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PhD Courses
Medical and Biomedical Sciences

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

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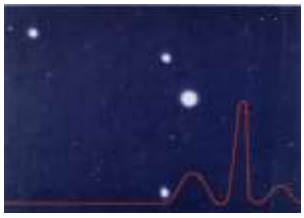

INDEX

University of Padua
PhD Courses
Medical and Biomedical Sciences

PhD COURSE "CLINICAL AND EXPERIMENTAL ONCOLOGY AND IMMUNOLOGY"

COORDINATOR: Prof. Paola ZANOVELLO

NON-GASTROINTESTINAL TUMORS SHAPE THE GUT MICROBIOTA COMPOSITION IN ORDER TO IMPACT THE ANTITUMOR IMMUNE RESPONSE

PhD Student: Dr. Silvia BEGHI
TUTORS: Prof. Andrea FACCIABENE and Prof. Antonio ROSATO
Ph.D. Course: Clinical and Experimental Oncology and Immunology

The human gut microbiome is the population of bacteria, viruses, fungi and eukaryotic microbes that colonize our intestine. Recent work suggests that the microbiome can influence carcinogenesis and the efficacy of cancer therapies, particularly the success of cancer immunotherapy. Immune dysfunction is commonly observed in patients with cancer contributing to tumor progression. While previous works have established a connection between the gut microbiota and the immune system, the mechanisms by which microbiota contribute to cancer-associated immune dysfunction are not well understood. The aim of this project is to understand how the presence of a non-gastrointestinal tumor is able to lead to gut dysbiosis and eventually impact the immune system response against the tumor itself. Using two different tumor models (lung and ovarian cancer) *in vivo*, we demonstrated a robust alteration of the gut microbiota bacterial composition in tumor-bearing mice compared to healthy mice using bacterial 16S gene sequencing technique. Thanks to metagenomics analysis of fecal bacterial DNA coming from tumor-bearing mice, we observed an increase in the relative abundance of *Firmicutes Ruminococcus* in both in vivo models, therefore we identified a possible bacteria candidate as key promoter of immune dysfunctions and tumor development. In order to unravel the mechanism used by a non-gastrointestinal tumor to shift the bacterial composition of the gut, we studied the expression of two well-known antimicrobial peptides: alpha defensin 5 and beta defensin 1 which are able to kill bacteria and are produced by gut epithelial cells. Indeed, we found a significant shift of these AMPs both at RNA and protein level. At this point, to demonstrate the impact of the gut microbiome composition on the antitumor immune response, we assessed by flow cytometry analysis the presence of IFN-γ T cells in tumor-bearing mice treated with or without a cocktail of antibiotics able to deplete their gut flora. Indeed, we observed an increase in IFN-γ production by T cells in tumors from antibiotic treated tumor bearing mice compared to non-treated one. In conclusion, we demonstrated that non-gastrointestinal tumors are able to shift the gut microbiota composition through alteration of AMPs expression at the intestinal level. This phenomenon leads to a shift in the relative abundance of specific bacteria as *Ruminococcus.* Moreover, antibiotic treatment of tumor-bearing mice manifested the impact of the gut microbiome on the antitumor immune response. Overall these findings reveal an uncover new mechanism of immune modulation utilized by tumors to undermine the immune responses and promote tumor progression.

PEMBROLIZUMAB IN RECURRENT HIGH-GRADE GLIOMA PATIENTS WITH IMMUNOHISTOCHEMICAL LOSS OF MISMATCH REPAIR (MMR) PROTEINS: A PILOT STUDY

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

Background

Pembrolizumab demonstrated promising results in hypermutated tumors of diverse origin. Immunohistochemical loss of mismatch repair (MMR) proteins has been suggested as a surrogate of hypermutation in high-grade gliomas (HGG). We evaluated efficacy and safety of pembrolizumab in relapsing HGGs with immunohistochemical loss of at least 1 MMR protein. Molecular biomarkers of pembrolizumab activity were also analyzed.

Material and Methods

Consecutive patients with recurrent HGG and immunohistochemical loss of MMR proteins were prospectively enrolled, after receiving RT and CT; MMR status was analyzed by immunohistochemistry (IHC), including MLH1, MSH2, MSH6 and PMS2 markers. Immunohistochemical loss of MMR proteins was defined as partial or total loss of IHC expression of at least one of the 4 proteins analyzed. Other inclusion criteria were: ECOG PS 0-2, histologically confirmed dignosis of high-grade glioma, dexamethasone ≤4 mg. Pembrolizumab was administrated at 200 mg every 3 weeks until progression disease or unacceptable toxicity. Tumor response was evaluated by brain MRI every 10 weeks according to the RANO criteria. OS and PFS were evaluated by Kaplan-Meier curves. CTCAE v4.0 was used for toxicity. Primary endpoint was disease control rate (DCR). Post hoc exploratory analyses included next-generation sequencing (Oncomine Tumor Mutational Load Assay and ACC GBM panel) and tumor mutational burden (TMB) evaluation.

Results

Between May 2017 and May 2019, 310 patients with HGG were screened by IHC for MMR proteins expression. Thirty-seven HGG (12%) had the immunohistochemical loss (partial or complete) of at least 1 MMR protein; among these, 17 had an ECOG PS >2 and 7 were taking dexamethasone >4mg/day. Therefore, 13 patients were finally enrolled and treated with pembrolizumab: 8 glioblastoma, 4 anaplastic astrocytoma, and 1 anaplastic oligodendroglioma. In 4 cases (3 glioblastomas, 1 anaplastic astrocytoma), IHC and molecular analyses were carried out on the primary tumor, and in 9 cases on the recurrent tumor. At the time of analysis, median follow up was 20.6 months. Patients were treated for 1 to 23 cycles of pembrolizumab (median, 3 cycles). All patients discontinued pembrolizumab due to disease progression. The DCR was 31%: 4 patients had stable disease and no patient had complete or partial response. Nine patients (69%) showed progressive disease. Median PFS was 2.2 months (95% CI 1.6-2.8); the 6-month PFS was 23%. Median OS for all patients was 5.6 months (95% CI 0.1-11.9); the 12-month OS was 38%. Mutation were found in at least one gene in all 12 cases. The most frequent somatic mutations were TP53 (8/12;67%), IDH1 (4/12;33%). ATRX, NF1, PTPN11 and RET mutations were found in 2 cases. TMB in the 12 evaluable patients ranged between 6.7 and 26.9 (median: 10.02) and 8/12 cases (67%) had >9 muts/Mb and were considered hypermutated. All of these values as well as the complete or partial loss of MMR protein expression were not significantly different between patients with PD and SD; thus, no molecular factor was predictive of pembrolizumab efficacy.

Conclusions

Pembrolizumab showed no apparent benefit in HGG patients with immunohistochemical loss of MMR proteins. No molecular biomarker was found to be associated to pembrolizumab activity. Further molecular analyzes are underway for a better characterization of this subgroup of patients

PROTEIN KINASE CK1α CONTROLS PLASMA CELLS AND STROMAL CELLS RUNX2 EXPRESSION POTENTIALLY AFFECTING THE MULTIPLE MYELOMA-ASSOCIATED BONE DISEASE

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

Background
Multiple myeloma (MM) is a malignant plasma cell neoplasm which displays organ damage including pathological bone involvement. The bone marrow (BM) microenvironment sustains the myeloma associated bone disease (MMABD). BM dependent survival pathways, such as Wnt/β-catenin, Hedgehog (Hh) and NF-κB signaling support MM clonal expansion and bone homeostasis imbalance. In particular, β-catenin regulates the RUNX2 osteogenic marker expression sustaining bone formation. Moreover, it has been shown that the overexpression of RUNX2 in MM cells could be responsible of osteoblastogenesis inhibition within the BM microenvironment, accelerating MMABD. Recently, we demonstrated that the Ser/Thr Protein Kinase CK1α is essential for MM plasma cell growth, regulating β-catenin and AKT survival signalling. In particular CK1α, phosphorylates β-catenin on Ser 45 promoting its proteasomal dependent degradation. Therefore CK1α inactivation could be therapeutically relevant also in MMABD to sustain β-catenin, RUNX2 and the osteogenic program.
To assess CK1 in MMABD, we silenced CK1α in BM stromal cells (BMSC) and analyzed osteoblastic differentiation to check if CK1α inactivation could contribute to ameliorate the bone homeostasis. We next studied the role of CK1α on MM plasma cell dependent regulation of RUNX2, in a BM microenvironment culture model, where CK1α silencing was achieved in plasma cells or in stromal cells.
Material and Methods
RNA interference for CK1α was obtained through the generation of IPTG-inducible CK1α shRNA MSC-HTERT-GFP stromal cells and MM INA-6 clones. BM microenvironment models were obtained by plating INA-6 CK1α silenced MM cells on a layer of MSC-HTERT stromal cells. MM cells and stromal cells populations were obtained through cell sorting. We evaluated the osteogenic markers *RUNX2* and *ALP* expression by quantitative RT-PCR or Western blot. Alizarin Red Staining was performed to identify calcium deposits upon differentiation towards osteoblastic lineage.
Results
CK1α silencing in BMSC did not cause cell apoptosis, but induced an osteoblastogenic transcriptional phenotype, leading to the stabilization of β-catenin, increased mRNA expression of the osteoblastic markers *RUNX2, ALP* and increased calcium deposits. In the MM-BMSC co-colture model, we found that the oncogenic role of CK1α on plasma cells sustained RUNX2 expression in MM, decreasing the level of RUNX2 in BMSC. Oppositely, the presence of MM plasma cells in co-colture with CK1α deficient MSC-HTERT was sufficient to block the stromal cells differentiation program achieved by CK1α inhibition.
Conclusions
Our data suggest that CK1α participates in the MM-BMSC crosstalk and could be beneficially therapeutically targetable both in malignant plasma cells and in stromal cells with the potential of ameliorating not only the MM plasma cell disease but also MMABD.

DETECTION OF *ERBB2* AMPLIFICATION BY NEXT GENERATION SEQUENCING IS HIGHLY CONCORDANT WITH STANDARD ASSAYS IN UTERINE SEROUS CARCINOMA

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Background
Uterine serous carcinoma is an aggressive subtype of endometrial cancer that accounts for fewer than 10% of endometrial carcinomas but is responsible for about half of deaths. A subset of cases has HER2 overexpression secondary to *ERBB2* gene amplification, and these patients may benefit from anti-HER2 targeted therapies, such as trastuzumab. HER2 protein overexpression is routinely assessed by immunohistochemistry (IHC) and *ERBB2* gene amplification by fluorescence in situ hybridization (FISH). Targeted next-generation sequencing (NGS) is increasingly used to routinely identify predictive and prognostic molecular abnormalities in endometrial carcinoma, including microsatellite instability and *POLE* mutations. To investigate the ability of a targeted NGS panel to detect *ERBB2* amplification, we identified cases of uterine serous carcinoma and compared HER2 expression by IHC and copy number assessed by FISH with copy number status assessed by NGS.
Material and Methods
The study was carried out on the formalin-fixed and paraffin-embedded specimens of uterine serous carcinoma (n=93) retrospectively identified from cases obtained at Brigham and Women's Hospital (Boston, Harvard Medical School) between 2014 and 2019. *ERBB2* copy number status using a combination of IHC (SP3 clone) and FISH was interpreted using the 2018 ASCO/CAP clinical practice guidelines for breast carcinoma. The IHC was scored independently by three pathologists. Discrepancies were resolved via consensus. Two areas of tumor with the highest expression were marked for ISH evaluation. The NGS panel included the coding regions of at least 275 genes, including *ERBB2*. *ERBB2* amplification by NGS was determined by the relative number of reads mapping to *ERBB2* in tumor DNA compared to control non-neoplastic DNA. Cases with copy number ≥6 were considered amplified and copy number <6 non-amplified.
Results
By IHC, 70 specimens were classified as negative (0 or 1+), 19 were classified as equivocal (2+), and 4 were classified as positive (3+). Using combined IHC/FISH, *ERBB2* amplification was observed in 8 of 93 cases (9%). NGS identified the same 8 cases with copy number ≥6; all 85 others had copy number <6.
Conclusions
In this series, NGS had 100% concordance with combined IHC/FISH in identifying *ERBB2* amplification. NGS is highly accurate in detecting *ERBB2* amplification in uterine serous carcinoma and provides an alternative to measurement by IHC and FISH.

HDAC6 INHIBITION MODULATE NOTCH PATHWAY IN TUMORS WITH NOTCH3 OVEREXPRESSION

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

Background

Histone deacetylases (HDACs) are enzymes involved in the remodeling of chromatin. In recent years, inhibition of HDACs has emerged as a potential strategy to reverse aberrant epigenetic changes associated with cancer. In fact, HDAC inhibitors (HDACi) promote apoptosis, induce cell cycle arrest and differentiation of tumor cells, by mechanisms which remain in part unknown. Differently from other HDACi, HDAC6 inhibitors mainly target cytoplasmic proteins: for instance they mediate α tubulin acetylation, that is associated with NOTCH3 elimination via lysosomal vescicles in T-Acute Lymphoblastic Leukemia (T-ALL). *(Pinazza M. et al, Oncogene 2018)*. Notch pathway is known to be essential in T-ALL and Pinazza M. et al. showed that NOTCH3 inhibition mediated by HDAC6i Tubacin in T-ALL impairs Notch signalling, reducing NOTCH3 surface levels. NOTCH3 results amplified and/or overexpressed also in some cases of female carcinoma such as triple negative breast cancer (TNBC) and ovarian cancer and is associated with poor prognosis *(Yamaguchi N, Cancer Research 2008) (Park, Cancer Res 2006) (Hu W, Cancer Res 2016)*. Unfortunately, therapies preventing Notch3 activation in female cancer are still missing, thus starting from T-ALL as model, we tested HDAC6i Rocilinostat in TNBC to efficiently block Notch signalling, since it is already in clinical trial in Multiple Myeloma and Lymphoma. As additional tool, we recurred to its combination with Bortezomib, in light of previous studies which described both the efficacy of combinations in reducing Notch3 protein in T-ALL model *(Pinazza M. et al, Oncogene 2018)* and cytotoxic effects in hematological malignancies *(Santo et al., 2012) (Amengual et al., 2015)*.

Material and Methods

Breast cancer cells MDAMB-468 and HCC1143 were selected due to high NOTCH3 protein expression and they were plated and treated with Rocilinostat (ACY1215) according to cell line sensibility. After 24h of treatment, NOTCH3 protein expression was evaluated by both flow cytometry and western blot, while Notch3 target gene was evaluated by RT-qPCR. α tubulin acetylation was considered as read out of efficacy of the treatment. Combinations of low doses of Rocilinostat plus Bortezomib as proteasome inhibitor were also tested. Cell growth reduction was assessed after 48h of treatment by MTS assay and apoptosis was determined by flow cytometry via Annexin V/Propidium Iodide (PI) staining after 24-48h of treatment.

Results

Breast cancer cell lines bearing NOTCH3 overexpression treated with HDAC6i Rocilinostat show enhanced cell death, decreased NOTCH3 protein level and gene target expression (HES1), thus resulting sensitive to its action. Despite to MDAMB-468, HCC1143 only showed protein reduction at higher concentration (>IC50). Concerning combinations and their impact on cell growth, Bortezomib results a good combination partner for Rocilinostat. Low doses of ACY1215 in combination with ineffective doses of Bortezomib highly reduce cell growth in both cell lines, leading to modest apoptosis after 24-48h of treatment.

Conclusions

Rocilinostat is an innovative tool to use in Notch3 overexpressing breast cancer cells considering its impact on Notch3 protein and on target gene, even if further studies concerning its modulation need to be performed. Combination strategy represents an additional and promising therapeutic instrument for TNBC due to the strong cytotoxic effects achieved at very low concentrations and it could stand for a good option in place of classic chemotherapy. Supplementary studies will evaluate their impact on Notch3 protein. Finally, *in vivo* experiments will be performed to confirm its efficacy and its ability in imparing tumor growth, increasing overall survival.

A SERUM-FREE PROTOCOL FOR THE *EX VIVO* EXPANSION OF CYTOKINE-INDUCED KILLER CELLS USING GAS-PERMEABLE STATIC CULTURE FLASKS

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

Background

Cytokine-Induced Killer (CIK) cells are *ex vivo* expanded T cells with NK cell phenotype. They express both CD3 and CD56 antigens, and exert a potent antitumor activity against a variety of tumors. Several clinical trials demonstrated the safety and the feasibility of CIK cell therapy, with very low side effects and minimal graft-versus-host toxicity. In this study, we developed a Good Manufacturing Pratice (GMP)-compliant protocol for robust large-scale expansion of CIK cells using G-Rex® gas-permeable static culture flasks.

Material and Methods

CIK cells were obtained by stimulating healthy donor PBMCs with GMP-grade IFN-γ, IL-2 and CD3 mAbs, and were cultured in G-Rex6 or G-Rex6M well plates. CIK cells in G-Rex6 were split only once at day 7 to reduce cell density (G-Rex6-6M), whereas the number of CIK cells culterd in G-Rex6M was not adjusted. In both culture conditions, fresh IL-2 was provided every 3-4 days. We compared these two culture protocols with the culture in standard flasks. Phenotype was analyzed by flow cytometry and cytotoxicity was assessed against several tumor cell lines by calcein-release assay.

Results

CIK cells cultured in G-Rex6-6M well plates showed an outstanding cell expansion compared to G-Rex6M well plates or standard culture flasks, with a 752-fold expansion and a mean of 10^9 total cells obtained per single well in 14 days, starting from just $2.5x10^6$ cells per well. In the first 2 weeks of culture, the percentage of $CD3^+CD56^+$ cells was comparable among the four different culture systems, but this subpopulation expanded more efficiently in G-Rex6–6M, resulting in the highest percentage of $CD3^+CD56^+$ CIK cells at day 21 (mean: 45.19 ± 14.53%) and day 28 (mean: 40.01 ± 17.38%). CIK cells generated in G-Rex showed a less differentiated phenotype, with a significantly higher expression of naïve-associated markers such as CD62L, CD45RA and CCR7. Importantly, G-Rex6-6M CIK cells showed a cytotoxicity that was overall similar to CIK cells cultured in all other conditions.

Conclusions

We propose a GMP-compliant protocol for robust large-scale production of CIK cells. G-Rex® system allows to obtain large amounts of CIK cells highly enriched in the $CD3^+CD56^+$ subset with a more pronounced immature phenotype, which correlates with a remarkable expansion potential in culture and could lead to a longer persistence and a more sustained anti-tumor response *in vivo*. Overall, this protocol has strong advantages over the existing procedures as it allows an easier, time-saving and cost-effective production of CIK effector cells, fostering their clinical application.

THE ROLE OF GLYCOLYSIS AND MITOCHONDRIAL RESPIRATION (OXPHOS) FOLLOWING ANTIANGIOGENIC THERAPY IN OVARIAN CANCER

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

Background

Ovarian cancer (OC) is the seventh most commonly diagnosed cancer among women in the world. Nearly all benign and malignant ovarian tumors originate from one of three cell types: epithelial cells, stromal cells, and germ cells. In epithelial ovarian cancer, increased VEGF expression has a prognostic value and it is associated with tumour stage, grade and patients' survival. Our laboratory conducted several studies about resistance to antiangiogenic therapy and one key mechanism of resistance is associated with tumour metabolism alterations due to the impairment of oxygen, glucose and ATP levels and the induction of a stably glycolytic phenotype. What we are currently investigating is the impact of mitochondrial DNA mutations on the metabolic features of ovarian cancer cells and their possible selection by antiangiogenic therapy. As already described in the literature, mutations in mitochondrial genes, including both nuclear genes and mitochondrial DNA (mtDNA) mutations, are related to increased cancer risk and drug resistance. The aim of this project is to study the impact of mitochondrial DNA mutations on tumor metabolism, growth and in the resistance to antiangiogenic therapy.

Material and Methods

Generation of ovarian xenografts. PDX were obtained and propagated by injecting 1 × 106 tumor cells intraperitoneally into eight-week old female NOD/SCID mice and housed in our specific pathogen-free animal facility. Animals developed ascitic component at different time points depending on the kinetics of tumor growth. Once mice developed tumors, they were anaesthetized with isoflurane/oxygen and sacrificed by cervical dislocation.

Antiangiogenic therapy experiments set up: PDXs were injected intra peritoneally (i.p) on NOD/SCID mice. We started treatment at 20% of the estimated time to sacrifice. Antiangiogenic therapy was performed with the anti-VEGF antibodies B20-4.1.1 (which neutralizes both human and murine VEGF) or Avastin (human VEGF-specific neutralizing antibody) twice per week.

Cell blocks and Immunohistochemistry. Cell blocks were obtained by the inclusion of 5x106 cells recovered from ascites and included in agarose. Sequentially, cell blocks were fixed in formalin, embedded in paraffin and subsequently processed for IHC analysis. Three-micron-thick formalin-fixed, paraffin-embedded (FFPE) tumor samples were stained either with hematoxylin and eosin or processed for IHC. IHC was performed by using an automatic stainer BOND III, (Leica Microsystems), by using anti-MCT4 Rabbit Polyclonal Ab, Santa Cruz Biotechnology (dilution 1:300). Slides images were acquired with Aperio CS2 and MCT4 signal was quantified with Aperio ImageScope v12.4.0.708.

Results & Conclusions

We performed mtDNA sequencing (in collaboration with Leonardo Caporali – Neurological science institute of Bologna) for twenty PDXs that are available in our laboratory. We selected five PDXs that have interesting mitochondrial DNA mutations and one additional PDX as control without mutations. Moreover, these PDXs express different level of MCT4, a marker related to glycolysis metabolism. Highly glycolytic models are good responders to anti-VEGF therapy and develop resistance later compared with poorly glycolytic PDXs.

We aim to verify the impact of mtDNA mutations on tumor metabolism by analysing the OXPHOS system functioning and oxygen consumption rate by Clark electrode/Seahorse analysis.

Finally, we are planning to test the sensibility of our PDXs to OXPHOS inhibitors to confirm the hypothesis that PDXs cells carrying pathogenic mtDNA mutations are more sensitive to these drugs.

FOCAL ADHESION KINASE (FAK) CONTRIBUTION TO THE PATHOGENESIS OF CHRONIC LYMPHOCYTIC LEUKEMIA

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Background

Chronic Lymphocytic Leukemia (CLL) is a neoplastic disorder characterized by the clonal expansion of B lymphocytes. CLL pathogenesis involves both intrinsic and extrinsic factors, essential for the transformation and the growth of CLL cells, as well as for the progression of the disease, besides being sustained by a tonic B-Cell Receptor (BCR) signaling activation. CLL is a clinically and biologically heterogeneous disease. Thus, it is important to identify novel biological and cytogenetic features to predict prognosis at the time of diagnosis and to monitor disease progression. In this context, we focused on the role and functions of Focal Adhesion Kinase (FAK), a molecule so far considered marginal in oncohematology, although it has long been known for its involvement in several solid tumors. Despite this, there is evidence that it interacts with players involved in the BCR signal transduction pathway, such as the Src-kinase Lyn, which was demonstrated to be upregulated in CLL cells. For these reasons, FAK is regarded as a protein worth of investigation for its putative role in CLL pathogenesis and/or maintenance.

Material and Methods

CD19+/CD5+ leukemic B cells were collected from 142 CLL patients; normal B cells were obtained from 10 healthy subjects (controls). FAK expression was analyzed by western blotting (WB) and the expression data were correlated with patients' prognostic parameters, i.e. mutational status of IGHV genes. WB membranes were stained with antibodies anti-total FAK to detect its expression; anti-phospho-FAK Tyr397, for FAK activation; anti-PARP to assess cell viability. β-actin was used to normalize the data. Cell viability was evaluated also by flow cytometry using the AnnexinV/PI staining kit. Statistical analysis was performed using Student's *t* test.

Results

By WB analyses, full-length (125kDa) FAK expression was evaluated in 142 CLL patients, and the result was not statistically relevant compared to the 10 healthy controls (0.56±0.55 *vs* 0.67±0.18, p: ns). Disease heterogeneity may be accountable for this result. In fact, we could observe that poor prognosis patients, i.e. $IGHV^{unmut}$, presented decreased FAK levels (0.39±0.36) compared to the $IGHV^{mut}$ counterpart (0.73±0.72; $p<0.001$). Moreover, bands of lower molecular weight (92/94, 84 and 50 kDa), which were also phosphorylated at activatory Tyr397, were particularly present in poor prognosis patients.

Defactinib is a small molecule inhibitor of FAK, currently being evaluated for potential combination therapies for various solid tumors. Defactinib inhibits FAK phosphorylation at the activatory tyrosine. B-cells of 46 CLL patients were cultured with 5μM Defactinib. After 24 hours, leukemic cells underwent significant apoptosis (33±24% of viable cells after treatment *vs* 62±17% untreated control; $p<0.0001$). In WB analysis, Defactinib led to an increased PARP cleavage. In CLL cells treated with Defactinib, FAK expression was analyzed by WB. We noticed a lower amount of 125kDa FAK in the treated condition (0.47±0.39) compared to untreated cells (0.91±0.90; $p<0.001$). Similarly, the level of phosphorylated FAK at activatory Tyr397 was decreased with Defactinib (1.16±0.80 *vs* 1.81±0.94; $p<0.0001$).

Conclusions

Our results suggest that FAK may have a role in CLL pathogenesis, especially in the poor prognosis patients with unmutated IGHV. Moreover, inhibition of FAK activation by the small molecule Defactinib led to a highly significant level of apoptosis in leukemic cells. These results suggest that FAK may be involved in a pathway promoting the malignant phenotype which deserves further investigation.

EXTENSIVE MOLECULAR PROFILING OF ADVANCED SQUAMOUS CARCINOMA OF THE ANAL CANAL (MSCAC) TREATED WITH AVELUMAB OR AVELUMAB AND CETUXIMAB PROGRESSED AFTER AT LEAST ONE LINE OF TREATMENT: THE TRANSLA-CARACAS STUDY

Ph.D. Student: Dr. Alessandra Anna PRETE - TUTOR: Dr. Sara LONARDI
Ph.D. Course: Clinical and Experimental Oncology and Immunology

Background In recent years, immune checkpoint blockade (ICB) therapy demonstrated promising activity in terms of response and survival in gastrointestinal (GI) malignancies. In advanced squamous cell anal carcinoma (mSCAC), encouraging results were reported with pembrolizumab in patients with PD-L1 positive tumors. As well, remarkable response rate was obtained with Nivolumab in a mSCAC population unselected by PD-L1 or other biomarkers. In this study, significantly higher expression of PD-1 on T cells and PD-L1 on tumor cells were found in responder compared to non-responders, further supporting the possible predictive role of PD-L1 for ICB therapy in mSCAC. PD-L1 upregulation and expression on cancer cells is at the basis of the adaptive immune resistance; PD-L1 expression is known to be promoted by IFNγ secreted by NK and CD8+ T cells into the tumor microenvironment. In vitro studies demonstrated that the anti-EGFR cetuximab could downregulate the IFNγ-mediated expression of PD-L1, thus counteracting the adaptive immune resistance and supporting the hypothesis of synergism between ICB and anti-EGFR therapy. Consolidated evidence showed that GI tumors with high microsatellite instability (MSI-H) are more responsive to ICB: unfortunately, no data are available about MSI-H role in predicting benefit from ICB in mSCAC. MSI-H tumors are characterized by high neoantigen load, PD-L1 expression and tumor mutation burden (TMB). High TMB proved positive predictive value for ICB in several solid tumors. In mSCAC, low TMB was reported, in line with other HPV-related squamous tumors, suggesting that other factors could determine the immunogenicity of these tumors. In head and neck squamous cell cancer, significant association was described between objective response and high TMB in case of HPV and EBV negative tumors; on the contrary, this association was not confirmed in HPV+ or EBV+ tumors. This observation supports the theory that in virus-related squamous neoplasms, viral neoepitopes more than somatic antigens might play a decisive role in the process of immunoescape.
Moving from this background, we conducted an open-label, prospective, multicenter randomized phase 2 trial to evaluate safety and activity of the anti PD-L1 avelumab alone or in combination with the anti-EGFR cetuximab in pretreated mSCAC: the CARACAS study (NCT03944252). In parallel, we designed a prospective multicenter translational study to identify new biomarkers predictive of efficacy for ICB in mSCAC: the TranslaCARACAS study.
Material and Methods In the CARACAS study, patients were randomized in a 1:1 ratio to receive either avelumab 10 mg/kg q2w ev (arm A) or avelumab 10 mg/kg and cetuximab 500 mg/mq q2w ev (arm B) until PD, unacceptable toxicity or patient refusal. For the present study, tumor samples from all the patients enrolled in the CARACAS study were collected in our institution. Diagnosis of SCAC was confirmed for each sample by central revision. Next generation sequencing (NGS) will be performed on each sample to assess the main biomarkers with potential predictive value for ICB therapy. On the remaining tissue after NGS analyses, HPV testing and PD-L1 expression analysis will be centrally performed. Primary objective of this stduy is to describe the result of the CARACAS study according to molecular analyses. Secondary objective is to assess survival in terms of PFS and OS according to molecular characteristics, to individuate new biomarkers predictive of efficacy for the two treatments, to individuate new biomarkers predictive of toxicity for the two treatments and to analyze the expression of HPV and PD-L1 and describe their correlation with response to the two treatments
Results From 18 September 2018 to 2 July 2019, 60 patients in total were enrolled in the CARACAS study, 30 in each arm, representing the ITT population. Each characteristic was well balanced between the two groups, with the only exception of sex: males were 19 while females were 41 in total. Median age was 63 years. 12 out of 30 pts in each arm had distant metastases; 7 in arm A and 10 in arm B received > 1 previous lines of treatment. At data cutoff (July 2020), investigator-assessed ORR was 10% and 17% in arm A and B respectively; in arm B, five patients showed PR, thus reaching the primary endpoint. Disease control rate (DCR) was 50% in arm A and 57 in arm B. No major safety concerns were observed in each arm. Survival analyses are ongoing. The analyses planned for TranslaCARACAS study are expected to be completed by December 2020. However, since follow up of patients in the CARACAS study will be performed up to 31 March 2021, final results will be available from April 2021
Conclusions The CARACAS study is the first trial to assess the double EGFR and PD-L1 blockade in mSCAC, documenting promising activity in terms of objective response rate. The TranslaCARACAS study will explore the correlation between response and molecular characteristics, HPV status and PD-L1 expression.

ASSOCIATION OF GUT MICROBIOME DIVERSITY AND COMPOSITION WITH PATHOLOGICAL COMPLETE RESPONSE (pCR) AFTER NEOADJUVANT CHEMOTHERAPY IN TRIPLE NEGATIVE BREAST CANCER

Ph.D. Student: Dr. Grazia VERNACI - TUTOR: Prof. PierFranco CONTE
Ph.D. Course: Clinical and Experimental Oncology and Immunology

Background:
The gut microbiome is the largest component of the host immune system and has been shown to influence response to anti-cancer therapies such as chemotherapy and immunotherapy. Little is known regarding the role of gut microbiome in the field of breast cancer.
Methods:
In this pilot prospective study, we characterized the gut microbiome of 18 triple negative breast cancer patients undergoing neoadjuvant chemotherapy. All patients received anthracycline and taxane-based chemotherapy, including carboplatin in 16 patients. Pre-treatment fecal samples were analyzed by 16S RNA sequencing. Shannon index was used to evaluate alpha-diversity. Differentially abundant bacterial OTUs between groups were determined using Linear Discriminant Analysis (LDA) Effect Size (LEfSe).
Results:
Patients characteristics were as follows: premenopausal 44%, ductal infiltrating carcinoma 100%, histologic G3 100%, cN+ 67%, cT>2cm 89%. No significant association between Shannon diversity index and baseline characteristics was shown. As expected, the most abundant taxa at the phylum level were Bacteroidetes and Firmicutes. Ten patients achieved a pCR. The Shannon diversity index was significantly higher in pCR vs non-pCR patients: median 5.057 (95% CI 4.923-5.339) vs 4.639 (95% CI 4.427-4.913), p=0.016. At the genus level, Alistipes and Ruminococcaceae UCG-002 were significantly enriched in pCR vs non-pCR patients (LDA score >3.0, p<0.05).
Conclusions:
The results of this pilot study show preliminary insights into the potential implications of gut microbiome in chemotherapy efficacy for breast cancer. As immune checkpoint inhibitors have become part of the therapeutic algorithm for triple negative breast cancer patients, a complete characterization of the host immune system is crucial in order to identify potential prognostic and predictive biomarkers.

ACUTE KIDNEY INJURY IN DECOMPENSATED CIRRHOSIS IS ASSOCIATED WITH BOTH HYPO-COAGULABLE AND HYPER-COAGULABLE FEATURES

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

Background:
Recent evidence suggests that acute kidney injury (AKI) is the main predictor of post-paracentesis bleeding in patients with cirrhosis. To assess factors responsible for bleeding tendency in AKI, we performed a prospective study comparing all three aspects of hemostasis (platelets, coagulation, fibrinolysis) in patients with decompensated cirrhosis with and without AKI.

Material and Methods:
Primary hemostasis assessment included platelet aggregation and secretion (platelet function markers) and von Willebrand factor (VWF). Secondary hemostasis assessment included pro-coagulant (Factor VIII, Factor XIII) and anti-coagulant (protein C, protein S, and antithrombin) factors and thrombin generation (TG). Tertiary hemostasis assessment included fibrinolytic factors and plasmin-antiplasmin complex (PAP).

Results:
Eighty patients with decompensated cirrhosis were recruited (40 each with and without AKI). Severity of cirrhosis and platelet count were comparable between groups. Median serum creatinine was 1.8 mg/dL and 0.8 mg/dL in patients with and without AKI, respectively. At baseline, patients with cirrhosis and AKI had lower platelet aggregation and secretion, indicative of impaired platelet function (increased bleeding tendency), without differences in VWF. Regarding coagulation factors, FVIII was higher, while protein C, protein S, and antithrombin were all lower which, together with increased TG, indicate hypercoagulability. In contrast, FXIII was lower in AKI (increased bleeding tendency). Finally, while both hypo and hyper fibrinolytic changes were present in AKI, a higher PAP indicated a hyper-fibrinolytic state. After AKI resolution (n=23/40), platelet function and coagulation improved to levels observed in cirrhosis patients without AKI, however fibrinolysis remained hyper-activated.

Conclusions:
In patients with decompensated cirrhosis, AKI is associated with both hypo and hypercoagulable features that can potentially increase the risk of both bleeding and thrombosis.

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PhD COURSE "DEVELOPMENTAL MEDICINE AND HEALTH PLANNING SCIENCES"

COORDINATOR: Prof. Carlo GIAQUINTO

CURRICULUM "ONCOHEMATOLOGY, MEDICAL GENETICS, RARE DISEASES, EPIDEMIOLOGY AND PREDICTIVE MEDICINE"

COQ10A AND *COQ10B* GENES AND COENZYME Q_{10} BIOSYNTHESIS IN HUMAN CELLS

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

Background Coenzyme Q_{10} (CoQ_{10}) is a redox-active lipid, which functions as electron carrier in the mitochondrial respiratory chain during oxidative phosphorylation and whose deficiency causes severe human diseases. Primary CoQ deficiency is a clinically and genetically heterogenous disorder, associated with fatal neonatal encephalopathy; Steroid-resistant nephrotic syndrome (SRNS); Hypertrophic cardiomyopathy (HCM); retinopathy and sensorineural hearing loss[(1)].
Yet, CoQ biosynthesis has not been fully defined in any organism[(2)]. Among genes involved in CoQ biosynthesis in mammals, there are the two paralogues *COQ10A* and *COQ10B*, which show a different expression pattern: *COQ10A* is constutively expressed and exists in two isoforms (different for the first exon), while *COQ10B* expression is regulated by circadian rhythms [(3)]. These two genes were studied in *Saccharomyces cerevisiae,* in which COQ10 gene encodes for a START domain protein, thought to be required for the proper localization of CoQ within the inner mitochondrial membrane[(4)]. However neither human *COQ10A*, nor *COQ10B* could efficiently complement yeast COQ10 mutants, suggesting a possible different role or mechanism of action of these two proteins in mammalian cells. To study their precise function, we have generated cell lines lacking either or both genes using CRISPR-Cas9 technology.
[(1)] M. J. Acosta, *et al.*, Biochim Biophys Acta. 2016 Aug;1857(8):1079-1085. [(2)] J. A. Stefely and D. J. Pagliarini, Trends in Biochemical Sciences, October 2017, Vol. 42, No. 10 [(3)] K. A. Dyar, *et al*., PLoS Biol 16(8): e2005886, 2018. [(4)] H. S. Tsui, *et al*., *J. Lipid Res. 2019. 60: 1293–1310.*
Material and Methods As *in vitro* model we used primary fibroblasts isolated from a skin biopsy of a patient with a missense mutation in *COQ10A* gene and Human Embryonic Kidney (HEK) 293 cells. HEK 293 were genetically modified by CRISPR-Cas9 technology, to obtain the knockout (KO) of either or both genes of interest. For the functional characterization of cell lines we measured CoQ intracellular content by HPLC; the activity of mitochondrial respiratory chain complexes through a single-wavelength spectrophotometer and ATP production levels by ATPlite Luminescence Assay System. We performed the analysis of mitochondrial supercomplexes by Blue Native PAGE and mRNA content of KO cell lines was sequenced by NGS. For transient transduction of cells we used Lipofectamine 2000, while for stable transduction we used ViraPower HiPerform T-Rex Gateway® Expression System, based on replication-incompetent lentiviruses. We performed also a mass spectrometry-based metabolomic analysis of cellular and extracellular metabolites and the monitoring of Reactive Oxygen Species (ROS) production through the use of an oxidant-sensitivefluorescent probe (Invitrogen).
Results We obtained three KO HEK 293 cell lines, respectively lacking *COQ10A*, *COQ10B* or both genes. The functional characterization of KO cell lines pointed out some interesting differences compared to wild type cells, in particular in the activity and in the assembling of mitochondrial respiratory complexes. We also confirmed that the defective phenotype observed in KO cells is rescued by the re-introduction of the corresponding missing wild type gene.
Conclusions Overall our results indicate that COQ10A an COQ10B are relevant for human pathology and that the data obtained in yeast on COQ10 function cannot be translated to mammalian cells. In human cells, *COQ10A* and *COQ10B* seem to be important for the activity of some mitochondrial respiratory chain complexes. However our data suggest that COQ10 proteins have also other important roles in mitochondrial homeostasis, but at the moment their relationship with CoQ metabolism is unclear.

METABOLOMICS APPLIED TO BIOMARKER DISCOVERY IN BRONCHOPULMONARY DYSPLASIA IN PRETERM INFANTS: RELIMINARY RESULTS

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

Background
Bronchopulmonary dysplasia (BPD) is a chronic lung disease that most commonly occurs after preterm birth. BPD is the most common chronic lung disease of infancy in developed countries. In the last years, the incidence of BPD remains stable.
BPD has been defined as an "injury syndrome superimposed on the essential lung growth and maturation required for survival". The injury resulting in BPD is multifactorial and it seems to begin before delivery, to continue at birth, and to be amplified in the postnatal period, but the specific pathogenetic mechanisms associated with the development of this condition are not completely understood.
Metabolomics can be defined as the analysis and interpretation of global metabolic data of a complex biological system, reflecting the effect of the environment on genetic patterns.
The main aim of the study is to apply the metabolomic approach in order to establish whether a biomarker profile exists at birth, capable of predicting the development of bronchopulmonary dysplasia (BPD) in preterm infants.
Material and Methods
This is a monocentric, observational study comparing the plasma and urinary metabolomic profile between preterm infants with BPD (cases) and without BPD (controls), carried out at Neonatal Intensive Care Unit of the Department of Women's and Children's Health, Hospital-University of Padua. Infants borned <32 gestational weeks were included in the study.
Results
177 patients were enrolled, 83 (46.9%) with BPD (cases) and 94 (53.1%) without BPD (controls). Among cases, 41 (49%) had mild BPD, 19 (23%) moderate BPD and 23 (28%) severe BPD. Neonates with BPD were born with lower gestational weeks and birth weight and needed more frequently resuscitation at birth, with a lower Apgar index at 5^{th} minute. The BPD patients group was associated with a higher incidence of others comorbidities of prematurity, in particular necrotizing enterocolitis, high-grade intraventricular haemorrhages and retinopathy of prematurity.
Preliminary analyzes were performed on urine samples collected within 48 hours of birth from 14 patients (7 cases and 7 controls). From the results there appears to be a different urinary metabolomic profile between subjects who subsequently developed BPD and controls.
Conclusions
The preliminary analyzes performed seem to indicate that a different metabolomic profile already exists at birth among newborns who will subsequently develop pulmonary bronchodysplasia compared to patients who will not develop it.

FAMILY CLUSTERS OF COVID-19 INCLUDING CHILDREN: AN ITALIAN EXPERIENCE

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Background. SARS-CoV-2 infection has been spreading, worldwide. This study analyze characteristics and impact of Coronavirus disease 2019 (COVID-19) in family clusters.
Material and Methods. A prospective observational study started in Veneto Region (Italy), in April 2020. Families including children (0-14 years) were enrolled if one or more members had COVID-19, confirmed by positive SARS-CoV-2 molecular assay at nasopharyngeal (NP) swab, for clinical follow-up and detection of serum SARS-CoV-2 IgG/IgM and plaque reduction neutralization test (PRNT). Chi-square and one-way ANOVA were used for either categorical or quantitative variables; simple linear regression assessed relationship between PRNT and age. P-value <0.01 was considered statistically significant.
Results. Forty-two family clusters analyzed included 69 children (M 40, F 29) with mean age 7.38 years (SD 5.79) and 83 parents (42 F, 41 M) aged 41.16 years (SD 8.33). 31(45%) children and 55(66%) parents had confirmed COVID-19. Among 28 symptomatic children with COVID-19, the majority had fever (n=21), diarrhea (6), cough (5), asthenia (5); 6 (20%) were admitted to Hospital and all recovered. Table 1 reports characteristics of children. Positive SARS-CoV-2 serology (IgG>1.100 kAU/L) was detected in 91.3% of paediatric COVID-19, 61.5 days (IQR 44-71) after 1st NP swab. In addition, 46.2% of children with negative swab had positive serology, 59.5 days (IQR 35-70.5) after 1st swab. Median IgG was 5.427 (IQR 1.702-11.790) in children with COVID-19 and 0.3665 (IQR 0.027-5.288) in those with negative swab; parent's median IgG was 1.108 (IQR 0.14-5.62). Median PRNT log of children (n=31) and adults (n=34) were 5 (IQR 4-6) and 4 (IQR 3-4), respectively. At linear regression, younger age was correlated with higher PRNT log (R^2=0.288, p-value<0.0001).
Conclusion. Children of COVID-19 family clusters have mild disease, however they may develop high levels of SARS-CoV-2 antibodies.

Table 1. Characteristics of children included in the study.

	POSITIVE NP-SWAB (n=31)	NEGATIVE NP-SWAB (n=33)	Swab not done (n=5)	p-value*
Sex (female)	14 (45.2%)	11 (33.3%)	4 (80%)	0.128
Ethnicity (caucasic)	30 (96.7%)	32 (96.9%)	5 (100%)	0.677
Age, years (mean, SD)	7.1 (5.9)	7.4 (4.8)	5 (4.5)	0.66
Siblings (yes)	22 (70.9%)	24 (82.8%)	4 (80%)	0.548
History of confirmed COVID-19 among other family members (n,%)	24 (77.4%)	32 (96.9%)	5 (100%)	0.036
Previous vaccinations, any (yes)	30 (96.7%)	33 (100%)	4 (80%)	0.045
Flu vaccination for 2019/2020 season (yes)	1 (3.3%)	6 (18.2%)	0	0.213
Comorbidities (yes)	7 (22.6%)	4 (12.1%)	1 (20%)	0.537
Any symptom at 1st swab (yes)	28 (90.3%)	10 (30.3%)	2 (40%)	<0.001
ER evaluation/Hospitalization (yes)	6 (20%)	4 (12%)	0	0.007

*Chi-square for categorical variables, One-way ANOVA for quantitative variables

DEVELOPMENT OF INNOVATIVE *IN VIVO* PRECLINICAL TOOLS FOR AN EFFECTIVE THERAPEUTIC STRATEGY IN PEDIATRIC ACUTE MYELOID LEUKEMIA

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

Background

Acute leukemia is the most common cancer in childhood and the acute myeloid leukemia (AML) accounts for 20% of all cases. Despite AML therapy achieved numerous improvements in the last decades, still 30% of patients experience relapse, this latter limiting chance of survival for the absence of reliable second line treatments. The urgency to identify new drugs represents the main unmet clinical need. The scientific world identifies the rare incidence of aggressive tumors in childhood and the lack of predictive *in vivo* models as the main limits to the development of novel strategies that may support or reduce the use of toxic chemotherapy and hematopoietic stem cell transplantation. This research aims to develop Patient-Derived Xenograft (PDX) models, as innovative and predictive tool to overcome old-fashioned therapies, moving toward a targeted personalized medicine. PDXs are widely used in cancer research due to its retained features of the patient's tumor, in fact overcoming the high homogeneity of traditional tumor cell lines, PDXs more closely resemble patient's heterogeneity.

Material and Methods

PDX models generation starts with in the inoculation of primary AML cells in mice. The engraftment of these cells induces tumor onset in the animals, generating the so called PDX-P0; leukemia cells derived from PDX-P0 are inoculated in other mice, called PDX-P1, and so on for subsequent passages, till PDX-P3 engraftment, passage at which the model can be considered stabilized. We have implanted 50 AML primary samples in immunodeficient mice (NSG) by using several inoculation strategies to improve the engraftment. In particular, we directly injected $1x10^6$ blasts intrafemoral, intravenous or intrahepatic or we subcutaneous implanted humanized 3D scaffold pre-seeded with AML blasts and mesenchymal stromal cells (MSCs). Leukemia onset has been monitored by hCD45 expression by flow cytometry in peripheral blood samples. At each PDX passage (P0-P1-P2-P3) immunophenotypic profile and Next Generation Sequencing (NGS) have been performed to assess primary tumor and PDXs similarity.

Results

To date, 9 AML-PDXs have been obtained using different inoculation methods, suggesting that there is not a preferential technique and the engraftment may depend from AML primary cells aggressiveness (risk class) and viability at the inoculation time; 11 P0 cases are still under evaluation for engraftment. We documented primary AML immunophenotype and genetic markers identified at diagnosis being maintained in the AML-PDXs. Three models underwent to whole exome and transcriptome sequencing. Results are currently under evaluation and strong similarity between patient-AML and mice tumor has been observed. We preliminary evaluated a series of new combination therapies on our AML-PDXs supporting these models as a reliable tool for *in vivo* drug efficacy validation.

Conclusions

We are producing a biobank of pediatric AML-PDX models able to represent different pediatric AML subtypes. AML-PDXs largely display primary tumor biology, and are suitable for new drug testing in phase II-like clinical trials. The NGS data will improve cancer driver mutation identification, with the aim to generate innovative targeted therapies and improve patient outcome.

NOVEL APPROACHES TO DIAGNOSIS AND THERAPY OF SEPSIS

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Background Sepsis is a life threatening condition and a main cause of mortality and morbility in newborns, consisting in body's response to infection causing injuries to its own tissues and organs, and it can be classified as early-onset sepsis EOS (infection ≤ 3 days) and late-onset sepsis LOS (infection > 3 days). neonatal sepsis is mainly suspected based on clinical presentation of symptoms (fever, hypothermia, respiratory distress, and others) that could be manifestation of other clinical conditions. To date, there is no specific molecule to diagnose sepsis, but only some non-specific classic clinical biochemistry parameters that suggest the diagnosis of sepsis in newborns to the clinician. The objectives of the study consists in the research and identification, through the use of analytical techniques of chromatography and mass spectrometry, of new markers that allow early diagnosis of neonatal sepsis and prediction of its evolution. The identification of new possible markers will therefore allow, on the one hand, to promptly therapy in affected patients, before clinical conditions worsen irreversibly, and on the other to avoid unnecessary and potentially dangerous therapy in uninfected patients. This result can be achieved by analyzing the characteristic metabolic profile of the patients under examination (newborns born preterm), present in biological samples such as plasma and urine. Part of the project consisted in the LC-MS analysis of samples collected from preterm neonates, applying a two-steps metabolomic approach to EOS neonates in order to investigate metabolic perturbations that could lead to the discovery for new early biomarkers. Firstly, an untargeted metabolomic approach was used to compare the metabolic profiles of urine samples from preterm neonates with and without EOS. Secondly, the results obtained guided the targeted metabolomic analysis of plasma samples, investigating the metabolic perturbation induced by sepsis.

Material and Methods Plasma samples were collected at birth, while urines within 24 hours of birth, and were stored at -80°C until analysis. Plasma and urines samples have been specifically prepared for different analysis. Metabolic profiling was performed in positive and negative ionization mode on an Acquity UPLC system (Waters, U.K.) coupled to a Quadrupole Time-of-Flight (QTof) Synapt G2 HDMS mass spectrometer (Waters MS Technologies, Ltd., Manchester, U.K.). Chromatography was performed using an Acquity HSS T3 (1.7 µm, 2.1 x 100 mm) column (Waters Corporation, Milford, U.S.A.). Target analysis was carried out using a Xevo TQ-S triple-quadrupole mass spectrometer, coupled to an Acquity UPLC (Waters Milford, MA, USA), with an Electrospray Ionisation (ESI) source. The ESI operated in positive ion mode with multiple reaction monitoring (MRM). Chromatography was performed on a Acquity HSS T3 (1.7 µm, 2.1 x 100 mm) column (Waters Corporation, Milford, U.S.A.) using specific mobile phases for different classes of metabolites. Instrument control, acquisition and the analysis of data were provided by MassLynx software (version 4.1, Waters). Quantification was performed using the TargetLynx function of the same software.
Univariate and multivariate statistical analysis (t-test, PCA,PLS-DA) were then applied to untargeted and targeted data. Most relevant variables were annotated searching in Human Metabolome Database HMDB and the METLIN database and investigated using pathway analysis.

Results Untargeted metabolic profiling has been applied to urine samples to discover the metabolic pathways that are perturbed by sepsis, and that are analyzed more in depth in the second step of the study by targeted analysis on plasma, obtaining two dataset, one for each ionization mode. Univariate data analysis highlighted 44 variables in the NEG data set and 332 variables in the POS data set as relevant. Specifically, pathways related to aminoacyl-tRNA biosynthesis, phenylalanine, tyrosine and tryptophan biosynthesis, nitrogen metabolism, cysteine and methionine metabolism, taurine and hypotaurine metabolism and phenylalanine metabolism resulted influenced. In the second step, 39 metabolites belonging to the families of amino acids, 9 neurotransmitters associated to tyrosine and tryptophan metabolism, 7 polyamines and 10 metabolites associated to the kynurenine pathway were quantified. Pathways associated to glutathione metabolism, aminoacyl-tRNA biosynthesis, phenylalanine, tyrosine and tryptophan biosynthesis and tryptophan metabolism resulted perturbated.

Conclusions Neonates with early-onset sepsis EOS showed a different metabolic profile at birth and, through LC-MS analysis, it has been possible to distinguish them from neonates without sepsis, highlighting that tryptophan and glutathione metabolic pathways were highly perturbed in septic neonates. The results show the potential of metabolomics in understanding biological pathways and pathophysiological mechanisms of sepsis. This could be the starting point for discovering new early biomarkers and therapeutic targets of neonatal sepsis.

LEFT ATRIAL DECOMPRESSION IN PATIENTS SUPPORTED WITH ECMO FOR FAILURE TO WEAN FROM CARDIO-PULMONARY-BYPASS AFTER PEDIATRIC CARDIAC SURGERY: A PROPENSITY WEIGHTED ANALYSIS

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

Background
LA-decompression on-ECMO may reduce left ventricular distension allowing myocardial rest and recovery, and protect from lung injury secondary to cardiogenic pulmonary edema. However, long-term benefits are still discussed. We sought to evaluate the association between LA-decompression and in-hospital mortality in patients who failed to wean (FTW) from cardiopulmonary bypass (CPB) after cardiac surgery.

Material and Methods
Children <18 y who required ECMO for FTW after cardiac surgery, during 2000-2016, were extracted from the Extracorporeal-Life-Support-Organization-Registry. Cardiac surgical procedural complexity was assigned using Risk-Adjustment-in-Congenital-Heart-Surgery-1 (RACHS-1). An inverse-probability-of-treatment weighting based on a propensity-score was used to balance selection biases. Weighted logistic regression was used to test the unadjusted and adjusted association between LA-decompression and in-hospital-mortality.

Results
Of the 2,915 patients supported with ECMO for FTW from CPB, 1495 had biventricular physiology, and 217 underwent LA-decompression. LA-decompressed patients were older ($p<0.001$), had more frequently baseline arrhythmic-conditions ($p=0.004$), underwent lower surgical-risk procedures (by RACHS-1, $p<0.001$), but requested longer CBP time ($p=0.001$) and more frequently an aortic-cross-clamp ($p<0.001$). Covariates were well-balanced in the propensity-weighted cohort. Mortality rate was 46% in LA-decompressed patients and 54% in others. Weighted logistic regression analyses showed LA-decompression to be protective for in-hospital mortality (unadjusted OR 0.843 [CI 0.719-0.988], adjusted OR 0.704 [CI 0.568-0.872]). Longer ECMO duration, need for other procedures on-ECMO, and ECMO-complications independently increased the risk of mortality. A lower FiO2 at 24h post-ECMO and heart transplantation on-ECMO reduced the risk of mortality.

Conclusions
LA-decompression independently reduced the risk of mortality in patients supported with ECMO for FTW from CPB, confirming that a longer-term benefit exists in this selected population. Further high-evidence studies are needed to clearly define the role of LA-decompression in this population.

LINKING CIRCRNA TO LEUKEMOGENESIS: UNDERSTAND REGULATORY ROLES IN HEMATOPOIESIS AND UNRAVEL ONCOGENIC FUNCTIONS

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

Background

B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is the most common malignancy observed in pediatric patients. In this study, we focus on MLL-AF4 positive leukemia characterized by the t(4;11)(q21;q23) translocation and associated with a very poor prognosis. Hematopoiesis is a process also regulated by an emerging new class of RNAs represented by circRNAs: covalently closed RNA molecules, in which the 3′- and 5′-ends are linked in a non-collinear way by a process called back-splicing. For specific circRNAs it has been shown that they can act as microRNA sponges inhibiting/enhancing downstream RNA pathways. In some cases, circRNAs were also shown to have coding capacity, to produce peptides or to interact with RNA-binding proteins (RBPs) and can be also formed by exons of different genes generating fusion-circRNAs. Therefore, the study of circRNAs roles in carcinogenesis and their possible use as new tumor therapeutic targets is becoming an highly impact topic.

Material and Methods

To identify MLL-AF4 circRNA expression, we performed RNA-seq using TruSeq Total RNA library protocol with RiboZero method and Illumina sequencer. CircRNA were quantified and annotated data by back-splice identification and analyzed using the CirComPara bioinformatic pipeline. CircRNAs were validated by PCR and Sanger sequencing using divergent primers. To verify the expression of the circRNAs we performed retrotrascription experiments using random primers followed by rq-PCRs using Platinum SYBR Green Q-PCR Super Mix (Thermo Fisher Scientific, Waltham, MA) from RNA treated with RNase R enzyme and without it. For *in vitro* functional investigation of circRNA role a semi-massive plate assay with 2 custom small interifering RNAs (Silencer® Select Custom Designed siRNAs, Thermo Fisher Scientific) for each selected circRNA was designed to specifically silence the backsplicing site. We transfected SEM and RS4;11 cell lines using Mirus Solution reagent and Amaxa elettroporator. Transfected cells were then cultured for 24-48-72 hours and analyzed for silencing efficiency by rq-PCR, cell growth by tripan blue count and resazzurrine viability assay. Cell death was assessed by annexin-PI. Moreover, divergent primers were designed on MLL and AF4 exons to detect the presence of f-circRNAs formed from the translocation site and assessed by PCR; in parallel Fcirc pipeline was applied to RNA-seq data.

Results

We successfully generated RNA-seq libraries from 4 MLL-AF4+ infant patients, identifying 51,104 expressed circRNAs. We focused on 85 common circRNAs differentially expressed in MLL-AF4 patients in comparison with CD34+ and B-cells from healthy donors generated previously by RNA-seq. We validated backsplicing site of 5 of them in MLL-re cell lines. We prioritized 29 circRNAs based on their expression levels in patients vs healthy cells, on the predicted ORF sequences and miRNA binding sites and used for *in vitro* investigation. Functional studies revealed for a circRNA an efficient silencing effect at 24 and 48h with 30pmol/ul. A significantly reduced level of cell growth respect to the control was observed, at 24, 48 and 72h after transfection. In contrast, we observed an absence of changes on cell death suggesting that the silencing of the identified circRNA can induce a cell cycle arrest.

Furthermore, we discover a new fusion circRNA made by MLL ex4 and AF4 ex11 and 6 f-circRNAs by *in silico* prediction. We also deeply investigate the expression of circAF4 generated from AF4 gene involved in MLL translocation in 22 MLL-AF4 patients.

Conclusions

We described the circRNAome of MLL-AF4 B-ALL infant patients and compared it with normal hematopoietic cells one, identifying the dysregulated circRNAs. Ongoing silencing experiments are elucidating the impact of specific circRNAs and pointing toward an extremely intriguing cell cycle regulatory role of selected circRNA, in MLL-AF4 leukemia together with the attractive role of circAF4 and the possible one of the just discovered f-circRNA from the MLL-AF4 translocation.

ACCURACY AND RELIABILITY OF MUM-1 IMMUNOHISTOCHEMISTRY FOR THE IDENTIFICATION OF PLASMA CELLS IN CHRONIC ENDOMETRITIS: HEAD TO HEAD COMPARISON WITH CD-138

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Background: Chronic endometritis (CE) is a persistent inflammatory condition of endometrial mucosa characterized by the presence of plasma cells in endometrial stroma. This condition may account for infertility, recurrent pregnancy loss and repeated embryo implantation failures. Diagnosis of CE is a challenge. The current gold standard for CE diagnosis is immunohistochemistry (IHC) for CD-138 (a proteoglycan found on the surface of plasma cells). However, CD-138 immunostaining is not exempt from diagnostic limitations, as endometrial epithelial cells are known to constitutively express CD-138 on the basolateral sides of their plasma membrane, potentially leading to deceitful interpretation of IHC results and misdiagnosis. For this reason, histo-pathological analysis is often combined with hysteroscopic examination for improving the rate of CE diagnoses through the identification of peculiar endometrial changes due to chronic inflammation. Multiple myeloma antigen 1 (MUM-1) is normally expressed in plasma cells, melanocytes, some B cells, and activated T cells. Immunohistochemistry (IHC) for MUM-1 has been widely used for the diagnosis of lymphomas and plasma cell tumours. To date, no study has still evaluated the potential use of MUM-1 IHC for the diagnosis of CE. Over that background, our aim was to evaluate the accuracy and reliability of MUM-1 IHC, as compared with CD-138, for the diagnosis of CE.

Material and Methods: We conducted an observational study on consecutive patients referred due to infertility at three IVF Units (Unit of Gynecology and Obsetrics, University of Padua, Italy; Hillel Yaffe Medical Center, The Rappaport Faculty of Medicine, Technion, Israel; II Unit of Ginecology and Obstetrics, Department of Biomedical Science and Oncoloy, University of Bari, Italy) from August 2017 to January 2020. All patients were submitted to hysteroscopy plus endometrial biopsy (for histological and IHC analyses). All samples were fixed in neutral formalin and later embedded in paraffin for histological analysis. Diagnosis of CE at hysteroscopy was based on our previously published criteria. Histological diagnosis of CE was performed by traditional H&E staining, CD138 and MUM-1 IHC (presence of ≥1 plasma cells/hpf). In a subgroup of patients, the number of immunoreactive cells was counted in the same number of stromal areas by three pathologists independently, and results were compared. Primary outcome was to evaluate the diagnostic accuracy of MUM-1 IHC for CE, as compared with CD-138 IHC (by using the combination of histological and hysteroscopic diagnoses as reference standard). Secondary outcome was to assess the reliability of MUM-1 IHC as compared with CD-138 IHC for the detection of plasma cells.

Results: A total number of 169 patients were analysed. The absolute number of immunoreactive cells was computed only in 86 patients, while inter-observer variability between pathologists was assessed for 28 patients. The reference standard identified CE in 107 women (63.31%), while CD-138 IHC was positive for CE in 96 cases (56.80%) and MUM-1 IHC was positive for CE in 103 cases (60.95%). Sensitivity and specificity of CD-138 and MUM-1 were respectively 85.9%, 93.5% versus 89.7% and 88.7%. The overall diagnostic accuracy of MUM-1 and CD-138 were similar (AUC= 0.892 vs AUC=0.898). The intercorrelation coefficient for single measurements was high between the two techniques (ICC=0.831, 0.761-0.881 95%CI). However, among CE positive women, MUM-1 allowed the identification of higher number of plasma cells/hpf than CD-138 (5.76 [SD 4.77] vs 4.66 [SD 3.51]; p=0.026). Additionally, MUM-1 showed higher inter-observer agreement as compared to CD-138 (ICC=0.905 versus ICC=0.724).

Conclusions: IHC for MUM-1 and CD-138 showed comparable accuracy for detecting endometrial stromal plasma cells. Importantly, MUM-1 showed lower inter-observer variability in the paired comparison of the individual samples than CD-138. Therefore, basing on our experience, MUM-1 may represents a novel, promising alternative to CD-138 for the diagnosis of CE.

SARS-COV-2 INFECTION AND REPLICATION IN HUMAN FETAL AND PEDIATRIC GASTRIC ORGANOIDS

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

Coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection is a global public health emergency. COVID-19 typically manifests as a respiratory illness but an increasing number of clinical reports describe gastrointestinal (GI) symptoms. This is particularly true in children in whom GI symptoms are frequent and viral shedding outlasts viral clearance from the respiratory system. By contrast, fetuses seem to be rarely affected by COVID-19 although the virus been detected in placentas of affected women. These observations raise the question of whether the virus can infect and replicate within the stomach once ingested.

Fetal and pediatric stomachs were isolated and characterized. Following stem cells isolation from the mucosal layer, gastric organoids were derived and cultured embedded in extracellular matrix (ECM). ECM was subsequently removed to allow the organoids to reverse polarity aiming to expose apical surface and to enhance SARS-CoV-2 infection. The revere polarity organoids are fully susceptible to a robust infection with SARS-CoV-2, while the efficiency of infection is significantly lower in fetal organoids and cells are undergoing programmed cell death.

The first expandable human gastric organoid culture across fetal developmental stages was established to support the hypothesis that fetal tissue seems to be less susceptible to SARS-CoV-2 infection. However, the virus can efficiently infect gastric epithelium in pediatric patients, suggesting that the stomach might have an active role in fecal-oral transmission of SARS-CoV-2.

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PhD COURSE "DEVELOPMENTAL MEDICINE AND HEALTH PLANNING SCIENCES"

COORDINATOR: Prof. Carlo GIAQUINTO

CURRICULUM "HEALTH PLANNING SCIENCES"

REGIONAL PERIPARTUM DEPRESSION PROGRAM: A NETWORK FOR PREVENTION, DIAGNOSIS, TREATMENT AND SURVEILLANCE. STATE OF ART ON THE SCREENING CAMPAIGN IN VENETO REGION

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Ph.D. Course: Developmental Medicine and Health Planning Sciences
Curriculum "Health Planning Sciences"

Background Mental illnesses are some of the most significant puerperal complications and outcomes impacting on women's health and on their whole family and community. A consistent literature agrees that perinatal depression (PD) is a frequent and ubiquitous problem, and that major risk factors are history of mental illnesses, lack of social support, isolation and substance abuse. Moreover, there are good chances with specific interventions of prevention and early action. The treatment choice depends on the severity of the symptoms and can be psychological and/or pharmacological ones. Although this knowledge is available and many interventions have been conducted around the world, there are few experiences of actively offered services aimed at the entire population on a permanent basis. For this reason, the Ministry of Health has launched a call to which the Veneto Region has responded with a project for the implementation of interventions in the following 4 areas: 1) Regional surveillance system for maternal depressions; 2) Information campaign for the general population and pregnant women; 3) Training of the staff; 4) Early screening of women and their early care. Within an ongoing Regional Project on preventing and taking care of women with PD, a broad network of dedicated services has been set up: in the maternity wards midwives administer to all women screening tests for PD, consisting in Edinburgh postnatal depression scale - EPDS and questionnaire on risk factors. Screening scores divide women according to their risk of developing depression: if a woman results at high risk, Departments for Mental Health will confirm or not diagnosis and start a treatment; if a woman is at medium risk, she is addressed to the Family Counselling services for an assessment, home visiting and treatment. This abstract reports the activities carried out and the main results regarding points 1 and 4.

Material and Methods

We built up a surveillance system using a web-based information system dedicated to PD and record linkage between this and current data flows (birth register, drug register, hospital discharge records, and psychiatric records).We run a pilot study to test the regional screening program. We administered a screening questionnaires to women who delivered in five maternity wards of Veneto Region (Camposampiero, Cittadella, Feltre, Treviso, Vicenza) between 20 July and 14 August 2020. These women experienced part of their pregnancy during the Coronavirus pandemic and lockdown. Screening is made of EPDS, an internationally validated questionnaire consisting of 10 items (cut off for high risk 15 without any other risk factors, or 12 with some risk factors), and an ad hoc risk factors questionnaire (14 items investigating past psychiatric pathologies, substance abuse and social aspects). An algorithm in a web-based information system divides women in groups of risk (low, medium and high). Some other questions were also asked concerning the experience during the pandemic (feelings, fear, access to NHS).

Results

1. The surveillance system pointed out that prevalence in Veneto of women with perinatal depression is 2%. Two out of three of them take psychiatric drugs, even in association, without the supervision of a specialist of NHS. About one woman a year committed suicide in the postpartum period. About one out of four women who had had depression during one pregnancy, relapsed the same problem in the following pregnancy, and in one out of three depression became chronic. The use of drugs without supervision and the absence of specialistic follow-up are serious risk factors for both relapse and chronicity of the problem.
2. We proposed screening to 105 women; 98 agreed to participate (93% adherence rate). 21,4% of women interviewed were at high risk of developing depression and 19,4% were at medium risk. About risk factors, 10% of women had a pre-existing psychiatric pathology, 4% history of addiction, and 5% loneliness. This last factor certainly increased during the quarantine period for the Covid-19 epidemic: 72% of women during pregnancy have restricted social contacts even among their closest family members also after the lockdown, and 45% went out exclusively for medical examinations.

Conclusions The known high frequency and severity of perinatal depression is therefore confirmed also for Veneto Region, as well as the importance of intercepting as many women as possible at risk of developing perinatal depression from the very first signs through a screening campaign.

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PhD COURSE "MOLECULAR MEDICINE"

COORDINATOR: Prof. Stefano PICCOLO

CURRICULUM "BIOMEDICINE"

CHARACTERIZATION OF THE SIGMA FACTOR σ^E REGULATORY NETWORK IN *MYCOBACTERIUM SMEGMATIS* UNDER SURFACE STRESS CONDITION.

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Ph.D. Course: Molecular Medicine
Curriculum "Biomedicine"

Background

Mycobacterium tuberculosis is the causative agent of tuberculosis and it is the most deadly of human bacterial pathogens. Specifically, in our laboratory, one of the main research field is about the role of sigma factors that are involved in the capability to survive in different environmental conditions through the modulation of global transcriptional profile. The alternative extracytoplasmic sigma factor SigE allows the survival in conditions of different stress conditions as surface stress, low pH and oxidative stress and is involved in virulence. The regulatory network of *sigE* is very complex and includes several regulatory nodes. We decided to study the activation of this network in detail to evaluate the role and hierarchy of the main regulators, which play a role in it: MprAB, ClgR and RseA. Since the *sigE* regulatory network of *M. tuberculosis* is very similar to that of the model organism *Mycobacterium smegmatis*, we used this bacterium which is easier to manipulate and faster to grow.

Material and Methods

Surface stress was obtained by the exposure to sodium dodecyl sulfate (SDS) 0,05% that is an anionic detergent able to modify bacterial surface. Through Real-time PCR analysis, we evaluate the dynamic of transcription of several genes *(sigE, sigB, rseA, clpP2)* along time after SDS addition in *M. smegmatis* wild type, mutant strain lacking *mprAB,* mutant strain lacking *clgR* and mutant strain in which RseA is not recognized by the ClgR-dependent protease.

Results

In the wild type strain, surface stress response is activated in the first 5 minutes after exposure to stress. However, in the mutant strain missing the two-component system MprAB the response is not activated at all, showing that MprAB has a fundamental role in starting the SigE-mediated stress response.

In the mutant strain missing ClgR the activation of the stress response was delayed and unstable. ClgR role in the SigE regulatory network is that to induce a protease that degrade the anti-sigma factor RseA. This degradation is essential for the full activity of SigE. The fact that in the absence of ClgR the activation is weaker and delayed suggest that the role of RseA is that to sustain the activity of SigE. After that, MprAB activates the stress response.

Finally, in a mutant strain in which RseA is not recognized by the ClgR-dependent protease due to a point mutation, the transcriptional level of sigE is similar compared to wild type but the expression of *sigB* and *clgR* (which depend on SigE) is very low.

Conclusions

We demonstrated that the two component system MprAB has the primary role in the detection of surface stress and in the activation of the *sigE* network, while ClgR and RseA have a secondary role in sustaining the network activation during time.

DISSECTING THE MOLECULAR FUNCTION OF MUTANT HUNTINGTIN WITH STEM CELLS

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Ph.D. Course: Molecular Medicine
Curriculum "Biomedicine"

Background

The Huntington's disease (HD) is an incurable, dominant neurodegenerative condition caused by a trinucleotide repeat expansion in the huntingtin gene. A fairly broad range of trinucleotide repeats (9-35) has been identified in normal controls, and repeat numbers in excess of 40 have been described as pathological. Mutant huntingtin (mHTT) misfolds and aggregates, leading to impaired proteasome function, loss of calcium homeostasis, mitochondrial dysfunction, transcriptional deregulation, and altered vesicular transport, that results in degeneration of specific types of neurons. To the present state of the art, it is not known whether these changes are the cause of HD, or simply consequences of a general impairment in the cellular function.

The main objectives of this research are understanding how Huntingtin (Htt), the protein mutated in Huntington Disease (HD), controls the cell behaviour, focusing mainly on cell survival.

Material and Methods

An unbiased genome-wide screening has been performed, looking for genes functionally involved in cytotoxicity induced by mHtt. Mouse Embryonic Stem cells expressing mHtt - which recapitulated some of the molecular defects associated with HD - have been generated, and used for a gain-of-function screening based on insertional mutagenesis. Such screening allowed the identification of over 100 candidate genes that are able to reduce the mHtt toxicity. Available gene-expression data from patients and animal models have been used to prioritize some of the newly identified genes for further functional characterization both in *in vitro* and in *in vivo* models of HD.

Results

Murine Embryonic Stem (mES) cells expressing either mutant Htt (an allele containing 128 CAG repeats, called Q128) and wild-type Htt (containing 15 CAG repeats, called Q15) have been successfully generated by electroporation of plasmids and selection to obtain cell lines stably expressing the two constructs. To generate our genetic mutants, we performed electroporation of piggyBac (PB) vectors, optimized for gain-of-function screens. We collected entire populations of resistant clones and used Splinkerette-PCR followed by Next-Generation Sequencing to map the integration sites in large populations of mutants. We analysed in total 17 mutant populations obtained from 4 independent experiments. We detected more than 10,000 integration events, corresponding to 804 unique integration sites and, after stringent statistical analysis, we identified 107 genes as candidate suppressors of cytotoxicity induced by mutant Htt.

Conclusions

We have performed a genome-wide, gain-of-function screening in Pluripotent Stem cells (PSCs) and identified 107 candidate genes able to suppress toxicity induced by mutant Huntingtin (mHtt). A fraction of these were further validated in vitro and in vivo in a Zebrafish model of HD. Such genes will be also tested in more relevant model systems, such as immortalized striatal neurons expressing mHtt and animal models and will represent novel promising therapeutic targets for HD.

INHIBITION OF HCMV REPLICATION BY SMALL MOLECULES INTERFERING WITH THE DIMERIZATION OF DNA POLYMERASE PROCESSIVITY FACTOR UL44

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Ph.D. Course: Molecular Medicine
Curriculum "Biomedicine"

Human cytomegalovirus (HCMV) is a leading cause of severe disease in immunocompromised individuals, and in congenitally infected newborns. Despite the availability of several drugs, pharmacological treatment is associated with toxicity and the emergence of resistant strains. Therefore, it is essential to identify new potential targets of therapeutic intervention. One of those is represented by the dimerization of HCMV DNA polymerase processivity factor UL44. Indeed, UL44 plays an essential role in viral replication by tethering the DNA polymerase holoenzyme to the DNA and its dimerization is absolutely required for DNA binding and OriLyt-dependent DNA replication since point mutations disrupting protein self-interaction also prevent DNA binding and abolish viral replication. The aim of this study is therefore to identify small molecules (SMs) that hinder viral replication by interfering with UL44 homodimerization. To this end, we first validated UL44 crystal structure by a variety of in vitro (GST-Pull Down and Thermal Shift) and in cells (Fluorescence and Bioluminescence Resonance Energy Transfer) assays and used it to perform a virtual screening to identify SMs potentially interfering with UL44 homodimerization. Based on cluster analyses and commercial availability, 18 out of 140 identified SMs were tested for their ability to impair replication of the TB4-UL83-EYFP recombinant HCMV by means of Fluorescent Reduction Assay (FRA). Four SMs reproducibly inhibited viral replication in the absence of evident cytotoxicity. Subsequently, MTT and FRA assays allowed to calculate the 50% cellular toxicity (CC50) and effective dose (ED50) relative to each hit. The 3 compounds with the highest selectivity index (ranging from ~5 to ~20) were further tested for their ability to inhibit the replication of an AD169-GFP recombinant virus and a GCV-resistant derivative, resulting in similar ED50. The most active compound inhibited AD169 replication by Plaque Reduction Assays with an ED50 of ~ 15 μM, and specifically impaired expression of late genes, as accessed by Western Blotting assays. Overall, our data suggest that SM-mediated impairment of UL44 dimerization and viral replication could be employed as a valuable therapeutic approach for the treatment of infections caused by drug resistant HCMVs, and the SMs identified here could represent a starting point for the development of new, highly-needed antiviral compounds.

Circulating miR-21and miR-122: prognosis prediction and correlation with HIF-1α in hepatocellular carcinoma patients treated with transarterial chemoembolization

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Ph.D. Course Molecular Medicine
Curriculum "Biomedicine"

Background
Several MicroRNAs (miRNAs) have been proposed as biomarkers in hepatocellular carcinoma (HCC) and two of the most promising are miR-21 and miR-122, with pro- and anti-oncogenic properties respectively. We aimed at evaluating miR-21 and miR-122 as prognostic biomarkers in HCC patients treated with trans-arterial chemoembolization (TACE), a treatment that delivers chemotherapy locally within the tumour(s) and induces liver ischemia (consequently stimulating angiogenesis). For this reason, we also investigated the correlation of these two miRNAs with the circulating levels of Hypoxia inducible factor-1α (HIF-1α).

Materials and Methods
In this retrospective study, 12 healthy subjects, 28 cirrhotics and 54 consecutively collected HCC patients (tested before and four weeks after TACE) were included. Whole blood miR-21 and miR-122 levels were measured by quantitative Real Time (qRT)-PCR, while serum HIF-1α was assessed by an Enzyme-Linked Immunosorbent Assay (ELISA) test. The value of the two miRNAs as prognostic markers was evaluated both for miRNAs quantified before TACE (at t_0) and for miRNAs ratio (miRNA measured after/before TACE).

Results
The highest level of miR-21 was found in cirrhotics, while HCC patients had the highest level of miR-122 (which was even higher in "viral" HCC, p=0.006). miR-21 levels dropped after TACE (p=0.03), while there were no significant differences between miR-122 levels measured before and after the treatment. miR-21 ratio and miR-122 at t_0, when below their respective cut-offs, identified patients with longer progression-free survival (p=0.0002 and p=0.02, respectively). The combined assessment of alpha-fetoprotein (the traditional biomarker for HCC) and miR-21 ratio, that both were demonstrated as being independent prognostic predictors, identified early progressors even among patients with complete or partial radiological response. miR-21 levels positively correlated with HIF-1α before (r=0.34 [95% CI 0.00 – 0.61]; p=0.045) and after TACE (r=0.35 [95% CI 0.02 – 0.61]; p=0.035), while no correlation was demonstrated for miR-122.

Conclusions
miR-21 ratio and miR-122 are useful prognostic markers after TACE and identify patients with positive outcome, even among those with radiologic disease control. miR-21 correlates with HIF-1α and probably has a role in modulating angiogenesis also in HCC, as it was demonstrated for other types of tumours.

ONCOLYTIC HSV-1 VECTORS FOR THE IMMUNOTHERAPEUTIC TREATMENT OF SOLID TUMORS AND EVALUATION OF MONOCYTES AS CARRIER CELLS

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Curriculum "Biomedicine"

Background

Oncolytic viruses (OVs) exploit molecular defects of cancer cells in intrinsic cellular antiviral pathways to achieve selective replication. OVs have a double antitumoral mechanism of action, which includes both direct cell lysis and eliciting an antitumoral immune response. Herpes simplex virus type 1 (HSV-1) is one of the most «popular» platforms for OV development due to its wide cellular tropism, high lytic activity and immunogenicity, and its ~160kb DNA genome which allows extensive manipulation and insertion of therapeutic genes. Also, the only OV approved for clinical use so far (T-VEC, for the treatment of melanoma) has been derived from HSV-1.

OVs need to be potentiated to tackle solid tumours with an immunosuppressive microenvironment (TME) such as pancreatic adenocarcinoma, glioblastoma, breast cancer (selected as our model tumor). Furthermore, to achieve a systemic antitumoral effect two strategies can be pursued, either intratumoral viral injection relying on an «in situ vaccine» effect or systemic injection, possibly by means of carrier cells to avoid antibody-mediated neutralization and to achieve targeted delivery. The aim of the project is to generate OVs with therapeutic genes that can subvert the immunosuppressive features of the TME and to evaluate the potential of monocytes as carrier cells for systemic delivery. Autologous patient monocytes can be easily recovered, moreover monocytes are actively recruited by many tumours in the TME as precursors of tumour associated macrophages.

Material and Methods

HSV-1 DNA embedded in a bacterial artificial chromosome (BAC) was further modified by BAC mutagenesis in a strain of *E.Coli* bacteria expressing the lambda-Red recombinase system. Mutagenesis was followed by reconstitution, achieved by lipofectamine-mediated transfection of BAC DNA in HEK293T cells. Expression of therapeutic genes was checked by reverse transcriptase PCR (RT-PCR) and ELISA assay, while EGFP expression was verified by fluorescence microscopy. oHSV-1 titration was performed by plaque assay on green monkey kidney VERO cells. In the coculture experiment, THP-1 cells were infected with multiplicity of infection (MOI) 1 PFU/cell EGFP-oHSV, washed three times with PBS 1X, and then cocultured with adherent uninfected MDA-MB-231 breast cancer cells at a carrier cell/cancer cell ratio of 1:1 or 2:1.

Results

Oncolytic HSV-1 viruses with the T-Vec backbone (Δγ34.5/ΔUs12) and different immunotherapeutic genes including human interleukin 12 (hIL12), mouse IL-12 (mIL12), soluble programmed cell death 1 (sPD1), FMS-like tyrosine kinase 3 ligand (Flt3L) were generated. Insertion of transgenes in the UL55-UL56 intergenic regions is not detrimental for oHSV replication, as shown with a virus harbouring the reporter gene EGFP (EGFP-oHSV). Expression of hIL12 and mIL12 was verified. Human monocytic cells THP-1 cells infected with EGFP-oHSV with MOI 1 PFU/cell could transmit the OV to uninfected cocultured human breast cancer cells (MDA-MB-231). PBS 1X from the third washing of THP-1 cells was titrated to ensure that no residual extracellular virions were present.

Conclusions

In conclusion, we generated oncolytic HSV-1 viruses which are able to express immunotherapeutic genes in cancer cells *in vitro*. Furthermore, preliminary experiments indicate that monocytes are suitable cells for the delivery of oHSV-1. This mode of delivery can be further characterized and tested on an animal model in comparison with intratumoral injection.

EXPLORING THE LINK BETWEEN METABOLIC ACTIVITY AND FERROPTOSIS

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Ph.D. Course: Molecular Medicine
Curriculum "Biomedicine"

Background

Ferroptosis (FPT) is a form of regulated cell death induced by iron-dependent lipid peroxidation. Dysregulation of ferroptosis is associated with cancer, neurodegeneration and ischemia-riperfusion injury[1]. It is linked to the missing or insufficient activity of the ubiquitous Selenoperoxidase glutathione peroxidase 4 (GPx4), that catalyzes a glutathione (GSH)-dependent reduction of membrane hydroperoxides to corresponding alcohols. Besides GPx4 inactivation, ferroptosis occurs when other conditions are satisfied: - oxygen metabolism leading to the continuous formation of traces of LOOH from phospholipid-containing polyunsaturated fatty acids; - availability of ferrous iron from the labile iron pool[2].

In this study, we used as a suitable model of ferroptotic cell death, that primed by erastin in HT1080 cells. Erastin, by preventing cystine import, decreases the cellular level of GSH and, consequently, GPx4 activity. Our previous results failed to establish a connection between mitochondrial electron transport chain and ferroptosis, but confirmed the important role of glutamine, via glutaminolysis, and other intermediate of TCA cycle in mediating ferroptosis upon erastin treatment. Importantly these data identified the activity of mitochondrial α-keto acid dehydrogenases as crucial for sensitivity to ferroptosis.

Material and Methods

Cell cultures and reagents. HT1080 cells were cultured in DMEM, low glucose, 4 mM GLN, 1 mM Sodium Pyruvate containing 10% (v/v) FBS, 1% (v/v) penicillin/streptomycin. Where indicated, medium was changed with DMEM without glucose, GLN, pyruvate and treated with erastin +/- ferrostatin-1.

Cell viability. Cells were seeded onto a 96-well plate (5000 cells/well) and treated with indicated compounds. Cell viability was assessed at different time points after treatment using resazurin sodium salt (Sigma-Aldrich).

SiRNA. Cells were plated at 50% of confluence in 10 mm dish and silenced according to Manufacturer instruction. For cell viability assays, 24 hours after the silencing cells were plated onto a 96-well plate (5000 cells/well) and treated the next day with indicated compounds. Silencing was verified by Western Blot or by qRT- PCR.

Results

To explore the role of single α-keto acid dehydrogenases, first we subjected HT1080 cells to erastin in a minimal medium, deprived of any source of metabolite that could sustain TCA cycle, i.e. glucose, GLN, pyruvate. As expected, cells didn't undergo ferroptosis despite the depletion of GSH similar to that observed in complete medium, where the ferroptotic response is maximal. Then we added single substrate in order to force single enzymatic activity: GLN and its product α-ketoglutarate (α-KG) restored erastin induced ferroptosis, underlying the importance of α-KG dehydrogenase complex. Glucose, by acting at multiple level, had the same effect. Importantly pyruvate efficiently recapitulated the role of GLN in ferroptosis, indicating that also pyruvate dehydrogenase complex (PDH) is involved. To test if pyruvate can substitute GLN for the production of oxidant radicals in membranes, we applied BODIPY C11 fluorescent probe and observed an increased oxidation signal in respect to control cells. To note, the ferroptosis inhibitor ferrostatin-1 abrogated the observed results.

To validate these findings, we silenced the pyruvate dehydrogenase complex (PDH) subunit E1 (obtaining a 75-80% of gene silencing) and evaluated the response to erastin treatment by resazurine assay. siRNA to E1 protected cell from ferroptosis induced by erastin in all the conditions tested.

Conclusions

Our evidence that mitochondrial oxidation of α-keto acid is indispensable to ferroptosis primed by GSH depletion, links metabolic activity of the cell to ferroptosis. In this respect, the essential contribution of PDH will be further examined by different approaches, first of all by measuring its activity and its regulation in the different metabolic conditions.

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PhD COURSE "MOLECULAR MEDICINE"

COORDINATOR: Prof. Stefano PICCOLO

CURRICULUM "REGENERATIVE MEDICINE"

PERIPHERAL NERVE INJURY AND BIOACTIVE POLYMERS DEVELOPMENT FOR AXONAL REGENERATION

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Ph.D. Course: Molecular Medicine
Curriculum "Regenerative Medicine"

Background

In case of peripheral nerve injuries (PNI) requiring neurorrhaphy, wrapping the repair-site with resorbable protectants assure for an adequate recovery microenvironment. In this study, two novel biodegradable wraps based on synthetic oxidized polyvinyl alcohol (OxPVA) and natural leukocyte-fibrin-platelet membrane (LFPm), respectively, were compared in repairing PNI in an animal model of disease.

Materials and methods

After sciatic nerve transection and neurorrhaphy, thirty Sprague-Dawley rats were randomly implanted with: a) NeuraWrap™ (control group); b) OxPVA; c) LFPm wraps. At 2 weeks and 12 weeks (prior to euthanasia) from surgery, functional recovery tests (Sciatic Function Index – SFI) were performed; thus, the explanted nerves underwent morphological analyses. The coaptation site was evaluated by histology (Hematoxylin and Eosin staining – HE) and immunohistochemistry (anti-CD3, -F4/80, -S100 -β-tubulin stainings); the proximal/distal stumps were investigated for ultrastructure (Toluidine-Blue staining/Transmission Electron Microscopy -TEM). Morphometric studies were also performed.

Results

SFI data showed recovery in function in all groups, with no significant differences at both endpoints. At dissection, no signs of inflammation/scar-tissue were visible in any group and only OxPVA and NeuraWrap™ residues were still recognizable. According to HE, CD3 and F4/80 analysis, no significant inflammatory infiltrate was observed in all groups, confirming wraps biocompatibility. Then, S-100 and β-tubulin staining and TEM analysis proved the specific nervous origin of the repaired tissue. Morphometry data showed significant difference ($p < 0.01$) for total cross-section areas which was higher at the proximal stump in the LFPm-wrap group versus NeuraWrap™ but not compared to OxPVA; while at distal level, both OxPVA and LFPm experimental groups had significantly higher ($p<0.05$) values of mean total cross-section area versus the commercial product. Considering fascicular area, OxPVA guaranteed significantly higher ($p<0.01$) mean values compared to NeuraWrap™ at both proximal stump and distal level; conversely, no significant differences were recorded for LFPm wraps at the two levels. Total axons density was significantly higher ($p<0.01$) for NeuraWrap™ versus OxPVA wraps but not versus LFPm wraps at proximal level; while, considering the distal portion, no differences among the groups aroused. Total number of axons was significantly different ($p<0.01$) only between OxPVA wraps and NeuraWrap™ at distal level with higher values for OxPVA.

Conclusions

OxPVA and LFPm wraps were effective in promoting nerve regeneration especially in the distal portion. Bioengineered OxPVA and LFPm wraps promoted lesion recovery and may be considered as an interesting alternative to the commercial NeuraWrap™.

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PhD COURSE "PHARMACOLOGICAL SCIENCES"

COORDINATOR: Prof. Nicola FERRI

CURRICULUM "MOLECULAR AND CELLULAR PHARMACOLOGY"

THE USE OF PHARMACOGENOMIC MARKERS TO INCREASE ANTIBLASTIC TREATMENTS SAFETY AND EFFICACY WITHIN THE PGX-GUIDELINES IMPLEMENTATION MULTICENTRIC PREPARE STUDY

Ph.D. student: Dr.ssa Alessia BIGNUCOLO
Tutor: Dr. Giuseppe TOFFOLI/Prof. Guglielmina FROLDI
Ph.D. Course: Pharmacological Science
Curriculum: "Molecular and Cellular Pharmacology"

Background: In recent years, multiple randomized controlled trials for a variety of drug-gene interactions have strongly associated pharmacogenetic-guided dose adjustment of drugs with improved patient outcomes and reduced toxicity. At present numerous PGx-based guidelines are available and continuously updated and translated (DPWG, CIPC, RNPGx, AIOM-SIF, PharmGKB). Nevertheless, despite the proven clinical validity of many PGx variants and the feasibility of several commercially available PGx panel tests, the implementation of PGx into routine care remains limited due to the lack of standardized guidelines and infrastructure and technologies for supporting the clinical decision. To overcome these barriers "The Ubiquitous Pharmacogenomics Consortium" has carried out a randomized clinical trial (PREPARE: *PREemptive Pharmacogenomic testing for prevention of Adverse drug Reactions*) funded by European Commission's Horizon-2020 program (G.A. n° 668353). This project, coordinated by Leiden University, encompasses seven European countries (The, Spain, UK, **Italy**, Austria, Greece, Slovenia) supporting the clinical validity of PGx guideline implementation. The aim of this prospective study is to demonstrate that a preemptive PGx test will both reduce the frequency and the severity of ARDs and result in a cost-effective approach to the therapies. The Experimental and Clinical pharmacology of CRO in Aviano represents one of the implementation site for this model of tailored therapy with regard to oncological field.

Material and Methods: The seven European countries were randomly assigned to control arm or to study arm for 18-month time-lag. Our site was recruiting in the first time block in control arm in which patients were enrolled with the standard of care and underwent a toxicity surveillance program. Data regarding ADRs were recorded in eCRF developed by PROMISE (The Netherlands). On October 1st 2018 our site switched to the study arm in which patients received a PGx-guided prescription of drug. Concurrently they underwent an identical ADRs surveillance program to the control arm in order to assess the safety of the therapies in terms of both ADRs frequency and severity reduction. The panel of 47 variants in 13 pharmacogenes was performed using the KASP assays by LGC Genomic, based on allelic discrimination technology. For each patient in the study arm a PGx report and a Safety Code Card were generated for guiding the dose and drug selection allowing patients to have a benefit in terms of a safer therapy.

Results: A total of 1224 patients (606 in control arm and 618 in study arm) were enrolled from March 2017 to June 2020 with an average of 29 patients per month, thus reaching the expected patients number for our center. 78% of patients showed oncological diseases whereas about 20% of the total were patients with cardiovascular disease (enrolled in San Filippo Neri Hospital in Rome). 50% of patients received a fluoropyrimidine based therapy both in control and study arm. Likewise irinotecan and warfarin distribution was quite homogeneous in both arms except for tamoxifen that showed a 10% increased prescription in the study arm. Focusing on PGx results about 4/5% of patients had not actionable genes, by contrast at least 95% of the patients showed at least 1 or more mutated gene for which a dosage/drug modification is relevant.

Conclusion: After the closure of the enrollment in June 2020, we are revising all toxicity data collected in the eCRF platform in order to perform the endpoint analysis. Since the majority of patients presents at least one actionable genotype supporting the importance of PGx, an effort is ongoing to implement preemptive PGx tests in the clinical practice. This innovative model of personalized medicine is valuable especially in oncological field in which narrow therapeutic window drugs are employed and the toxicities could be life-threatening.

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Medical and Biomedical Sciences

PhD COURSE "PHARMACOLOGICAL SCIENCES"

COORDINATOR: Prof. Nicola FERRI

CURRICULUM "PHARMACOLOGY, TOXICOLOGY AND THERAPY"

THERAPEUTIC DRUG MONITORING OF PALBOCICLIB, RIBOCICLIB AND LETROZOLE IN WOMEN WITH BREAST CANCER BY LC-MS

Ph.D. Student: Dr. Ariana SOLEDAD POETTO
Tutors: Drs./Profs. Morena ZUSSO and Giuseppe TOFFOLI
Ph.D. Course: Pharmacological Science
Curriculum: "Pharmacology, Toxicology and Therapy"

Background
The cyclin-dependent kinase inhibitors (CDKis) palbociclib and ribociclib have been recently introduced in the treatment of women with hormone receptor positive (HR+), human epidermal growth factor receptor 2 negative (HER2-) locally advanced or metastatic breast cancer (mBC), in combination with endocrine therapy (ET) (mostly letrozole). This association has notably improved therapeutic benefit but in some cases, based on individual safety and tolerability, dose reductions or discontinuation of therapy are necessary. The loss of this therapeutic chance could be avoided and efficacy may be improved by therapeutic drug monitoring (TDM). A limiting factor for TDM studies is the paucity of fast and reliable bioanalytical methods, suitable in a clinical context.
Materials and Methods
A bioanalytical method for the simultaneous quantification of palbociclib, ribociclib and letrozole has been developed and fully validated according to FDA/EMA guidelines. The method is based upon the use of LC-MS/MS technology and therefore highly sensitive and specific. Plasma samples were collected from patients with breast cancer, treated with palbociclib or ribociclib and eventually letrozole, according to a clinical study protocol.
Results
The validation process revealed high recovery and selectivity and a neglectable matrix effect for the three analytes. The assay is linear for the three analytes in the tested concentration ranges, which were 0.3-250 ng/mL for palbociclib, 10-10000 ng/mL for ribociclib and 0.5-500 ng/mL for letrozole, covering all the plausible in vivo plasma values. The carry over phenomenon was notably reduced by setting up a dedicated washing method. Applying the method for the analysis of incurred samples, it was confirmed to be reliable and reproducible.
Conclusions
The first LC-MS/MS method for the simultaneous quantification of palbociclib, ribociclib and letrozole in human plasma was developed and fully validated. Its wide analytical range enables the evaluation of all the pharmacokinetic parameters. Given the competitive runtime and the simple and fast sample preparation protocol, this method would allow to perform TDM in a short turn-around time.

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

COORDINATOR: Prof. Annalisa ANGELINI

CURRICULUM "*BIOSTATISTICS AND CLINIC EPIDEMIOLOGY*"

STATISTICAL METHODS FOR ESTIMATING PERSONALIZED RISK PROFILES BASED ON PRECISION MEDICINE TOOLS IN ONCOLOGICAL PATIENTS

Ph.D. Student: Dr. Fabiola GIUDICI - TUTOR: Prof.ssa Giulia BARBATI
Ph.D. Course: Traslational Specialistic Medicine 'G.B. Morgagni'
Curriculum "Biostatistics and Clinic Epidemiology"

Background:
In order to provide better treatment for patients and more effective targeted drugs, it is vital to understand disease mechanisms and to identify informative markers for patient susceptibility, disease progression and treatment response. The process for using statistical inference to establish personalized treatment strategies requires specific techniques for data-analysis that optimize the combination of competing therapies with candidate genetic features and characteristics of the patient and disease. During this PhD's second year I'm dedicated to analyse oncologic data with adequate statistical methods in order to identify prognostic and predictive biomarkers

Material and Methods: Statistical methods used

-LINEAR MIXED EFFECTS MODELS (LMM): LMMs are appropriate statistical tools to assess the prognostic value of serial biomarker evaluation (longitudinal studies). As independent variable fixed effects, time point and outcome status were entered into the model. As random effects, intercepts for subjects as well as per-subject random slopes for the effect on dependent variables were employed. (*R-package lme4-lmer*)

CUT-POINT FINDING METHODS FOR CONTINUOUS BIOMARKERS
Cut-point finding is a crucial step for clinical decision making when dealing with continuous biomarkers. A time-dependent ROC curve analysis was performed for determining the prognostic accuracy of biomarkers for predicting recurrence (R-package: *survivalROC, timeROC*)

LANDMARK ANALYSIS: this analysis was used to avoid a type of distortion called "immortal time bias". Landmarking is one of the most used approaches for the analysis of time-dependent covariates in time-to-event data. The estimated effect of the time-dependent covariate in a landmarking analysis is based on the value of the time-dependent covariate at the landmark time point, after which the time-dependent covariate may change value.

COMPETING RISK ANALYSIS: Cumulative incidence function model and multivariate Fine-Gray regression for proportional hazards modeling of the subdistribution hazard (SH) model were used to estimate the prognosis of patients in presence of competing risks. (*R-package cmrsk*)

Results: I report here some results about research projects involving biomarker analysis

-*BREAST CANCER BIOMARKERS*:

1) **ΔKi67** score evaluated between basal and residual tumor at definitive surgery showed to be highly predictive of clinical complete response, and a potential parameter to be used for predicting disease-free survival and overall survival in luminal breast cancer (BC) treated with neoadjuvant endocrine-based therapy;

2) we found a threshold of Δ**SUV** that is capable of precociously identifying hormone positive (HR+) HER2- metastatic breast cancer (mBC) patients who are much more likely to benefit from everolimus-exemestane therapy in the long-term period. These results could have an impact on treatment personalization in HR+ HER2- mBC patients;

3) the quantitation of cell-free DNA (**cfDNA)** long with the **LINE1** had a predictive role for BC diagnosis able to detect BC in its early phases

-*ANAL CANCER BIOMARKERS:* we demonstrated that an hyperspression of **PD-L1** by tumor cells and a **high presence of intraepithelial CD3**, sign of local immune response to neoplastic invasion, can be considered the 3- months response predictors and best survival prognostic factor.

Conclusions: As biostatistician, I translated medical questions into a precise data-based questions. I developed approaches to optimally collect and use data and I applied adequate statistical methods to perform prediction models of treatment success (personalised or stratified medicine) and future outcomes (prognosis).

INCIDENT study
hIddeN CovID-19 casEs Network estimation

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Ph.D. Course: Traslational Specialistic Medicine 'G.B. Morgagni'
Curriculum "Biostatistics and Clinic Epidemiology"

Background
Recent literature has reported a high percentage of asymptomatic or paucisymptomatic cases among subjects with COVID-19 infection. This proportion can be hard to count, therefore it constitutes a hidden population. This study aims at developing a proof-of-concept study for estimating the number of undocumented infections of COVID-19

Material and Methods
This is the protocol of the INCIDENT (hIddeN CovID-19 casEs Network estimation) study, an online cross-sectional survey with a snowball sampling based on the Network Scale Up method (NSUM). The original personal network size estimation method was based on a fixed effect maximum likelihood estimator. We propose an extension of a precedent Bayesian estimation methods to estimate the unknown network size via Markov Chain Monte Carlo algorithm.

Results
The study is still ongoing. On the 6th of May 2020, 1961 questionnaires had been collected, 1703 were completed except for the random questions, and 1652 were completed in all the three sections. The respondents were mainly female (1037, 61%) and prevalently residents the areas most affected by COVID-19, Veneto, Lombardia, and Piemonte. The algorithm has been initialized at the first iteration and will be applied to the whole dataset.

Conclusions
Knowing the number of asymptomatic COVID-19 cases is extremely important for reducing the spread of the virus. Our approach reduces the number of questions to pose. This allows to speed up the filling of the questionnaire, with a subsequent reduction of non-response rate

DEEP LEARNING FOR PREDICTING URGENT HOSPITALIZATIONS IN ELDERLY POPULATION USING ADMINISTRATIVE ELECTRONIC HEALTH RECORDS

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Ph.D. Course Translational Specialistic Medicine ¨G.B. Morgagni¨
Curriculum "Biostatistics and clinical epidemiology"

Background
In the elderly population, defined as people aged 65 and over, a rising prevalence of polypharmacy, the use of multiple medications by the same individual, has been observed. Polypharmacy is closely related to multi-morbidity and it has been associated with poor adherence and potentially preventable hospital admissions.
The aim of this study is to explore the relationship between polypharmacy and medical history using medication purchases and hospitalization diagnoses from elderly north-western Italian population using Deep Learning (DL) on administrative Electronic Health Records (EHRs) .

Material and Methods
Data was drawn from the Piedmont Longitudinal Study (PLS). The Italian 2011 census and the administrative EHRs were linked for about four million of individuals resident in Piedmont, a north-western Italian region. More in detail, we extracted from administrative databases the hospital admissions and the drug prescriptions, of subjects resident in Piedmont region at the 1st of January 2015 and aged 65 years or more. So, the cohort was composed by 1,159,141 individuals.
Three different scenarios were analyzed: including only medications prescriptions, including only hospitalization diagnoses and including both.
The Bidirectional Encoder Representations from Transformers (BERT) is a Natural Language Processing DL approach originally developed by Google. It was applied to learn the history of polypharmacy and hospital admissions using medication purchases and hospitalization diagnoses from administrative EHR in 2015-2018. Medications were represented with 4-digit ATC codes and diagnoses were collected from ICD-9-CM codes in hospital discharges records. To reduce dimensionality, diagnoses were grouped using the Single-level Clinical Classifications Software (CCS) for ICD-9-CM.
Masked language model (MLM) and next sentence prediction (NSP) were used to pre-train BERT in a self-supervised way, and results are reported in terms of accuracy.
To evaluate the goodness of embeddings, a t-SNE graphic evaluation was performed. In particular, the embedding results of the top 10 occurring elements and their nearest neighbours in terms of cosine similarity will be shown.
Finally, the attention mechanism was considered to enable prediction interpretation.
The DL model was tested on the prediction of urgent hospitalizations within 3 months.
For computational limits, we were not able to use the entire dataset but 100,000 samples were randomly allocated in the training set, 25,000 samples in the validation set, and 25,000 in the testing set.

Results
We choose an architecture of BERT composed by 6 layers, 12 attention heads, 512 intermediate layers and 288 hidden size layers.
When both hospitalizations diagnoses and medications prescriptions were considered, the highest accuracies in both MLM and NSP were reached (respectively 97% and 99%).
The t-SNE on embeddings shows clusters that are potentially reflecting co-occurring conditions and/or the belonging to the same clinical group.
In the prediction task, results show that the best scenario is considering medication prescriptions, hospitalization diagnoses, and demographics information, reaching a precision of 61%, Recall 89%, F1 score 73% and AUROC 97%. Medication prescriptions result as the most informative data.

Conclusions
Results suggest BERT is able to embed medication purchasing records and diagnoses, collected for administrative purposes, into patients' medical history for predicting future hospitalizations, providing a tool that could help to plan the allocation of future healthcare resources.

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

COORDINATOR: Prof. Annalisa ANGELINI

CURRICULUM "CARDIOTHORACIC AND VASCULAR SCIENCES"

"EXPERIMENTAL" ARTERIOSCLEROSIS
History of the first fifty years of experimental studies on the lipid pathogenesis of atherosclerosis

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Ph.D. Course: Traslational Specialistic Medicine "G.B. Morgagni"
Curriculum "Cardiothoracic and Vascular Sciences"

Background
Nowadays, atherosclerosis represents one of the most explored bio-medical fields of research in both basic and clinical sciences, outlining one of the most significant examples of translational medicine. At the end of the nineteenth century, the first attempts to reproduce atherosclerotic lesions through experiments on animals began in order to understand the mechanisms of atherosclerotic pathogenesis in humans. Our research aims to reconstruct the experimental process that led to the understanding of the role of lipids in the pathogenesis of atherosclerotic plaque.
Materials and Methods
The development of this historical-medical study has been conducted through the typical research methodology of the History of Medicine, involving the analysis of direct and indirect sources. The approach to the sources was "anatomo-pathological" and "physiopathological" in order to understand the evolution of the etiopathological descriptions of atherosclerotic plaque.
Results
Between the end of the nineteenth century and the beginning of the twentieth century, arteriosclerosis of the large arteries (*atheromatous arteriosclerosis*) and the arteriosclerosis of the small arteries (*fibrous arteriosclerosis*) were traced back to the generic term of *arteriosclerosis* (Banti, 1907). These two entities presented some fundamental morphological and etiological common characters on one side and specific peculiarities on the other one. At the end of the nineteenth century, numerous laboratory experiments were conducted to recreate these anatomopathological lesions in animal models. Among the first experiments we find the adrenaline injections in rabbits at the level of the aorta, which would have led to a calcification of the same one according to the anatomopathological confirmation carried out by the experimenters (Josué, 1903). In 1908, some physician, including Ignatowski, altered the usual herbivorous diet for experimental rabbits by adding animal albumin (derived from eggs, milk and red meat). Following this adulteration, they found "*alterations of parenchymatous organs*" and "*sclerosis of the aorta with necrosis of the media and calcification of the intima*". Afterwards, Steinbiss added the rabbit's diet with dried albumin. Later (1912), Anitschkow and Chalatow from one side and Wacker and Huek on the other, attributed these alterations to the "*cholesterin*" (cholesterol) present within the food. They therefore decided to feed experimental rabbits with pure cholesterol dissolved in sunflower oil: the autopsy finding showed arterial lesions such as those found previously. Due to these evidences the need to investigate the relationship between arteriosclerosis and *cholesterin* arose, especially in consideration of the fact that human arteriosclerotic lesions were rich in cholesterol. Thus, animal models with "*experimental cholesterol disease*" began to be recreated. The experiments also extended to omnivorous animals, with a diet similar to the human one. These experimental models represented an important confrontation for human pathology. Upon human, therefore, an attempt was made to examine the relationship between arteriosclerosis and the serum cholesterol level. Christianson's researches, still based on animals and on the relationship among atherosclerosis, fat and cholesterol, were published; then Hueper's ones followed (the early Forties of the twentieth century) on the injection of so-called "*macromolecular*" substances and the development of *foam cells*; the research on the "*dysmetabolic theory*" of arteriosclerosis by JR Moreton which attributed to chylomicrons a fundamental role in atherosclerotic pathogenesis came out. Finally, the fundamental researches by JW Gofman, F. Lindgren and Colleagues (1950) on the so-called "*lipid micelles*" and the "*lipoproteins*" -smaller than "*chylomicrons*"- which were associated with the appearance of atherosclerotic events in the arteries, were released. In fact, the role of lipids and lipoproteins in atherosclerosis was established.
Conclusions
The creation of these experimental models thus represented a powerful tool for understanding the human arteriosclerosis and it definitively paved the way for contemporary lipidology.

SHORT DUAL ANTIPLATELET THERAPY FOLLOWED BY ASPIRIN OR $P2Y_{12}$ INHIBITOR MONOTHERAPY VERSUS PROLONGED DUAL ANTIPLATELET THERAPY AFTER PERCUTANEOUS CORONARY INTERVENTION WITH SECOND-GENERATION DRUG-ELUTING STENTS: A SYSTEMATIC REVIEW AND META-ANALYSIS OF RANDOMIZED CLINICAL TRIALS

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Curriculum "Cardiothoracic and Vascular Sciences"

Aims
Whether very short (≤3 months) dual antiplatelet therapy (DAPT) followed by aspirin or monotherapy with a $P2Y_{12}$ receptor inhibitor confers benefits compared with standard, prolonged DAPT (12 months) is unclear.

Methods
Multiple electronic databases, including PubMed, Scopus, Web of Sciences, Ovid, and ScienceDirect, were searched to identify trials comparing ≤3 vs. 12 months of DAPT after PCI with second-generation DES implantation. The primary safety endpoint was major bleeding 12 months following randomization; the primary efficacy endpoint was stent thrombosis 12 months following randomization. Secondary endpoints included all-cause death, myocardial infarction, and stroke. Summary hazard ratios (HRs) and 95% confidence intervals (CIs) were estimated by fixed-effect and random-effects models. Differences between trials employing aspirin monotherapy following DAPT and trials employing $P2Y_{12}$ receptor inhibitor monotherapy following DAPT were defined and treatment-by-subgroup interaction assessed.

Results
A total of eight randomized clinical trials including 38877 patients were available for analysis. Major bleeding was significantly lower in patients assigned to short DAPT compared with those assigned to prolonged DAPT (HR 0.63, 95% 0.48-0.83, p<0.001). No significant differences between treatment groups were observed in terms of stent thrombosis (HR 1.21, 95% CI 0.91-1.62, p=0.196). The secondary endpoints of all-cause death (HR 0.85, 95% CI 0.70-1.03, p=0.091), myocardial infarction (HR 1.05, 95% CI 0.89-1.23, p=0.567), and stroke (HR 1.09, 95% CI 0.68-1.74, p=0.734) were not significantly different between treatment groups. There was not treatment-by-subgroup interaction according to the type of antithrombotic therapy used for each endpoint assessed.

Conclusion
After second-generation DES implantation, 1-to-3 months of DAPT followed by aspirin or $P2Y_{12}$ inhibitor monotherapy is associated with lower major bleeding and similar stent thrombosis, all-cause death, myocardial infarction, and stroke compared with prolonged DAPT.

A NON-TOXIC DECELLULARIZATION TREATMENT OF BIOCOMPATIBLE SCAFFOLDS WITH A SPECIAL FOCUS ON BIOLOGICAL AND BIOMECHANICAL EVALUATION OF SIS-ECM AIMING AT CARDIOVASCOLAR REPAIR

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Curriculum "Cardiothoracic and Vascular Sciences"

Background. In recent years, in the cardiovascular field, the production of biological matrices has become increasingly important, based on the decellularization of xenogeneic tissues. Small intestinal submucosa (SIS) physiologically presents two different portions: one smoother and one wrinkled. No companies clarify which portion performs better in terms of biological and biomechanical characteristics. For this reason, we investigated the fingerprints of both parts (smooth and wrinkled layer of the SIS) in order to define the specific biological and mechanical peculiarities of each layer. For this biological material is essential to treat the biological matrices through a decellularization process in order to isolate the ECM from the cellular component, keeping the structure and component of bioactive molecules intact, which support cell migration, proliferation and differentiation. The TRiCOL decellularization method was developed in the Cardiovascular Regenerative Medicine laboratory directed by Prof. Gino Gerosa in collaboration with Prof. Michele Spina. Very recently Triton X-100 has been included in the REACH list due to its degradation to a substance with endocrine disrupting properties. Starting from 2021 Triton X-100 will no longer be marketed hence an alternative detergent in the aforementioned decellularization method is needed. For this reason, Tergitol 15-S-9, a non-ionic detergent, with the same mechanism of action as Triton X-100 but not toxic, has been evaluated as an alternative.
Materials and Methods. We used pigs to extracted the small intestinal submucosa and native pericardia bovine (NBP) and porcine (NPP). TRiCOL decellularization was performed for SIS samples and two types of mechanical tests were performed on SIS-ECM and native SIS tissue in smooth and wrinkled part: uniaxial tensile tests at complete rupture and cyclic biaxial tests. For pericardia we used Tergitol 15-S-9 0.1–1% (*w/v*) and 10 mM sodium cholate. For all tissues (SIS and pericardia bovine and porcine) histology staining (He&Eo), quantification of DNA and immunofluorescence evaluation were performed in order to evaluate the effciency of decellularization. To analyze the ultrastructure differences in the different tissues, confocal microscopy analysis and multiphoton microscopy evaluation were also performed in both native and decellularized tissues.
Results. Biomechanical tests, uniaxial tensile tests showed that smooth SIS exhibited better mechanical performances, greater rigidity and resistance when compared to the wrinkled layer even after decellularization treatment, mainly along the longitudinal direction. The decellularization protocol based on Tricol, modified the mechanical behaviour of the tissue when compared to the native, both in the uniaxial and in the biaxial cyclic tests, by increasing the values of tension and stiffness of the smooth SIS decellularized along the longitudinal direction. Histological analysis on smooth SIS showed collagen fibers which appear more linear while appearing more curled in the wrinkled portion. In the wrinkled the presence of adipocytes and lymphovascular channels is greater in quantity and larger than in the smooth. In the wrinkled portion we detected a greater quantity of enterocyte nuclei, these data were also confirmed by the quantification of DNA. There were no qualitative differences in the label free collagen within the two parts: smooth and wrinkled, either for native and decellularized tissues. The treatment with Tergitol 15-S-9 decellularization did not modify the ECM structure and the main compound in both DBPs and DPPs. Histological analysis (He&Eo) and immunofluorescence demostrated the absence of nuclei and good preservation of extracellular matrix. These results were also confirmed by the DNA content below the threshold line (< 50 ng / mg) as suggested by Crapo et *al.*
Conclusions. According to our preliminary results, the mechanical behaviour of the two portions of the SIS exhibit considerable differences with the smooth SIS showing greater mechanical resistance. Decellularized smooth SIS exhibited significantly different performances when compared to native SIS unlike the wrinkled SIS. Further investigations, such as quantification of biochemical components and structural morphology of the tissue by scanning electron microscope (SEM), are required prior to application in the clinical arena. The treatment with Tergitol 15-S-9 decellularization did not modify the ECM structure and the main compound of extracellular matrix in both DBPs and DPPs. Histological analysis with Hematoxylin and Eosin staining demostrated the absence of nuclei and good preservation of extracellular matrix. These results were also confirmed by the DNA content below the threshold line (< 50 ng / mg) as suggested by Crapo et *al.*

ROLE OF CARDIAC SYMPATHETIC NEURONS IN DRIVING STRUCTURAL AND FUNCTIONAL REMODELING IN ARRHYTHMOGENIC CARDIOMYOPATHY (AC) HEARTS

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Ph.D. Course: Traslational Specialistic Medicine "G.B. Morgagni"
Curriculum "Cardiothoracic and Vascular Sciences"

Background. Arrhythmogenic Cardiomyopathy (AC) is a genetic cardiac disorder, predominantly caused by mutations in desmosomal genes, and represents one of the main causes of arrhythmic sudden death (SD) in the young and athletes. Pathologically, AC is hallmarked by fibro-fatty myocardial replacement, which generates the substrate for life-threating arrhythmias, typically triggered by physical/emotional stresses, associated with the activation of the sympathetic nervous system. At the time being, *AC is still an incurable disease, as several aspects of its pathogenesis remain obscure*. Current AC research has primarily focused on desmosomal gene mutations solely in cardiomyocytes, but desmosomal genes are ubiquitously expressed in all cells. Here, we are testing the **hypothesis** that ***AC is "a disease of the myocardial cellular network,"*** which is coordinated by cardiac sympathetic neurons (cSNs), through noradrenaline (NA) and the poorly studied Neuropeptide Y (NPY).

Materials and Methods. To test our hypothesis, we used a mouse model with ubiquitous expression of mutant murine Desmoglein-2 (*Dsg2*) (*Dsg2*$^{mut/mut}$, *Chelko et al. 2016 JCI Insight*), and a novel knock-in mouse, recently generated in our laboratory, carrying a mutation in murine Desmoplakin (*Dsp*^{S331A}, *Di Bona et al. unpublished).* Heart function was characterized by echocardiographic and electrocardiographic analyses. Confocal immunofluorescence (IF), multiphoton-imaging of tissue clarified heart blocks, and molecular analyses were used to assess cardiac structure and neural innervation. Additionally, FDA approved inhibitors and drugs were tested *in vivo for* their efficacy on the prevention of structural and functional remodeling. Data from AC murine models have been combined with those obtained from the analyses of autoptic human heart samples (in collaboration with Prof. C. Basso).

Results. We demonstrated that the morphology and topology of cardiac sympathetic innervation is significantly altered in both AC mouse models analyzed, albeit specific mutation-linked features. Such cSN alterations start to appear before myocardial remodeling occurs, thus supporting a direct effect of AC on cardiac sympathetic innervation. Interestingly, cSN alterations were additionally observed in heart samples from AC patients. Based on these results, we assessed the role of cSNs in disease pathogenesis and performed pharmacologic cardiac-denervation in neonatal *Dsg2*$^{mut/mut}$ mice *via* 6-OH-DA injection. Surprisingly, cSN ablation prevented myocardial structural and functional remodeling, prompting the use of β-blockers and FDA-approved NPY receptor (NPY-R) antagonists (i.e. BIBO3304 and MK-0557). In a parallel project, performed during my fellowship as a visiting research student at the Johns Hopkins University School of Medicine (Baltimore, USA), we evaluated the effect of psychosocial stress (*via* the resident-intruder paradigm) in *Dsg2*$^{mut/mut}$ mice. Notably, AC mice showed abnormally high in-trial mortality, compared to controls. Of the survivors, *Dsg2*$^{mut/mut}$ mice exhibited markedly aggravated cardiac dysfunction and remodeling, and increased incidence of arrhythmias. Despite similar baseline values, *Dsg2*$^{mut/mut}$ mice displayed more anxiety-like behavior, than controls following the psychosocial stress paradigm.

Conclusions: Taken altogether, our results indicate that: i) cSNs are additional cell types affected in AC (*Scalco et al., in preparation*); ii) cSNs have a direct role in AC pathogenesis, not only in triggering arrhythmias, but also structural cardiac alterations (*Scalco et al., in preparation*); and iii) psychosocial stress, in which SNs have a key role, has direct structural and functional cardiac repercussions (*Agrimi, Scalco et al., Scientific Report, under revision*). Additional experiments are in progress to assess whether pharmacological interference with SN signaling may be called upon as a promising therapeutic strategy to counteract disease progression and reduce the incidence of SD in the AC patient population.

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

COORDINATOR: Prof. Annalisa ANGELINI

CURRICULUM "CLINICAL AND TRASLATIONAL NEUROSCIENCES"

NANO ENGINEERING OF HUMAN EAR

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Curriculum "Clinical and Traslational Neurosciences"

Background

Medical research is currently focused on the use of nanoparticles, heterogeneous compounds with many characteristics and sizes in the order of nano-scale, to repair and stimulate tissues, to deliver drugs, to treat cancer etc.. Among the active research fields, there is bioengineering by which nano-compounds are challenge to create implantable devices in the human ear, more performing than a classic cochlear implant and with a lower impact on patient's quality of life.

Material and Methods

In this work we tested the *in vitro* biocompatibility of two piezoelectric nanoparticles, barium titanate and lithium niobate, possible candidates for the creation of new cochlear implant, on two different cell lines: OC-k3 and PC12. The OC-k3 are sensorial cells deriving from the Organ of Corti, while the PC12 are neuronal cells. The toxicity of nano-compounds was evaluated by cell viability, morphological changes, Cytochrome c distribution, ROS production and complexity of the neuritic network for the neuronal cell line.

Results

The results showed that the treatment with both nanoparticles did not affect the viability of OC-k3 and PC12 cells, as well as the morphology, the Cytochrome c distribution and the ROS production at all times and tested doses. The evaluation of the neuritc network didn't show any changes in PC12 cells treated with the nanoparticles.

Conclusions

In conclusion, barium titanate and lithium niobate nanoparticles seem to be biocompatible for the inner ear and neuronal cell lines, and could be therefore excellent candidates for the creation of devices that can replace the functions of damaged hair cells.

NEUROFILAMENT LIGHT CHAIN AS A POSSIBLE BIOMARKER IN ADULT SMA TYPE 2 AND 3 PATIENTS UNDERGOING NUSINERSEN TREATMENT

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Ph.D. Course: Traslational Specialistic Medicine 'G.B. Morgagni'
Curriculum "Clinical and Traslational Neurosciences"

Background: Spinal Muscular Atrophy (SMA) is an autosomal recessive disorder due to progressive degeneration of lower motor neurons resulting in muscle atrophy, proximal limbs and axial muscle weakness and possible respiratory and bulbar involvement. Pathogenic hallmark is the loss of function of Survival Motor Neuron Protein (SMN), encoded by mutated or deleted SMN1 gene. SMN2, the centromeric paralogous gene of SMN1, produces only a small amount of functional SMN (Levin, 2019). Nusinersen, a SMN2 splicing modifier able to increase the production of functional SMN protein, is the only disease modifying therapy approved for SMA patients. Neurofilaments light chain (NfL) are intermediate filaments exclusively expressed in neurons. As NfL levels raise following axonal damage, they might be promising diagnostic and prognostic biomarkers in motor neuron diseases (Gaetani, et al., 2019). Limited evidence concerning adult SMA patients is currently available in literature, this study aims to investigate the role of NfL as disease and treatment-response biomarker in a cohort of adult SMA type 2 and 3 patients.

Material and Methods: 33 SMA type 2 and 3 patients were recruited at the Neuromuscular Center of Padua Hospital, where nusinersen treatment was administered from February 2018 to September 2019 in a loading phase (L1 baseline, L2 day 14, L3 day 28, and L4 day 63) and a maintenance phase (M1, M2 and M3, every four months). Cerebrospinal fluid (CSF) samples were collected at each administration; NfL was tested at each time point, additional neurodegeneration biomarkers total tau (t-Tau) and phosphorylated tau (p-Tau) proteins were tested at time points L1 and L3. Human Profilin-1 (PFN-1) was tested at each time point as an exploratory muscular biomarker. NfL concentration was determined with a commercial enzyme-linked immunosorbent assay (ELISA) kit (UmanDiagnostics, Umea, Sweden). T-Tau and pTau were measured with an automated Chemiluminescent Enzyme Immunoassay (CLEIA) analyzer (LUMIPULSE G600 II by Fujirebio, Japan); PFN-1 was measured with a commercial ELISA kit (Cusabio, China). Neuromuscular outcomes were tested at L1, L4, M1, M2 and M3 with appropriated validated motor scales (HFMSE, RULM, 6MWT, MRC and NSAA).

Results: Baseline CSF NfL, t-Tau and p-Tau levels were overall included in the reference ranges for healthy donors provided by testing assay manufacturers. Mean NfL was 211.97 ± 180.9 pg/ml in SMA patients, compared to 809.53 ± 1,065.26 pg/ml in controls. Correlation was found between baseline log[NfL] and age both in SMA patients ($r=0.69$, $p<0.0001$) and control group ($r=0.75$, $p<0.0001$). Also log[t-Tau] and log[p-Tau] correlated with log[NfL] ($r=0.48$, $p=0.005$ and $r=0.53$, $p=0.002$) at L1, but not at L3, although a slight significant increase was found in t-Tau ($p=0.022$) and p-Tau ($p=0.004$) at L3. NfL significantly increased in loading phase until L3 (mean increase 285.45 pg/ml). From L4 NfL started to decrease and no significant difference was found with baseline at M1, M2 and M3. PFN-1 at baseline was higher in SMA than in healthy controls (mean 989 vs 608 pg/ml, $p=0.0018$). PFN-1 showed a complex dynamic during loading phase, with a significant reduction at L4. No correlation was found between NfL and motor scores at each time point.

Conclusions: Our study partially reinforces recently published results in similar patients (Wurster, et al., 2019) (Wurster, et al., 2020) (Faravelli, et al., 2020), adding insights on NfL dynamic during the first month of treatment. Neurodegenerative biomarkers might inadequately relate to long disease duration. Results of shorter time points in our cohort might suggest a putative limited axonal remodelling, although a transient side effect of lumbar puncture might not be excluded.

WHOLE GENOME SEQUENCING IN A PAIR OF SIBLINGS AFFECTED WITH DUCHENNE MUSCULAR DYSTROPHY WITH DISCORDANT COGNITIVE PHENOTYPE

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Ph.D. Course: Traslational Specialistic Medicine "G.B. Morgagni"
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Background: Central nervous system (CNS) involvement in dystrophinopathies is variable, although loosely associated with mutations in the C-terminal domains of dystrophin. We aimed to identify candidate variants involved in the modulation of CNS phenotype in dystrophinopathies, by performing whole genome sequencing (WGS) in a pair of Duchenne muscular dystrophy (DMD) siblings with discordant cognitive phenotype and filtering variants with a dedicated bioinformatic algorithm.

Materials and Methods: Patient 1 was born of healthy, non-consanguineous parents. He was diagnosed with DMD at the age of 4 years (deletion *DMD* exons 45-52), and lost ambulation at the age of 11 (currently 29 y.o.). He had no speech delay nor intellectual disabilities. Patient 2, his younger brother, was diagnosed with DMD due to the same mutation, and lost ambulation at the age of 12 (currently 14 y.o.). However, he presented severe speech delay and an autism-spectrum disorder (ASD) with behavioral issues requiring neuroleptics. We performed WGS using DNA extracted from peripheral blood of Patient 1 and 2 and their parents. Structural Variants were analyzed with Breakdancer algorithm[1], while indel and SNPs (single nucleotide polymorphisms) were filtered exploiting our own Python scripts in according to the following criteria: not shared between the two siblings; frequency < 5% in European population and considering function of genes involved.

Results: WGS confirmed identical *DMD* deletion breakpoints in both brothers and their mother. The bioinformatic algorithm filtered a "short list" of 41 variants situated in 36 loci, reviewed individually for potential pathogenetic mechanisms and previous reports of involvement in neurodevelopmental phenotypes. The most notable variants were rs112339619, a G>A single SNP upstream of the ASD risk gene *ANK3*, previously reported in a WGS study of ASD twins[2]; and rs117696080, an intronic variant in *NRXN3*, encoding neurexin 3, a dystrophin-associated glycoprotein (DAPG), and also identified in the same ASD twin study. Patient 2 was heterozygous for both these variants, while patient 1 had a wild-type genotype at both loci. Both of these variants are in regions of strong conservation: vertebrate PhastCons of 0.96 e 1.00 respectively. BreakDancer shows an interesting homozygous deletion (2166 bp) in an intronic region of ASD risk gene NALCN. Moreover, a co-expression with DMD gene has been shown in literature.

Conclusions: Identified variants represent putative modifiers of the cognitive phenotype of DMD. We plan to validate these findings by sequencing a larger cohort of dystrophinopathy patients with and without cognitive issues.

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Medical and Biomedical Sciences

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

COORDINATOR: Prof. Annalisa ANGELINI

CURRICULUM "ENDOCRINE AND METABOLIC SCIENCES"

THE EFFECTS OF IODINE SUPPLEMENTATION IN PREGNANCY ON IODINE STATUS, THYROGLOBULIN LEVELS AND THYROID FUNCTION PARAMETERS: RESULTS FROM A RANDOMIZED CONTROLLED CLINICAL TRIAL IN A MILD-TO-MODERATE IODINE DEFICIENCY AREA

Ph.D. Student: Dr. Simona CENSI - TUTOR: Prof. Angelo AVOGARO
Ph.D. Course: Traslational Specialistic Medicine "G.B. Morgagni"
Curriculum "Endocrine and Metabolic Sciences"

Background: Iodine supplementation during pregnancy in areas with mild-to-moderate iodine deficiency is still debated. The detrimental impact of severe iodine deficiency during pregnancy is well known, since adequate thyroid hormone levels are fundamental to proper neurological development in the fetus. The effect of mild-to-moderate iodine deficiency in pregnancy (urinary iodine concentrations (UIC) between 50 and 150 ug/L) is less clear, however, and the benefits of supplementation in this case are less obvious. World Health Organization recommends iodine supplementation during pregnancy in areas with a median Urinary Iodine Concentration (UIC) <100 μg/L and use of iodized salt between 20 and 90% of households, like Italy and in particular Veneto region. However, according to recent Italian Consensus, gynecologists are only advised to suggest the use of iodized salt and foods rich in iodine and not to give a supplementation**.**
Material and Methods: A single-center, randomized, single-blind and placebo-controlled (3:2) trial was conducted. We enrolled 90 women before 12 weeks of gestation. From enrollment up until 8 weeks after delivery, 52 women were given an iodine supplement (225 μg/day, potassium iodide tablets) and 38 were given placebo. All women received an educational intervention on the use of iodized salt and dietary compounds rich in iodine. At recruitment (T0), in the second (T1) and third trimesters (T2), and 8 weeks after delivery (T3), we measured participants' urinary iodine-to-creatinine ratio (UI/Creat), thyroid function parameters (thyroglobulin (Tg), TSH, FT3, and FT4), and thyroid volume (TV). The newborns' TSH levels were obtained in all cases from the congenital hypothyroidism screening program. The newborns' urinary iodine concentrations were evaluated in 16 cases.
Results: Median UI/Creat at recruitment was 53.3 μg/g (IC 95% 43.72–67.20). Significant differences in UI/Creat emerged between the Iodine and Placebo groups at T1 (with median UI/Creat 183.23 and 65.54 μg/g, respectively; $p < 0.0001$), and at T2 (median UI/Creat 171.16 μg/g and 84.19 ug/g, respectively; $p < 0.0001$), but not at T3, though it was still higher in the Iodine group (median UI/Creat 104.88 μg/g and 58.24 μg/g, respectively; $p = 0.10$). TSH, FT4 and FT3 did not differ significantly between the Iodine and Placebo groups. Tg levels were lower in Iodine group at T2 (6.07 ng/mL in the Iodine group, and 9.80 ng/mL in the Placebo group) ($p=0.02$). On the whole, UI/Creat and Tg were negatively correlated throughout the study ($p=0.03$, $r = -0.12$). TV increased by +D7.43% in the Iodine group, and by +D11.17% in the Placebo group. No differences were found between the newborns' TSH levels on screening the two groups.
Conclusions: Despite running education programs about iodine, in the Veneto region pregnant women are still iodine-deficient. Iodine supplementation in our study proved effective to reach an iodine adequacy during pregnancy in areas of mild-to-moderate iodine deficiency. Educational intervention on the use of iodized salt and dietary compounds rich in iodine prompted a slight but statistically insignificant median increase in UI/Creat in our Placebo group, so it is not enough for iodine adequacy. Tg proved a good parameter for measuring iodine intake in our placebo-controlled series. Iodine supplementation did not prove harmful to pregnancy in areas of mild-to-moderate iodine deficiency, with no appreciable harmful effect on thyroid function.

IMPROVING TREATMENT OF TYPE 2 DIABETES: FROM PHARMACOGENETICS TO REAL-WORLD STUDIES, WHILE FACING THE COVID-19 PANDEMIC

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Ph.D. Course: Traslational Specialistic Medicine "G.B. Morgagni"
Curriculum "Endocrine and Metabolic Sciences"

Background: The main aim of my research activities is to identify new strategies to improve cardiovascular prevention and treatment of patients with type 2 diabetes. To pursue these goals, I am following two complimentary approaches. First, through pharmacogenetics studies identifying those patients with better response to CVD preventive treatments (e.g. fenofibrate). Second, through real-world studies improving the translation of randomized clinical trial into clinical practice, for those treatments with proven cardiovascular benefit. Moreover, since the beginning of COVID-19 pandemic, I have conducted study to dissect the relationship between diabetes and COVID-19 infection and disease progression.

Altogether, over the last 12 month I have published 13 papers, 9 of which as first (or co-first) Author. I have been invited as speaker to two European meetings (European Atherosclerosis Society – EAS 2020 Meeting, and European Association for the Study of Diabetes – EASD 2020 Meeting), and to two national meetings (Italian Society of Diabetes -SID-, and SID-SISA, Italian Society of Atherosclerosis). I have been the leading organizer in the scientific committee (for the third consecutive year) of the 2020 National Meeting of Young Researchers of SISA, SIIA and SIMI - this year host in an virtual edition). I have won in collaboration with the group of Prof. Doria (Joslin Diabetes Center, Harvard Medical School, USA) a National Institute of Health – NIH R21grant – to further studying the genetic variant that we have recently identified and that allow to distinguish subject with better cardiovascular prevention when treated with fenofibrate (role Collaborator with instrumental role in study design). These results have also allowed me to apply for a Ricerca Finalizzata Giovani Ricercatori grant (awaiting results) to conduct more study in University-Hospital of Padova and carrying on National and International Collaborations.

Among this year activities, given the primary role of COVID-19 pandemic in shaping this year activities for all of us, I'll detail here the results of one of the project on COVID-19: "*Newly-diagnosed diabetes and admission hyperglycemia predict COVID-19 severity by aggravating respiratory deterioration*" (accepted in Diabetes Research and Clinical Practice).

Aim: We investigated whether pre-existing diabetes, newly-diagnosed diabetes, and admission hyperglycemia were associated with COVID-19 severity independently from confounders

Material and Methods: We retrospectively analyzed data on patients with COVID-19 hospitalized between February and April 2020 in an outbreak hospital in North-East Italy. Pre-existing diabetes was defined by self-reported history, electronic medical records, or ongoing medications. Newly-diagnosed diabetes was defined by HbA1c and fasting glucose. The primary outcome was a composite of ICU admission or death.

Results. 413 subjects were included, 107 of whom (25.6%) had diabetes, including 21 newly-diagnosed. Patients with diabetes were older and had greater comorbidity burden. The primary outcome occurred in 37.4% of patients with diabetes compared to 20.3% in those without (RR 1.85; 95%C.I. 1.33-2.57; $p<0.001$). The association was stronger for newly-diagnosed compared to pre-existing diabetes (RR 3.06 vs 1.55; $p=0.004$). Higher glucose level at admission was associated with COVID-19 severity, with a stronger association among patients without as compared to those with pre-existing diabetes (interaction $p<0.001$). Admission glucose was correlated with most severity clinical indexes and its association with adverse outcome was mostly mediated by a worse respiratory function.

Conclusion. Newly-diagnosed diabetes and admission hyperglycemia are powerful predictors of COVID-19 severity due to rapid respiratory deterioration.

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

COORDINATOR: Prof. Annalisa ANGELINI

CURRICULUM "THORACIC AND PULMONARY SCIENCES"

EX VIVO PERFUSION TECHNIQUES IN LUNG TRANSPLANTATION

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Ph.D. Course: Translatıonal Specialistic Medicine "G.B. Morgagni"
Curriculum "Thoracic and Pulmonary Sciences"

Background

Ex-vivo lung perfusion (EVLP) in the clinical setting

- Alternative to static cold storage for organ preservation
- Reduction of lung injury caused by cold ischemic preservation
- Assess extended criteria donor lungs or non-heart beating donors
- Recover or "resuscitate" unsuitable lungs
  -Oedema
  -Thrombi
  -Infection

Animal models of EVLP

Valuable tool for translational research in transplantation, they allow:

- Evaluation of organ function
- Modeling of relevant pathologic conditions (acute lung injury, ALI)
- Trans-tracheal agent delivery
- Gene or molecule delivery

Which animal model?

- ✓ Small animal models (rats) are reliable enough and less expensive than large animal models (pig).

x Setting up an EVLP circuit in the rat model is technically challenging, since it requires downscaling of all components of the circuit that was developed for humans

Material and Methods

1 Experimental Surgery

Procurement of heart-lung blocks

- Requires rat's anaesthesia and intubation
- Surgical procedure performed with operating microscope

2 Assembly of the EVLP platform

Jacketed reservoir: 2 liters capacity; External chamber for thermostatic circuit
Rodent Ventilator: Tidal volumes between 50µl-5ml; Adjustable PEEP; Volume or pressure-controlled
Roller pump: 4 channels; Adjustable flow between 0,003-35 ml/min
Measuring system: Connects with transducers; Allows automatic constant-pressure perfusion
Organ chamber: Set temperature and pressure; Cannulae for left atrium and pulmonary artery
Data acquisition: Integrated software; Real time monitoring of main physiologic parameters

Future perspectives

- Setting up of a stable ex-vivo lung perfusion protocol on the rat model
- Comparisons of the effect of different preservation protocols on ischemia-reperfusion injury after lung transplantation
- Use of EVLP as a platform for administration of experimental treatments (gene therapy)

THE EFFECTS OF IODINE SUPPLEMENTATION IN PREGNANCY ON IODINE STATUS, THYROGLOBULIN LEVELS AND THYROID FUNCTION PARAMETERS: RESULTS FROM A RANDOMIZED CONTROLLED CLINICAL TRIAL IN A MILD-TO-MODERATE IODINE DEFICIENCY AREA

Ph.D. Student: Dr. Mariaenrica TINE'- TUTOR: Prof. Marina SAETTA
Ph.D. Course: Translatıonal Specialistic Medicine "G.B. Morgagni"
Curriculum "Thoracic and Pulmonary Sciences"

Background Cell-to-cell communication is an essential component for mammalian development, preservation of homeostasis and efficient responses to threats within the surrounding environment. Beyond signalling through cell-cell contact, microvesicles (MVs) have recently emerged as important information shuttles that can disseminate homeostatic and disease signals. Few studies have described MVs in blood in chronic obstructive pulmonary disease (COPD), a condition due to smoking in which innate and adaptive immune inflammation play an important mechanistic function. Indeed, epithelial and endothelial cell-derived-MVs were found to be increased in the blood of COPD patients, suggesting a promising role for MVs as biomarkers for the diagnosis and prognosis of the disease. However, no study has so far investigated MVs in the bronchoalveolar lavage (BAL) of COPD subjects. The potential presence of MVs in tissue fluids, like BAL from the lung, offers the possibility of focusing specifically on the presence, origin and roles of these vesicles in human lung pathology.
The purpose of this study was to investigate the presence and source of MVs in BAL of smokers with and without COPD compared to non-smoking controls.
Material and Methods BAL samples were obtained in 9 non-smokers, 16 smokers without COPD, and 19 smokers with COPD, undergoing clinically indicated bronchoscopy. For the identification and analysis of the MVs, BAL was filtered and centrifuged and 20 µl of supernatant were incubated with monoclonal antibody against cell-type specific antigens. A new generation of flow cytometer, that can detect MVs to a size as low as 150 nm, was used to identify and separate the MVs population according to the dimension (ranging from 150 to 900 nm) and to the cell-specific antibody.
Results Using flow-cytometry in BAL, we identified the presence of endothelial and alveolar macrophage-derived MVs, and found that the number of alveolar macrophage-derived MVs was higher in smokers with COPD compared to smokers without COPD [440 (49-952) vs 91 (56-567); $p < 0.05$], and non-smokers [440 (49-952) vs 43 (29-583); $p < 0.05$]. Moreover, the number of alveolar macrophage-derived MVs correlated with the amount smoked ($r = 0.46$; $p = 0.05$) and with the degree of airway obstruction measured by the FEV1 % predicted ($r = -0.56$; $p = 0.01$).
Conclusions Endothelial and alveolar macrophages-derived MVs are present and measurable in human BAL fluid. In response to smoking and to the development of COPD, the number of alveolar macrophages-derived MVs increases and is related to the amount smoked and to the decrease in lung function. These results open the opportunity for future investigation of these microvesicles as biomarkers and possible mechanistic guides in COPD.
Next project Parallel to the research on COPD, I will study "The mechanisms of inhaled corticosteroid resistance in severe Th2 asthma" in Oxford, joining the Nuffield Department of Medicine. Cell culture, RNA sequencing and flow cytometry will be applied on sputum and BAL samples obtained from severe asthmatics and controls to sort out the lymphocyte subsets involved in the development of steroid resistance in asthmatics with Th2 high airway inflammation.

AUTHORS' INDEX