suitable for understanding the decisions made, especially in a clinical context; Network analysis shows an effective understanding of the biological interactions in the different phenotypes, to investigate the essential role of biological processes and disease mechanisms. These methods have the potential to be a reliable system to improve heart transplant patient follow-up.

CARDIAC SYMPATHETIC NEURONS ARE ADDITIONAL CELLS AFFECTED IN GENETICALLY DETERMINED ARRHYTHMOGENIC CARDIOMYOPATHY

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Background
Arrhythmogenic Cardiomyopathy (AC) is a familial cardiac disease, mainly caused due to mutations in desmosomal genes, which accounts for most cases of stress-related arrhythmic sudden death, in young and athletes. AC hearts display fibro-fatty lesions that generate the arrhythmic substrate and contractile dysfunction. Correlation between physical/emotional stresses and arrhythmias supports the involvement of sympathetic neurons (SNs) in the disease, but this has not been proven before.
Methods
Confocal immunofluorescence and multiphoton imaging of clarified heart blocks assessed sympathetic innervation in hearts from Desmoglein-2 mutant (*Dsg2*$^{mut/mut}$) mice, at different disease stages. *In vitro* analyses assessed the role of AC-linked DSG2 downregulation on SN biology.
Results
SNs express DSG2 implying that DSG2-mutation carriers would harbor the mutant protein in SNs. Analyses of thin heart sections and three-dimensional reconstruction of the neuronal network revealed significant changes in the physiologic SN topology, with massive hyperinnervation of the intact subepicardial layers and heterogeneous distribution of neurons in fibrotic areas. cSNs isolated from *Dsg2*$^{mut/mut}$ neonatal mice, prior to the establishment of cardiac innervation, show alterations in axonal sprouting, process development and distribution of varicosities. Consistently, virus-assisted DSG2 downregulation replicated in normal SNs the phenotypic alterations displayed by *Dsg2*$^{mut/mut}$ primary neurons, corroborating that AC-linked *Dsg2* variants may primarily affect SNs.
Conclusions
Our results uncover that altered sympathetic innervation is an unrecognized feature of AC hearts, which may result from the combination of cell-autonomous and context-dependent factors implicated in myocardial remodeling. Our results favor the concept that AC is a disease of multiple cell types also hitting cSNs.

COMPREHENSIVE ANALYSIS OF DESMOGLEIN-2 VARIANTS IN ARRHYTHMOGENIC CARDIOMYOPATHY: INSIGHTS FROM GENOMIC DATA, FAMILY SCREENING, AND CELLULAR MODELS

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Background
Desmoglein-2 (DSG2, MIM#610193) is a transmembrane desmosomal glycoprotein associated with neoplastic diseases and arrhythmogenic cardiomyopathy (ACM) (OMIM#107970; ORPHA247). ACM pathognomonic features include electrical instability and fibro-fatty replacement of the myocardial tissue. An undefined number of *DSG2* variants is submitted in Genome Aggregation Database (gnomAD) or ClinVar archive, and their role in ACM pathogenesis is still unclear. Specifically, the rare missense variant encoding p.(N266S), identified in an Italian heart transplant ACM proband, was among the pioneering findings linking this gene with the biventricular ACM phenotype, and it was recapitulated later in an animal model. However, its clinical significance remains an open question.
Aim
The aim of this study is to enhance the understanding of *DSG2* gene variants through a multi-phase approach that combines literature review, database search, genomic data analysis, family screening, and the development of cellular models.
Materials and methods
Systematic searches were conducted on PubMed looking for DSG2-related ACM articles, considering articles in English, original research articles, and compatibility of titles and abstracts with the topic. A reclassification of all genetic variants was performed according to the current American College of Medical Genetics and Genomics (ACMG) recommendations. DSG2 p.(N266S) variant effect was modeled using an *in vitro* system: 2 geneticin resistant-GFP plasmids, carrying either DSG2 Wild-type or the p.(N266S) variant fused to FLAG portion, were generated using site-directed mutagenesis. Transfection into HL-1 cells was obtained by the 4-D Nucleofector System (Lonza©) by optimizing the manufacturer's protocol (HL-1 Wt and HL-1 Mut). Moreover, CD34+ hematopoietic progenitors, isolated from patients and healthy donors, were reprogrammed into induced pluripotent stem cells (iPSCs) through the transient overexpression of key factors. Functional analysis and cell models characterization were performed by Sanger sequencing, Real-time PCR, electron transmission microscopy (TEM), and confocal microscopy.
Results
The literature search revealed 90 *DSG2* genetic variants, which were subsequently re-classified as follows: 34 as pathogenic (P) or likely pathogenic (LP), 23 as of uncertain significance (VUS), and the remaining as benign (B) or likely benign (LB). Further research in gnomAD and ClinVar databases listed 4278 variants, of which 8% were classified as pathogenic (P) or likely pathogenic (LP). The majority of P-LP variants are frameshift variants, with a higher prevalence in the extracellular (EC 1-4) and transmembrane domains, followed by splice site, nonsense, and missense variants spread throughout the protein. As such, the archetypal missense variant encoding p.(N266S) was found in gnomAD database as VUS. This variant was modelled in HL-1 cells showing its abnormal distribution throughout the cytoplasm via confocal microscopy and the desmosomal structure alteration via TEM. This specific missense variant was identified in 4 families within our ACM cohort, enabling the generation of 2 iPSCs lines (one from a healthy donor and one from a proband). Real-Time PCR confirmed stemness and Sanger sequencing identified the DSG2 p.(N266S) variant in the proband- derived iPSCs.
Conclusions
Literature review and databases analysis highlighted that the majority of *DSG2* variants are missense, which significance should be further clarified. Herein, HL-1 cell model revealed the abnormal distribution of the missense DSG2 p.(N266S) in cytoplasm and the alteration of desmosomes, demonstrating its pathogenicity. This finding was further confirmed by co-segregation analysis within families. Further, iPSC cells were used for a personalized cell model tailored to the patient's genetic background, with a remarkable impact on diagnosis and personalized management of the disease.

RIGHT VENTRICULAR TO PULMONARY ARTERY UNCOUPLING IS AN EARLY PREDICTOR OF POOR OUTCOME IN WILD-TYPE TRANSTHYRETIN AMYLOID CARDIOMYOPATHY

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Background
Non-invasive right ventricular to pulmonary artery (RV-PA) coupling assessment has prognostic value in patients with heart failure (HF). Little is known about its application in patients with wild-type transthyretin amyloid cardiomyopathy (wtATTR-CM).
Material and Methods
This single-centre retrospective study included consecutive patients with wtATTR-CM diagnosis undergoing 2D echocardiogram. RV-PA coupling was evaluated with the ratios between tricuspid annular plane systolic excursion (TAPSE), RV free wall longitudinal strain (RVFWLS) or RV four-chamber longitudinal strain (RV4CLS) and pulmonary artery systolic pressure (sPAP). Primary endpoint was the composite of all-cause mortality and HF hospitalisation.
Results
Overall, 100 patients (91% males, median age 81 years, 85% in National Amyloid Centre (NAC) stage $\leq$ 2, 18% in NAC stage Ia and 82% in New York Heart Association class $\leq$ II) were enrolled. During a 16-months follow up (Q1-Q3:12–24), the primary endpoint occurred in 37 patients (37%). TAPSE/sPAP (HR 0.04, 95% CI 0.01 – 0.24, $p < 0.001$), RVFWLS/sPAP (HR 0.07, 95% CI 0.01–0.41, $p = 0.003$) and RV4CLS/sPAP (HR 0.06, 95% CI 0.01–0.53, $p = 0.011$) emerged as independent predictors of the primary endpoint and showed incremental risk prediction compared with TAPSE, RVFWLS and RV4CLS, considered as separate parameters. No differences in outcome risk prediction were observed among TAPSE/sPAP, RVFWLS/sPAP and RV4CLS/sPAP ($p > 0.05$).
Conclusions
RV-PA uncoupling, as assessed by different echocardiography modalities, is an early predictor of poor outcome in patients with wtATTR-CM.

PhD COURSE "TRANSLATIONAL SPECIALISTIC MEDICINE «G.B. MORGAGNI»"

COORDINATOR: PROF. DARIO GREGORI

Curriculum "CLINICAL AND TRANSLATIONAL NEUROSCIENCES"

NEUROLOGICAL CLINICAL OUTCOME FOLLOWING OCCLUSION OR OCCLUSION TEST OF CEREBRAL AFFERENT VESSELS

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Background
Micro ischemic areas can occur in various types of invasive procedures and are known to occur with high frequency even during simple diagnostic angiographies. This study aims to assess the presence of these micro ischemic areas following an invasive carotid artery occlusion test, which can last up to 20 minutes.
Material and Methods
Thirty-five patients were recruited in 2023 and 2024 for this preliminary study. Each patient underwent a carotid artery occlusion test on one hemisphere, while the other hemisphere was explored angiographically without occlusion. MRI with Diffusion-Weighted Imaging (DWI) sequences was performed 24 hours post-procedure to detect new ischemic lesions. The lesions in the occluded hemisphere were compared to those in the non-occluded, angiographically explored hemisphere.
Results
Preliminary data indicate that micro ischemic lesions were detected in 18 of the 35 occluded hemispheres and in 16 of the 35 non-occluded hemispheres. No clinical events were recorded. The difference between the two groups was not statistically significant ($p = 0.81$), as determined by Fisher's exact test.
Conclusions
These findings suggest that, despite the invasive nature of the carotid artery occlusion test, when correctly performed, it does not cause more harm than a simple diagnostic angiography. Therefore, this procedure can be considered in selected cases without excessive concern. Further research is needed to confirm these preliminary observations and to refine patient selection criteria.

NEUROMUSCULAR DISORDERS

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Background

Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are both caused by mutations in the *DMD* gene, which encodes the dystrophin protein. DMD typically results from the complete absence of dystrophin, leading to severe muscle weakness and cardiorespiratory complications. In contrast, BMD patients exhibit a broad range of clinical presentations, from asymptomatic cases with elevated creatine kinase (CK) levels or isolated cardiac involvement to severe muscle weakness. The causes leading to phenotypic variability in BMD are not fully understood, but differences in the amount and quality of residual dystrophin are thought to be key factors. Similarly, heterozygous females with dystrophin mutations may present a broad phenotypic variability, but the underlying mechanisms are still not fully elucidated.

Material and Methods

1) BMD Characterization: A retrospective, observational study was conducted using data from 163 male patients with BMD followed at the John Walton Muscular Dystrophy Research Centre, Newcastle, UK. The study aimed to characterise the clinical features, genetic mutations, and disease progression in this cohort.

2) DMD Heterozygous Females: An observational and cross-sectional study was performed on 47 DMD heterozygous females at the Neuromuscular Centre, University of Padova, Italy. This study focused on the clinical and molecular characteristics of DMD carriers, examining correlations between X-chromosome inactivation and disease severity.

3) Dystrophin Quantitation: Skeletal muscle biopsy samples of 67 male dystrophinopathy patients followed up at the Neuromuscular Centre, University of Padova, were utilised to quantify dystrophin using Liquid Chromatography with Tandem Mass Spectrometry (LC-MS/MS) in collaboration with the Department of Pharmaceutical Sciences, Binghamton University, US. This study aimed to correlate dystrophin levels with clinical severity and loss of ambulation (LoA).

Results

1) Large deletions in the *DMD* gene were the most common mutation type (78%), followed by large duplications and small mutations (11% each). The mean age at the last neuromuscular assessment was 33.2 years (range 1.4-86.3). Twenty-three percent of patients were non-ambulant, with a mean age at LoA of 42 years. Cardiac involvement was observed in 52.3% of patients, whereas severe respiratory impairment was rare (13%), occurring predominantly in non-ambulatory patients. Neurocognitive issues were reported in 44.2% of cases.

2) Forty-three percent of female carriers showed muscle weakness, primarily involving proximal muscles. CK levels were normal in approximately 20% of symptomatic and 46% of asymptomatic patients. Cardiac involvement was present in 20% of individuals. X-chromosome inactivation significantly correlated with skeletal muscle involvement severity but not with cardiomyopathy.

3) A statistically significant correlation ($rho=0.78$, $p<0.0001$) was observed between dystrophin levels and skeletal muscle phenotypes across DMD, BMD, and individuals with no weakness. Higher dystrophin levels were associated with a protective effect on the age at LoA (HR 0.93 ± 0.02, $p<0.001$).

Conclusions

Observational data are crucial to inform clinical care for BMD, given the absence of international standards of care. BMD also serves as a valuable in vivo model for developing and refining dystrophin-restoring therapies. Accurately determining the level of dystrophin necessary to mitigate symptoms is crucial, with LC-MS/MS being the most reliable method for dystrophin quantification. The study of heterozygous females can offer key insights into the dystrophin levels to prevent symptoms and the potential for mosaic muscle to achieve genetic and biochemical normalization. Such findings may prove essential for advancing dystrophin gene therapies.

PhD COURSE "TRANSLATIONAL SPECIALISTIC MEDICINE «G.B. MORGAGNI»"

COORDINATOR: PROF. DARIO GREGORI

Curriculum "THORACIC AND PULMONARY SCIENCES"

ISCHEMIC REPERFUSION INJURY ON PULMONARY GRAFT FOR THERAPEUTIC TRANSPLANTATION PRESERVED WITH CONTINUOUS NORMOTHERMIC PERFUSION TECHNIQUE: A SWINE MODEL

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Background
Ex-Vivo Lung Perfusion (EVLP), represents a valid technique for lungs preservation at standard duration(6h), allowing to extend the donor pool. However, organ reconditioning is limited by perfusion time. The swine model is very similar to humans for studying the effects of prolonged ex-vivo perfusion. The aim of the research is to assess the efficacy of pEVLP system (portableEVLP), OrganCareSystem™ (OCS™), for prolonged perfusion times (24h) on swine model, evaluating the effects from a clinical and pathological point of view also after left lung implant.

Material and Methods
The research consists on two arms with different OCS perfusion times: 6h vs 24h. During the pEVLP, lungs were proned at 5h of perfusion in both the groups. At the end of the perfusion, the right lung was analyzed (pre-reperfusion sample) and the left lung was implanted in another pig. Transplantability was assessed on the view of the stability of perfusion parameters and with a final P/F at least > 300. The implant was performed with the same human technique. After transplantation at 1 hour from X-clamp opening a left upper and lower biopsy were performed (post-reperfusion sample) as well as Arterial Blood Gasanalysis from the left atrial (LA) cuff at 1-2-3 hours after transplantation. After 3 hours, the animal was sacrificed and the left lung analyzed. The examination was performed on haematoxylin and eosin slides, according to a modified scale of items based on the study by Matute-Bello et al.

Results
A total of 20 animals were studied (10 for each group). We found no differences in the animal weight ($p>0.99$), in donor P/F ($p=0.35$) and in recipient P/F ($p>0.99$) before the implant between the two groups. Lung function parameters were stable during OCS perfusion in both groups. We observed an higher increase in lungs weight after OCS in the 24h group, although not statistically significant. After transplantation, the P/F from the LA cuff at 1-2-3h was at least >340 without differences between the two groups.
In the pre-reperfusion samples no significant pathological differences were found between the two groups. The post-reperfusion biopsies (1h and 3h) showed a significant difference in the two groups for the parameter emphysema ($p<0.05$) and for hemorrhagic congestion ($p<0.05$) in the upper and lower lobes respectively.

Conclusions
The swine model was reliable in reproducing the single lung transplantation. The prolonged normothermic lung perfusion (24h) was safety and feasible and despite some microscopical alterations has shown encouraging results in the clinical setting also after implant, compared to the control group (6h).

USE OF THE CELLULAR CULTURES AND FLOW CYTOMETRY FOR THE STUDY OF CHRONIC LUNG DISEASES: A TRANSLATIONAL RESEARCH PROJECT

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Background

Eosinophils, key immune cells involved in chronic lung diseases like asthma and COPD, have two distinct phenotypes: resident IL-5-independent eosinophils (rEos) and inflammatory IL-5-dependent eosinophils (iEos). These phenotypes are found in steady-state and inflamed tissues, respectively. The presence of varying levels of both iEos and rEos in peripheral blood of patients with asthma and COPD has been reported, likely influenced by different microenvironmental cues modulated by macrophages. Macrophages are known to exhibit a proinflammatory M1 phenotype, more prevalent in COPD or an anti-inflammatory/pro-resolution M2 phenotype, more prevalent in asthma. Recently we have demonstrated that polarized macrophages can release extracellular vesicles (EVs), lipid bilayer-delimited particles, able to regulate other cells. Based on this, my PhD's second-year goals are to: 1) develop an in vitro model of mature eosinophils using the EoL-1 cell line to study rEos and iEos, and validate macrophage differentiation protocols to assess whether EVs from M1 or M2 macrophages can drive eosinophil differentiation; 2) evaluate the presence and proportion of eosinophils subtypes (iEos and rEos) in peripheral blood of asthmatic patients with varying ages of onset, particularly patients with early-onset asthma (EOA, onset before 12 years) and late-onset asthma (LOA, onset after 40 years).

Material and Methods

1) We developed an in vitro model of eosinophils starting from the commercial cell line EoL-1 that could be differentiated in mature eosinophils by the stimulation with butyric acid. We validated the protocol of macrophages differentiation to stimulate EoL-1 cells with EVs derived from M1and M2 macrophages. THP-1 monocytes were differentiated into macrophages by incubation with PMA (25 ng/mL) for 72 hours, followed by polarization with IFN-γ (20 ng/mL) and LPS (250 ng/mL) for M1 or IL-4 (20 ng/mL) for M2 over 48 hours, with macrophage polarization validated through the expression of mRNA markers IRF1 for M1 and ALOX15 for M2. Supernatants will be collected to stimulate EoL-1 eosinophils with macrophage-derived EVs as part of experiments planned for my third year of PhD.

2) 9 healthy donors and 22 asthmatic patients (12 EOA, 10 LOA) were enrolled. Clinical data and peripheral blood samples were collected for flow cytometric analysis of rEos and iEos (iEos: $CD45^{+}CD16^{-}Siglec8^{+}CD62L^{low}$; rEos: $CD45^{+}CD16^{-}Siglec8^{+}CD62L^{high}$).

Results

1) Stimulating 500,000 EoL-1 cells/mL with 500 μM butyric acid for 5 days optimally balances differentiation (90%) and viability (65%), while qPCR confirmed successful monocyte differentiation into M1 and M2 macrophages through a 100-fold increase in IRF1 and an 8-fold increase in Alox-15 expression, respectively.

2) The percentages of total eosinophils (8.6±4.9% and 10.9±8.9%) and iEos (7.8±5.3% and 7.2±5.2%) were significantly higher in both EOA and LOA compared to healthy controls (4.3±2.9%, p=0.033 and p=0.027; 3.6±3.1%, p=0.019 and p=0.041, respectively). Additionally, in asthmatic patients, total IgE (kUA/L) positively correlated with the percentage of iEos (r=0.593, p=0.033).

Conclusions

These preliminary results establish a foundation for further research into eosinophils' roles in asthma and COPD, highlighting potential links to allergic responses. Future studies will explore how macrophage-derived extracellular vesicles affect eosinophil differentiation, aiming to enhance understanding and therapeutic approaches.

AUTHORS' INDEX