UNIVERSITÀ DEGLI STUDI DI PADOVA

University of Padua
PhD Courses
Medical and Biomedical Sciences

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

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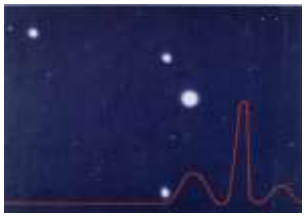

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COORDINATOR: Prof. Paolo BERNARDI

CRYOEM ATOMIC STRUCUTRE OF POTATO VIRUS X

Ph.D. Student: Dr. Alessandro GRINZATO - TUTOR: Prof. Giuseppe ZANOTTI
Ph.D. Course: Biomedical Science

Background

Potato virus X (PVX) is a filamentous plant virus belonging to the *Alphaflexviridae* family and the type-member of the genus *Potexvirus*. It infects several herbaceous plants and has been largely studied for its detrimental impact on the global potato economy. Moreover, the PVX capsid protein can be engendered to produce chimeric virus particles (CVPs) displaying heterologous peptides/chemicals of interest. By this approach, the virus particles can be used as platforms for nanotechnology/nanomedicine applications.

Material and Methods

The purified sample was applied to glow-discharged holey carbon grid and vitrified. Grids were imaged in a Titan Krios microscope. After beam-induced motion correction and Contrast Transfer Function estimation, the selected micrographs undergo for further particle picking and 2D classification, a simulated helical lattice was used as initial model for the 3D refinement.
The final resolution was set to 2.2Å according to the gold standard FSC. The initial atomic model for the PVX CP was generated by homology while the ssRNA chain was modelled as a polyU chain. The model was improved by iterative cycles of real space refinement and manual model rebuilding.

Results

We obtained a 3D structure of the PVX particle with a resolution of 2.2 Å. At the moment of the writing, this represents the highest resolution ever obtained for a flexible filamentous virus by cryoEM, that allow us to build a detailed model of the CP and a segment of the genomic RNA.
PVX is composed by single-stranded positive-sense RNA molecule coated by approximately 1300 copies of the same coat protein (CP). The repeating unit of PVX is formed by 8.8 CP protomers, arranged to form a left-handed helical structure. The CP is composed of three domains: a short N-terminal domain or domain I, a central core domain, or domain II, and a C-terminal domain, or domain III. Domains I and II are exposed on the external surface of the virus particle, whilst domain III and in part domain II build up the wall of a continuous internal tunnel. Domains II and III also generates a continuous crevice where the RNA is hosted and protected. Each CP subunit interacts with two nearby subunits and with subunits present in the lower and upper ring of the helical structure. The connection between subunits is further strengthened by the domain I.

Conclusions

The structure confirms that the flexible filamentous plant viruses present a universally conserved RNA binding pocket at the molecular level: the bound RNA interacts with protein residues mainly via its sugar/phosphate backbone, without specificity for a given RNA sequence. This potentially opens the way to the design of antiviral compounds that could bind to the CPs of the plant viruses belonging to the *Flexviridae* and *Potyviridae* family. In addition, a chimeric virion, formed by the sequence of PVX CPs and a synthetic RNA, could be used as a vector to inject a designed RNA sequence inside plant cells. Plant viruses have been also considered as nanoparticles carriers of other molecules, acting as drugs or vaccines. The atomic structure of the virus particle shows that the N-terminal region is flexible and exposed to the solvent, and so it is well suited for the transport and delivery of epitopes. The detailed knowledge of the atomic structure of the virion and of the CP offers the opportunity to identify more sites for chemical/genetic modification of the virus nanoparticle.

NEURON-GLIA INTERACTIONS IN NEUROVASCULAR COUPLING

Ph.D. Student: Dr. Mislav MAJNARIĆ – TUTOR: Dr. Giorgio CARMIGNOTO
Ph.D. Course: Biomedical Science

A large diversity of interneurons in terms of morphology, connectivity, molecular and functional properties warrants a signalling specificity to surrounding neurons that is crucial to generate the functional heterogeneity that characterizes brain circuits. Recently, we provided *in vivo* evidence of an interneuron type-specific signalling to astrocytes in the mouse somatosensory cortex (Mariotti et al. 2018). Astrocytes may, therefore, represent functional non-neuronal components of inhibitory circuits. The main goal of my project is to understand whether GABA-activated astrocytes are directly involved in neurovascular coupling mechanisms. Cerebral blood flow is controlled by the neuro-vascular unit which comprises different cells including astrocytes. The astrocytic fine processes and the end-feet are strategically positioned to contact neuronal synapses and cerebral blood vessels, respectively, and their Ca^{2+} changes in response to glutamatergic signalling have been reported to contribute to local cerebral blood flow (CBF) changes evoked by high neuronal activity (Zonta et al. 2003). We here advance the hypothesis that Parvalbumin and/or Somatostatin expressing (PV and SOM,respectively) interneurons - that are rapidly activated by sensory stimulation - signal directly to astrocytic end-feet where they activate GABA receptor-mediated Ca^{2+} elevations and, in turn, the release of vasoactive molecules. To address this hypothesis, in anaesthetized mice we apply a combination of optogenetics, electrophysiology and single/2-photon laser scanning microscope Ca^{2+} imaging in neocortical neurons and astrocytes expressing genetically encoded Ca^{2+} indicators. We address the following fundamental questions: i) do astrocytic end-feet respond with GABA-mediated Ca^{2+} elevations to optogenetic activation of PV or SOM interneurons? ii) do these Ca^{2+} elevations occur with a short delay and are they followed by a blood vessel response? iii) does a sensory stimulation that activates PV or SOM interneurons also induce GABA-mediated Ca^{2+} responses in astrocytes? Can these responses contribute to local CBF changes? Preliminary results suggest that optogenetic interneuron activation induces an overall increase in astrocytic Ca^{2+} signals at soma and small processes around capillaries and arterioles (4-11 um diameter), whereas no responses of endfeet in contact with larger arterioles (15-25 um diameter) were detected. These data needs to be validated in a larger number of experiments. In the forthcoming experiments, we will also use the Lck-GCaMP6, a membrane tethered Ca^{2+} indicator which may reveal subtle Ca^{2+} responses at the end-feet. After confirming that astrocytes are activated by either PV or SOM interneuron signaling, the CBF will be assessed by measuring the change in arteriole diameter and velocity of red blood cells. Future experiments will also evaluate CBF changes and astrocyte activity at the end-feet upon sensory stimuli that specifically activate neocortical interneurons. Inhibition of interneurons by halorhodopsin and the use of astrocytic $GABA_B$ receptor KO mice will then allow us to further assess the precise role of interneuron-to-astrocyte signaling in these phenomena.

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PhD COURSE "CLINICAL AND EXPERIMENTAL ONCOLOGY AND IMMUNOLOGY"

COORDINATOR: Prof. Paola ZANOVELLO

LACTATE POTENTIATES DIFFERENTIATION AND EXPANSION OF CYTOTOXIC T CELLS

Ph.D. Student: Dr. Laura BARBIERI - TUTORS: Proff. Antonio ROSATO, Randall S. JOHNSON
Ph.D. Course: Clinical and Experimental Oncology and Immunology

Background
Exercise has a range of effects on metabolism. In animal models, repeated exertion reduces malignant tumour progression, and clinically, exercise can improve outcome for cancer patients. The etiology of the effect of exercise on tumour progression is unclear, as are the cellular actors involved. Recent work has indicated a relationship between immune response and exercise-induced changes in cancer progression. In this study we investigate the association between exercise-derived metabolites and CD8+ T cell function.

Material and Methods
To assess exercise´s effect on tumor progression, mice were housed with access either to wirelessly recording running wheels or locked control wheels and tumour growth was monitored. Standard IHC as well as Mantra™ were used to identify infiltration of relevant immune populations. To assess a role of CD8+ T cells in mediating exercise anti-tumoral effects, antibodies against CD8 or isotype control were administered *in vivo*. To evaluate the effect of lactate on tumor growth, wild type mice were subject to daily intraperitoneal injections of Sodium L-Lactate prior to and during tumor growth. Murine CD8+ T cells were purified from spleens by positive magnetic bead selection. Purified CD8+ T-cells were activated with anti CD3/CD28 beads in the presence of Sodium L-Lactate or control compounds. For human CD8+ T cells, peripheral blood mononuclear cells were harvested from standard buffy coats of healthy donors, obtained from the Department of Transfusion Medicine at Karolinska University Hospital. Human naïve CD8+ T cells were purified by a two-step magnetic bead selection and activated as for the mouse. Phenotypic analysis of CD8+T cells was performed by flow cytometry. Metabolic activity, i.e. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), was analyzed in a Seahorse Extracellular Flux Analyzer XF96 (Agilent). Statistical analyses were performed with Prism 7 version 7.0.

Results
We show here that exercise-induced reduction in tumour growth is dependent on CD8+ T cells and that lactate, which is produced at high levels during exertion, alters differentiation and expansion of CD8+ T cells. Interestingly, we found that at elevated levels lactate is used as a fuel during T cell activation. ^{13}C isotope labelling showed that lactate almost completely displaced the contribution of glucose to metabolites downstream of glycolysis. We further found that injection of lactate into animals reduced malignant tumor growth in a dose- and CD8+ T cell-dependent manner *in vivo*.

Conclusions
These data demonstrate the unexpected importance of lactate as a carbon source for activated T cells. Increased lactate levels produced in well-perfused tissues like skeletal muscle could have profound effects on the differentiation and efficacy of a cytotoxic T cell response. Overall our results show that lactate could act to increase the antitumour activity of cytotoxic T cells, and in so doing, reduce cancer progression.

STAT3 MUTATIONS IMPACTS ON OVERALL SURVIVAL OF T- CELL LARGE GRANULAR LYMPHOCYTE LEUKEMIA PATIENTS

Ph.D. Student: Dr. Gregorio BARILA' - TUTOR: Prof. Gianpietro SEMENZATO
Ph.D. Course: Clinical and Experimental Oncology and Immunology

Background: T- cell Large Granular Lymphocyte Leukemia (LGLL) is a rare and chronic lymphoproliferative disorder characterized by the expansion of CD3+ T-LGLs. Patient's affected by T-LGLL can be asymptomatic or develop cytopenia, mostly neutropenia. Recently, somatic *STAT3* and *STAT5b* mutations were discovered in approximately 40% of patients.
The aim of this study is to analyse clinical and biological features of a large cohort of T-LGLL patients to identify prognostic markers affecting patients' outcome.
Material and Methods: From 1992 to 2018, clinical and biological data of 129 T-LGLL patients were collected. *STAT3* exon 21 and *STAT5b* exon 16-18 mutations analysis was performed by Sanger sequencing.
Results: In our cohort, median age at diagnosis was 59 years. According to CD4 and CD8 expression, T-LGLL was classified in CD4-/CD8+ LGLL (CD8+ T-LGLL, 84/129, 65.1%) and CD4+/CD8$^{dim/neg}$ LGLL (CD4+ T-LGLL, 45/129, 34.9%). No dominant Vβ was found within CD8+ LGLL while, in CD4+ LGLL Vβ13.1 was expressed in 13 out of 45 cases (28.9%), with a frequency significantly higher towards CD8+ LGLL (28.9% vs 3.6%, p<0.0001). Killer Immunoglobuline-like Receptor (KIR) expression was detected in 36 patients (27.9%), with CD8 LGLL displaying significantly higher KIR frequency towards CD4+ LGLL (34.5% vs 15.6%, p=0.0244). CD94/NKG2 receptor expression was also studied in the entire cohort, with 18 cases positive (14%). CD94 expression in CD8+ patients was significantly higher than CD4+ patients (20.2% vs 2.2%, p=0.0034).
Clinical features of the cohort were collected. Patients with CD8+ LGLL were characterized by higher frequency of neutropenia (Absolute Neutrophils Count, ANC<1,500/mm^3, 58.3% vs 2.2%, p<0.0001), severe neutropenia (ANC<500/mm^3, 34.5% vs 0%, p<0.0001) anemia (Hb<120 g/L, 17.9% vs 0%, p=0.0012), severe anemia (Hb<90g/L, 13.1% vs 0%, p=0.0082), splenomegaly (23.8% vs 4.4%, p=0.0059), concomitant autoimmune/inflammatory disease (29.8% vs 6.7%, p=0.0030) and treatment requirement (26.2% vs 0%, p<0.0001). DNA samples of 103 and 96 patients were available for *STAT3* and *STAT5b* mutations analysis, respectively. *STAT3* mutations were detected in 39 CD8+ patients (37.9%) while *STAT5b* mutations were found in 12 CD4+ patients (12.5%), therefore *STAT3* and *STAT5b* mutations were mutually exclusive in CD8+ and CD4+ T-LGLL.
With a median follow up of 8 years, median overall survival (OS) of our cohort was not reached. Major features associated to reduced OS were age > 65 years (median OS not reached, p=0.001) and presence of *STAT3* mutations (267 months vs not reached, p=0.0102), while no significant survival differences were found between *STAT5b* mutated and *wild-type* patients (not reached vs 267 months, p=0.3576).
Conclusions: In conclusion, we showed that CD8+ and CD4+ LGLL are two different entities with distinct clinical and biological features and, for the first time, we demonstrated the dismal impact of *STAT3* mutations in patients' survival, suggesting that this biological feature should be considered as a potential target of therapy. RNA sequencing of CD8+ and CD4+ LGLL samples, either *STAT3/STAT5b* mutated or wild-type, is ongoing to elucidate the pathways involved in these different clinical entities and related to *STAT* mutations.

LIQUID BIOPSY TO MONITOR RESPONSE TO TREATMENT IN ADVANCED NON-SMALL CELL LUNG CANCER PATIENTS

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

Background: Liquid Biopsy has the potential to monitor biological effects of treatment without invasive procedures. Tumor-specific genetic alterations can be detected in circulating cell-free DNA from plasma using high sensitivity molecular techniques, such as droplet digital PCR or next generation sequencing (NGS). The outcome of advanced non-small cell lung cancer (NSCLC) patients has been substantially improving in the latest years, thanks to the introduction of immune checkpoint inhibitors (ICIs) in parallel with an increasing knowledge of the tumor genetic profile. While 20% of patients respond well to ICIs, more than 50% do not respond and 10% experience hyper-progression, a rapid worsening of clinical status. Liquid biopsy could also help individuating pseudo-progression, a tumor enlargement due to immune infiltrate. Considering the cost of the treatment, predictive biomarkers of response are needed. Aim of this study is to validate liquid biopsy in advanced *EGFR-ALK-ROS1* wild-type NSCLC and explore the correlation of dynamic variation of tumor-specific mutations in plasma with outcome.
Material and Methods: Advanced NSCLC patients undergoing systemic anti-cancer treatment were prospectively enrolled in this mono-institutional trial (MAGIC-1). Tissue genetic alterations were screened by MassARRAY® (Sequenom MA) or NGS. Plasma samples were collected at baseline (T1), after the first cycle of treatment (T2), and at first radiological restaging (T3). The first cohort of patients, bearing *KRAS* mutations in tumor DNA (*KRAS*-m), was analyzed in plasma using droplet digital PCR. Semi-quantitative index of fractional abundance of mutated allele (MAFA) was used.
Results: From January 2017 to August 2018, 105 patients were prospectively enrolled and genotyped in tissue. *KRAS*-m cohort (n=62) included 24 immunotherapy-treated patients. At baseline, *KRAS* mutations in cfDNA were detected in 25 out of 50 samples analyzed (50%, 95% CI: 35.5%-64.5%). Results of liquid biopsy were considered both as a static parameter (presence *versus* absence of *KRAS* mutation at each time points) and as a dynamic parameter (increase of MAFA from baseline *versus* stable/decreasing value) and correlated with clinical outcomes. The presence of *KRAS* mutation at T2 was associated with increased probability of experiencing progressive disease (PD) as best radiological response (adjusted OR: 9.9; 95% CI: 2.4-40.9, p:0.001). Increased MAFA (T1-T2) predicted shorter progression free survival (PFS) (adjusted HR: 2.7; 95% CI: 1.4-5.5, *p*:0.005) and overall survival (OS) (adjusted HR: 5.7; 95%CI: 1.9-16.7, *p*:0.001). The effect on outcome was independent on treatment and among immunotherapy-treated patients increased MAFA (T1-T2) negatively affected PFS (HR: 6.2; 95%CI: 1.4-26.5, *p*:0.013).
Conclusions: Dynamic monitoring of *KRAS* mutation at early time-points during treatment was significantly associated with PFS and OS, indicating the feasibility of detecting *KRAS* mutation in clinical practice and opening new perspectives for the management of advanced NSCLC treatment. A more comprehensive study is ongoing on the *KRAS* wild type cohort using NGS targeted panel to explore dynamic variations of other mutations in plasma. A second trial is planned to further investigate dynamic change of *KRAS* and additional driver genes at earlier time points in advanced NSCLS patients treated with ICIs.

A FIRST HIGH-DEFINITION LANDSCAPE OF SOMATIC MUTATIONS IN CHRONIC LYMPHOPROLIFERATIVE DISORDER OF NK CELLS

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

Background

Chronic Lymphoproliferative Disorder of NK cells (CLPD-NK) is included in the 2017 WHO classification of mature T- and NK-cell neoplasms as provisional entity. Similarly to T-cell large granular lymphocytes leukemia (T-LGLL), CLPD-NK is characterized by an indolent clinical course and is clearly distinguished from the aggressive NK cell leukemia (ANKL), this latter behaving as an acute severe disease. CLPD-NK and T-LGLL patients mostly present cytopenias when symptomatic. Accumulating evidence on the genomic landscape of T-LGLL pointed to a key role of somatic mutations within JAK-STAT (mostly in *STAT3* and *STAT5B* genes) and RAS-MAPK pathwa*ys* (*Coppe et al. Leukemia 2017*) in this disorder. Recent data highlighted more heterogeneous molecular features in ANKL, involving *JAK2* and *STAT3* mutations, lesions of epigenetic modifiers, tumor suppressors and a putative role of Epstein Barr Virus (*Dufva et al. Nat Comm. 2018*). In our cohort of 57 CLPD-NK patients the frequency of *STAT* mutations has been found significantly lower (<10% of all cases) as compared to literature data (*Barilà et al. Blood Cancer J. 2018*). The genomic landscape of CLPD-NK patients negative for *STAT* mutations was thus investigated to better elucidate the molecular basis of the disease.

Material and Methods

Ten CLPD-NK patients were recruited according to negativity for *STAT* mutations and typical immunophenotype ($CD16^{+}/CD57^{\pm}/CD56^{\pm}$). WES profiling (Agilent SureSelect 60 Mbp, Illumina sequencing, paired end reads) of immunomagnetically purified leukemic clone (>95% purity) and of normal granulocytes, as control, was obtained for each patient. Sequencing data were analyzed by an in-house bioinformatic pipeline encompassing data cleaning, read mapping against the reference genome and somatic variant calling with complementary approaches (MuTect, MuTect2 and Strelka2). Variants were next filtered according to population allele frequency (gnomAD <5%), clinical significance (COSMIC and ClinVar) and predicted functional impact on proteins (SnpEff, MetaSVM and MetaLR).

Results

High sequencing depth was obtained with a mean coverage of 150 reads. From over 6,000 somatic variants detected in the cohort, 869 SNPs and 14 indels, rare and of high predicted impact were prioritized. Number of variants (88 per patient in average) and mutation spectra were very homogeneous in the cohort. Of note, the high discovery power of our settings disclosed an extensive subclonality of somatic variants, with over 78% with an allele variant frequency (VAF) lower than 5% in the leukemic clone. Nevertheless, 20 deleterious and high VAF somatic variants occurred in each patient in average. A very low recurrence of mutated genes in CLPD-NK patients was found. Pathway-derived mutation network analysis revealed functional connections among genes mutated in different patients. A major involvement of JAK-STAT pathway is likely to be ruled out, but all cases presented deleterious high VAF mutations of genes and pathways potentially impacting on cell survival, proliferation and chromatin-remodelling. In addition, somatic lesions in proteins involved in innate immunity and NK-cells activation seem particularly interesting.

Conclusion

Ongoing comparison of genes and pathways mutated in CLPD-NK with available data for T-LGLL and ANKL is providing new clues on biological similarities and differences of these clinical entities. This study contributes to better define the molecular mechanisms accounting for CLPD-NK, thus opening new areas of investigation.

ROLE OF IRON METABOLISM IN TUMOR-INFILTRATING MACROPHAGES FROM GLIOBLASTOMA PATIENTS

Ph.D. Student: Dr. Sara MAGRI - TUTOR: Prof. Susanna MANDRUZZATO
Ph.D. Course: Clinical and Experimental Oncology and Immunology

Background

Glioblastoma multiforme (GBM) is one of the most common and malignant primary brain tumors. Despite aggressive treatments, it is still a deadly disease with a poor prognosis. GBM is known to create an immunosuppressive microenvironment that hampers the efficacy of standard and immuno-therapies. We and others demonstrated that GBM microenvironment is infiltrated with a large proportion of bone marrow-derived macrophages (BMDMs). We performed a phenotypic and functional characterization of the immune infiltrate in GBM biopsies and observed that BMDMs possess an immune suppressive activity. We also determined that, following 5-aminolevulinic acid (5-ALA) administration to patients before surgery, BMDMs showed the highest protoporphyrin IX (PPIX) fluorescence emission, compared to other cells. Since PPIX is the precursor of heme we wondered if the presence of this metabolite could be the consequence of an altered iron metabolism in tumor-infiltrating macrophages, and also if this pathway could be linked to their immunosuppressive activity. Taking advantage from transcriptomic data of an external data set of 8 cases of GBM that were subjected to single-cell RNA sequencing, we analyzed the expression of *HMOX1*, the gene encoding for heme oxygenase-1 (HO-1), a central enzyme in the iron pathway and found that it was highly expressed in BMDMs.

Material and Methods

To evaluate the potential role of this gene in immunosuppression induced by BMDMs, we used an immune suppressive assay in the presence of HO-1 inhibitors. Cell suspension was obtained from the dissociation of GBM tissues and BMDMs were separated by immunomagnetic sorting and treated with HO-1 inhibitors (zinc protoporphyrin IX (ZnPPIX), tin protoporphyrin IX (SnPPIX) and OB24 hydrochloride). We also tested *HMOX1* siRNA before testing their immunosuppressive activity. BMDM immune suppressive activity was evaluated as the ability to suppress the proliferation of αCD3/αCD28 activated and CellTrace labelled allogeneic T lymphocytes. In order to optimize the experimental conditions, I set up a model of immunosuppressive macrophages derived from M-CSF treated monocytes isolated from healthy donors. Another *in vitro* model of immunosuppressive cells, previously developed in our laboratory, bone marrow derived-myeloid derived suppressor cells (BM-MDSCs) was used. The proper concentrations of inhibitors were established in viability assays. The efficacy of siRNA transfection was evaluated by flow cytometry and the efficiency of mRNA knockdown was assessed by RT-PCR.

Results

I found that the pre-treatment of BMDMs with ZnPPIX is able to restore T cells proliferation while SnPPIX and OB24 hydrochloride treatments showed a reduced effect on immunosuppression. I am now evaluating *HMOX1* gene silencing trying different methods to obtain a significant efficiency of silencing. Moreover, I am investigating if there is a link between HO-1 and itaconate, a metabolite that likely plays a role in macrophage-based immune response. From literature itaconate, that is increased in activated macrophages, boosts levels of transcription factor Nrf2, increasing the expression of downstream target genes, including *HMOX1*. I found that the addition of itaconate in immunosuppression assays increases immunosuppressive activity of macrophages and counteracts the effect of ZnPPIX. I also observed the same results in BM-MDSC model.

Conclusions

Our results show that the connection between cell metabolism and immune response could be exploited from a therapeutic point of view. In particular, I showed that HO-1 have a role in BMDM-induced immunosuppression and thus could represent a new target to relieve the immunosuppressive microenvironment present in GBM patients.

DETECTION RATE OF MPMRI/TRANSRECTAL US FUSION BIOPSY FOR THE DIAGNOSIS OF PROSTATE CANCER IN BIOPSY-NAÏVE PATIENTS

Ph.D. Student: Dr. Alessandro MORLACCO - TUTOR: Prof. Giacomo NOVARA
Ph.D. Course: Clinical and Experimental Oncology and Immunology

Background: Fusion prostate biopsy allows precise targeting of ultrasound-guided biopsy to multiparametric MRI (mpMRI) suspicious areas, thus overcoming the known limitations of standard random sampling. The PRECISION trial suggested the role of mpMRI-guided fusion biopsy (FB) as the optimal strategy to adopt, as compared to the standard random biopsy (SB), while, recently, the MRI-FIRST study suggest-ed that prostate cancer (PCa) detection rate is improved by using both techniques (SB and FB). The aim of the present study is to compare the detection rate of mpMRI/transrectal US FB to standard transrectal RB for PCa diagnosis.
Material and Methods: from November 2016 to February 2019, 100 patients with positive mpMRI (PIRADS v2 ≥3) and no prior prostate biopsy underwent mpMRI/transrectal US FB with Hitachi RVS system and concurrent transrec-tal 24-cores SB at a single institution. The two biopsy procedures were performed by two separate operators, keeping the SB operator unaware of the results of mpMRI and the location of the FB. All MRI scans were performed at our institution by two dedicated uro-radiologists or reviewed by one of the 2 dedicated uro-radiologist and PIRADS re-assigned if performed elsewhere. Each core was pro-cessed with sandwich technique in a single biobox and examined by a single dedicated uro-pathologist. Clinically significant PCA (CSPCa) was defined as in the PROMIS trial (i.e. Gleason score ≥4 + 3 or a maximum cancer core length 6 mm or longer). Statistical analyses were performed with SPSS v.24.0 software. Continuous variables were reported as median and interquartile range (IQR).
Results: Median age at biopsy was 64 years (IQR 60-70) and median total PSA was 5,59 ng/ml (IQR 4-8,7), with a median prostate volume at US of 54 ml (IQR 40,8-68). Overall, 64% had a PCa diagnosis at biopsy. FB only identified correctly 45 (70.3%) PCa cases. However, FB only would have missed the diagnosis in 18 cases (28,1%), of which 9 were CSPCa. On the other hand, SB correctly identified all but one cancer. Stratifying the results by PIRADS score, overall PCa detection was 47% in PIRADS 3, 56% in PIRADS 4 and 80% in PIRADS 5 lesions. CSPCa was diagnosed in 44% of the patients. Specifically, SB identified 39 out of 44 of these CSPCa (88.6%), failing to detect only 11.4%. Conversely, FB alone would have missed 25% of the CSPCa. Stratifying by PIRADS score, CSPCa detection was 24% in PIRADS 3, 43,4% in PIRADS 4 and 64% in PIRADS 5.
Conclusions: FB and RB showed a complementary role for detection of any PCa and CSPCa in biopsy-naïve, mpMRI-positive patients. The present data do not support the adoption of FB only in this clinical setting due to the risk of missing significant cancers.

CLOCK GENES POLYMORPHISMS AS PREDICTORS OF RISK AND PROGNOSIS IN PATIENTS WITH SOFT TISSUE SARCOMAS

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

Background: Dysfunction of the circadian clock and genetic variants in the clock genes are a potential risk factor for cancer development and progression. As for virtually all cancers, genetic variation of the host is believed to play an important role in the determinism of the risk of soft tissue sarcomas (STS). However, no information is currently available on the relationship between circadian genes polymorphisms and susceptibility or prognosis of patients with STS. The present study aimed to explore this association.

Patients and methods: We analyzed 14 single-nucleotide polymorphisms (SNPs) of 6 core circadian genes that have been linked to cancer risk and prognosis. SNPs genotyping was performed by quantitative real time PCR. We collected peripheral blood and clinic-pathological data from 162 patients with liposarcoma and leiomyosarcoma and from 620 cancer-free individuals. The relationship between the selected SNPs and sarcoma susceptibility and prognosis was tested evaluating additive, recessive and dominant genetic models. Subgroup analysis based on sarcoma histotype was carried out assuming the additive genetic model. Multivariate logistic regression and multivariate Cox proportional hazard regression analyses were used to evaluate the association between the selected SNPs with the risk of developing STS and prognosis of patients affected by STS. Pathway variation analysis was carried out using the Adaptive Rank Truncated Product model.

Results: Six SNPs resulted statistically significantly linked to susceptibility or prognosis of STS ($p > 0.05$). The minor allele of the CLOCK polymorphisms rs1801260 (C) and of PER2 rs934945 (T) were associated with a lower risk of developing STS (26% and 35% respectively, based on the additive model). In subgroup analysis this association was statistically significant in liposarcoma (33% and 41% respectively). Carriers of the minor allele A of NPAS2 rs895520 had an increased predisposition to develop sarcoma with an additive model (33%), with special regard to leiomyosarcoma (44%). RORA rs339972 C allele was associated with a decreased predisposition to develop sarcoma assuming an additive model (29%) and leiomyosarcoma (36%). PER1 rs3027178 was linked to a reduced predisposition only for liposarcoma (32%). As regards patient prognosis, the minor allele of rs7602358 located upstream PER2 was significantly associated with survival of patients with liposarcoma, assuming additive model (HR 1.98, 95% CI 1.02-3.58, P= 0.04). Germline genetic variation in the whole circadian pathway was associated with the risk of developing STS (P =0.035)

Conclusions: The present study is the first one to explore the relationship between Clock SNPs and STS predisposition and prognosis. Our results support this association and represent a starting point for further studies that are needed to clarify the molecular mechanisms underlying this relationship.

Keywords: Soft tissue sarcomas (STS), leiomyosarcoma, liposarcoma, Clock genes, Single-nucleotide polymorphisms (SNPs), Circadian pathway, risk, survival.

STUDY ON PERITUMORAL MICROENVIRONMENT OF CHONDROSARCOMA

Ph.D. Student: Dr. Giulia TROVARELLI - TUTOR: Prof. Pietro RUGGIERI
Ph.D. Course: Clinical and Experimental Oncology and Immunology

Background

Chondrosarcoma (CS) is a rare bone sarcoma, composed by cartilage cells, that affects adult people. Nowadays, the only treatment of these patients is surgery since high grade CS is resistant to chemotherapy and radiotherapy [1, 2]. However, despite an adequate surgical treatment, (wide resection) the incidence of local recurrence or metastases remain still high, compromising the patient's survival. Therefore, new therapeutic approaches are needed.

In our project we would like to study the microenvironment around chondrosarcoma cells in order to investigate which factors could influence the tumor progression, searching new molecules that could be used to introduce new type of chemotherapy effective again CS [3,4].

During the first year of PhD course, we analyzed concentration hyaluronan protein (HA), in the proximity of CS comparing peritumoral, intratumoral and healthy tissue after surgical excision in 12 patients. Our preliminary results demonstrate an increase of the amount of the HA in peritumoral and tumoral stroma compared to healthy tissue [5].

The aim of this PhD year was to enhance CS tissues collection and to obtain CS cell line continuing the study about the role of HA in CS.

Material and Methods

Healthy, peritumoral and tumor tissues were collected after surgical treatment of CS patients and stored at -80°C. In parallel, 5 cc of plasma was collected and stored at -80°C to evaluate new biomarkers of disease. Cell line of CS were obtained and stored in liquid nitrogen in order to plan and test new potential pharmacological metal-based compounds on CS cell.

Results

We increased the collection of CS cases: eight new CS patients were enrolled and both, tissues and plasma, were collected. Therefore, we have now 20 CS cases: 2 dedifferentiated CS, 2 clear cell CS and the remaining conventional CS.

Human chondrosarcomas cell line (JJ012 and FS090) were kindly donated by Prof. Joel A. Block (Rush University Medical Center, Chicago), thanks to international co-operations.

In collaboration with the Chemistry Department, we are selecting the most promising therapeutic metal-based compounds, which demonstrate to be effective on CS cell lines.

Conclusions

The role of HA will be assessed through the analysis of the new collected samples. The idea is to evaluate the total amount of HA (in tissues and in plasma), to discriminate between low and high molecular weight HA (which have different functions), to quantify both gene and protein expression of molecules involved in HA turnover and HA signaling pathway (hyaluronidase 1,2, hyaluronic acid synthetase-1,2,3, RHAMM and, CD44). Moreover, we will test metal-based compounds CS cell lines in monolayer and in 3D in collaboration with the Chemistry Department.

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PhD COURSE "CLINICAL AND EXPERIMENTAL SCIENCES"

COORDINATOR: Prof. Paolo ANGELI

CURRICULUM "CLINICAL METHODOLOGY, METABOLISM, ENDOCRINOLOGY, NEPHROLOGY AND EXERCISE"

EFFECTS OF A SINGLE DOSE OF PASIREOTIDE ON GLUCOSE METABOLISM IN PATIENTS WITH CUSHING'S DISEASE AND PREDICTORS OF DIABETES MELLITUS DEVELOPMENT DURING TREATMENT

Ph.D. Student: Dr. Mattia BARBOT - TUTOR: Prof. Carla SCARONI
Ph.D. Course: Clinical and Experimental Sciences
Curriculum "Clinical Methodology, Metabolism, Endocrinology, Nephrology and Exercise"

Background: Even though transsphenoidal pituitary surgery remains the first line therapy in Cushing's disease (CD), in the last decade the role of medical treatment has come back to the spotlight thanks to the development of new compounds. Among them, the only pituitary directed drug approved for the treatment of CD is pasiretoide (PAS), a novel somatostatin receptor multiligand. PAS has proven to be an effective treatment for CD in phase III trial, but its use is burdened by the high incidence of diabetes mellitus (DM). The aim of this study was to evaluate the effect of a single subcutaneous injection of PAS on glucose metabolism in CD patients, to identify factors predicting rapid deterioration of glycaemic control and assess PAS effectiveness in controlling hypercortisolism in our cohort.
Material and Methods: Fourteen patients (12 females/2 males, mean age 43±11.3 years) with CD were treated with PAS at the starting dose of 600 mcg twice daily. Before starting the therapy, all patients were submitted to an acute PAS test (600 mcg s.c.) with measurements of ACTH, cortisol, glucose, insulin, c-peptide, insulin, GIP, glucagon, GLP-1, at time 0' and then every 30 minutes for 2 hours to predict the development of DM2 during therapy. Patients were followed for a mean time of 17 months (range 2-63) with clinical and hormonal assessment.
Results: There was a significant reduction in urinary free cortisol (UFC, 582±456 vs 254±356 nmol/24h, p=0.001) and late night salivary cortisol (LNSC, 12.2±11.6 vs 3.8±1.7 nmol/L, p=0.003) in all cohort with concomitant improvement in clinical picture; both weight (74.5±16.2 vs 69.9±16.4 kg, p=0.01) and waist circumference (95.3±13.6 vs 93.6±13.4cm, p=0.02) decreased throughout PAS treatment. Overall 10/14 patients reached UFC normalization whereas LNSC was within normal range only in 4 cases. A single PAS dose produced a significant decrease of all hormonal parameters assessed (p<0.0001), except for glycaemia which reached the highest value 120' after the injection (baseline 4.65±0.52 vs peak 8.91±3.63 mmol/L, p<0.0001). Overall 7/14 patients developed DM within the first 2 months of therapy. Among baseline characteristics, patients who developed DM showed no differences in age, weight, visceral adiposity, HOMA-index, fasting glucose and severity of CD with those who did not have glucose metabolism alterations; however, compared to non-DM patients, DM patients displayed higher baseline fasting c-peptide (respectively 694.4±109.6 vs 947.4±318.4 pmol/L, p=0.05) and HbA1c levels (30±3.0 vs 37.1±1.8 mmol/mol, p=0.001), with the latter being normal in all cases. Finally, glucose peak tent to be higher in DM-patients than in those who did not develop hyperglycaemia (7.2±2.2 vs 10.6±4.1 mmol/L, p=0.06).
Conclusions: PAS confirmed to be a valuable tool for the treatment of recurrent or persistent CD after neurosurgery. It was able to rapidly suppress insulin secretion and the incretin system with a subsequent increase in glucose levels into the diabetic range producing also a concomitant decrease in glucagon values. Patients at higher risk of DM during PAS therapy were those with higher fasting c-peptide at elevated serum glucose peak during the acute test. Interestingly baseline HbA1c, seems to predict the risk of PAS-induced DM. CD patients candidate to PAS therapy who display these metabolic features should carefully be monitored especially in the first 2 months of treatment due to the high risk of develop DM.

PROTECTIVE EFFECT OF LICORICE ON DAPSONE-HYDROXYLAMINE-INDUCED MEMBRANE ALTERATIONS IN RED BLOOD CELLS FROM PATIENTS WITH ENDOMETRIOSIS

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Curriculum "Clinical Methodology, Metabolism, Endocrinology, Nephrology and Exercise"

Background
Endometriosis, an estrogen-dependent chronic gynaecological disease, is characterized by a systemic inflammation status. Previous evaluations, performed during my first year of PhD, confirmed that this pro-inflammatory status involves even red blood cells (RBC), which show an inability of counteracting adjunctive oxidative stress, represented by diamide and dapsone-hydroxylamine (DDS-NHOH), a mild and powerful oxidant, respectively. This impairment of antioxidant defences could play an important role in the pathogenesis and potential worsening of endometriosis. Previous studies evidenced that licorice and its constituents have a protective effect on oxidative-related RBC alterations.
During my second year of PhD, the aim of my researches was to investigate the effect of licorice intake on the oxidative-related alterations in RBC membranes from patients with endometriosis, and its protective properties against further prooxidant agents, represented by DDS-NHOH.
Material and Methods
Twelve patients with endometriosis, confirmed with histological exam, were enrolled and compared with 12 healthy volunteers, considered as control group. All women were treated with licorice extract (about 25 mg daily). After one week of treatment, blood samples were analysed for band 3 tyrosine phosphorylation (Tyr-P) in the presence or absence of diamide or DDS-NHOH. Baseline blood samples of all women enrolled were previously analysed for band 3 Tyr-P in the project of the last year. RBC membranes were analysed by Wester blotting and anti-Tyr-P immune-revealed bands were densitometrically estimated. Results were correlated with plasma glycyrrhetinic acid (GA) concentration, measured by HPLC-MS.
Results
After licorice intake both patients and controls showed a significant reduction of band 3 Tyr-P levels after diamide and DDS-NHOH compared with baseline values (p <0.005). Mean plasma GA concentrations were not significantly different in the two groups and presented a direct correlation with the reduction of both diamide and DDS-NHOH induced alterations, especially in patient group.
Conclusions
Licorice intake seems to decrease the high level of oxidative-related alterations on RBC membranes. The reduction of DDS-NHOH induced side effects in vitro suggests licorice as a new potential tool for preventing further switching into severe endometriosis.

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PhD COURSE "CLINICAL AND EXPERIMENTAL SCIENCES"

COORDINATOR: Prof. Paolo ANGELI

CURRICULUM "RHEUMATOLOGICAL AND LABORATORY SCIENCES"

PRESENTATION AND OUTCOMES BETWEEN 2000-2010 AND 2011-2018 DECADES IN GRANULOMATOSIS WITH POLYANGITIIS AND MICROSCOPIC POLYANGIITIS: A 20 YEARS FOLLOW UP MONOCENTRIC STUDY

Ph.D. Student: Dr. Mara FELICETTI- TUTOR: Prof. Paolo SFRISO
Ph.D. Course: Clinical and Experimental Sciences
Curriculum "Rheumatology and Clinical Sciences"

Background: Granulomatosis with polyangiitis (GPA) and microscopic polyangiitis (MPA) are systemic diseases with a wide spectrum of clinical presentation. Our study aims to analyze presentation and 5 years-outcomes of GPA and MPA patients diagnosed between 2000 and 2018 in the monocentric cohort of Padova Vasculitis Center.
Material and Methods: We retrospectively collected all GPA and MPA diagnosed between 2000-2018 and followed in our clinic. We focused on demographic and clinical features at diagnosis, 6 months mortality, 5 years-relapse rate and survival rate.
Results: We identified 110 patients (F/M 57/53, Caucasian 97%) that met the inclusion criteria. Only 39 patients were diagnosed between 2000-2010, while 71 between 2011-2018 (+180% of new diagnosis than in the previous decade).
82 (75%) were GPA, while 28 (25%) MPA. Interestingly, 21/28 MPA patients belonged to the 2011-2018 cohort (30% vs 18%, p=ns, Δ +12%).
Mean age at diagnosis of 58.4±16.4 years, without significant differences between the two decades, even if, in the last decade, we observed an increasing trend of AAV in young adults (age<30 years) and elderly patients (age>65 years) (respectively 14% vs 3%, p=0.054 and 35% vs 18%, p=0.057). Despite the increased frequency of elderly patients and MPA, the hospital admission rate at diagnosis slightly decreased over time without reaching the statistical significance (78% vs 91%, p=0.08).
In all cohort 97/109 were ANCA positive: 55% cANCA/PR3, 33% pANCA/MPO and 1% double positive without significant differences between the decades.
The patients more frequently presented with systemic symptoms (77%), ENT and lung involvement (respectively 62% and 66%). Renal vasculitis was reported in 66% of patients with a mean eGFR of 50±40 ml/min. Moreover, we registered 17 alveolar haemorrhages (AH), 4 cardiac involvement, 4 subglottic stenosis (SGS) and 3 gastrointestinal (GI) disease. Clinical presentation did not significantly differed over time, however, in the last decade, we observed 14/17 AH and all SGS, while the other more severe manifestations (CNS, cardiac and GI involvement) were evenly distributed
Overall, only 1 patient died within 6 months, while the 5-years survival calculated with Kaplan Meier method was 94.3% (6 events, 5 in the last decade). 5-years relapse rate was 31%. Neither the survival rate nor the relapse rate significantly changed over time.
Conclusions: Our data showed a consistent increase of AAV diagnosis over time, in line with the most recent literature. This could reflect a real increasing frequency of AAV in the population, but also a higher awareness of the disease among physicians. The comparison between the two decades, showed also an increasing trend of AAV in elderly patients and an increased presentation with MPA and AH, all markers of more severe disease and difficult management. This could explain the lack of the improving survival trend over time, that has been reported in the literature.

HEART HEMODYNAMICS IN SYSTEMIC SCLEROSIS: INSIGHT FROM A PRESSURE-VOLUME LOOP ANALYSIS

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Ph.D. Course: Clinical and Experimental Sciences
Curriculum "Rheumatology and Clinical Sciences"

Background: Despite cardiac symptoms occur in only 20-35% of patients, primary myocardial involvement is common in systemic sclerosis (SSc), representing one of the main causes of death. Ventricular-arterial coupling (VAC) reflects the interaction between ventricular performance and effective arterial load.
Objectives: We investigated alterations in VAC in SSc patients and their associations with survival free from cardiovascular hospitalizations.
Material and Methods: In this single institution cohort study, SSc patients (n = 65) without signs and symptoms of heart disease were retrospectively compared with healthy matched controls (n = 30) using noninvasively measured end-systolic ventricular elastance (Ees), arterial elastance (Ea), VAC (Ea/Ees), end-diastolic elastance (Eed) and ventricular efficiency (LVEff).
Results: SSc patients had significantly higher Ees (p=0.002), Ea (p=0.002), and Eed (p=0.01) than controls. VAC values were consequently comparable to controls (p=0.59). However, Ees was lower and VAC was higher in patients with diffuse cutaneous form (dcSSc) compared to patients with limited form (lcSSc) (3.2 vs 4.36 mmHg/ml, p=0.001 and 0.66 vs 0.56, p=0.02, respectively). Consequently, LVEff was lower in dcSSc patients (75% vs 79%, p=0.02) than in lcSSc patients. Over a median of 2 years (range 2 months-10 years), 4 patients died, 16 were hospitalized for angina and 18 for heart failure. Patients with VAC>0.63 (the median value) had poorer survival free from hospitalizations than patients with VAC ≤0.63 (7% vs. 34%; Log-rank p = 0.02), and was associated with risk of mortality (hazard ratio, 2.00; p = 0.03) compared with VAC ≤0.63. (Figure)
Conclusions: Our study suggests that ventricular-arterial function and coupling may be impaired in SSc patients without signs and symptoms of heart disease. VA coupling seems to have a prognostic role in SSc patients.

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PhD COURSE "CLINICAL AND EXPERIMENTAL SCIENCES"

COORDINATOR: Prof. Paolo ANGELI

CURRICULUM "HEMATOLOGICAL AND GERIATRIC SCIENCES"

IDIOPATHIC AND CONGENITAL ERYTHROCYTOSIS: STUDY OF A LARGE COHORT OF PATIENTS

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

Background. Absolute erythrocytosis occurs when there is an increase in the red-cell mass to more than 125% of the predicted value for the body mass of the patient, resulting in persistently raised Hemoglobin (Hb) and/or Hematocrit (Htc) levels. Differential diagnosis includes Polycythemia Vera (PV), secondary, hereditary (HE) and idiopathic erythrocytosis (IE). Mutations in genes members of the oxygen sensing pathway (OSP) (*EPOR, VHL, EPAS1*/HIF2α and *EGLN1*/PHD2) have been associated with HE. To date, about 30 mutations have been described in the *EGLN1* gene, the majority localized in the catalytic domain and impairing binding of HIF2α. The PHD2 p.C127S variant is frequently observed in Tibetans with D4E in cis, in linkage disequilibrium with other missense variants (in particular EPAS1). Surprisingly, the combination of *EGLN1/EPAS1* variants results in a gain-of-function effects that blunts the hypoxic response in Tibetans, protecting them from erythrocytosis at high altitude. At normoxic conditions and in low-landers, this variant is present in 15-30% of non-Tibetan controls with a still unclear effect. Recently, *Biagetti et al* demonstrated the recurrence of HFE single nucleotide variants (SNVs) in patients with IE, postulating a possible link between increased iron bioavailability and erythrocytosis.
Material and Methods: Using an appropriate algorithm to ruled out any possible cause of secondary acquired erythrocytosis (EPO levels, chest and abdomen imaging, spirometry, p50, arterial blood gas analysis) and PV (negative JAK2 mutation analysis for both V617F and exon 12), we collected 205 patients with erythrocytosis. HE mutations were analysed by Sanger sequencing of the following genes: *EPOR* exon 8, *VHL* coding region, *EGLN1* exon 1-3, *EPAS1* exon 12. HFE SNVs were studied using an allele-specific real-time PCR.
Results: Seventy-six patients were classified as HE, either for positive for any mutations in OSP genes, or for relevant family history of erythrocytosis even if wild-type. Within 30 patients carrying PHD2 variants, we found an erythrocytotic 55-year old male with a missense heterozygous PHD2 mutation (c.806 T>A, p.I269N), not previously described. His kindred evaluation shows that one daughter and one sister have erythrocytosis and both carry the same heterozygous mutation of the propositus. Furthermore, his father died with a diagnosis of PV in the pre-JAK2 era. In contrast, the patient's brother and the other daughter, both with normal Hb and Htc, had wild-type PHD2. Of note, the propositus and his two daughters were heterozygous for H63D in HFE gene. Interestingly, 11 patients carry the missense heterozygous variant (PHD2 p.C127S) previously reported in Tibetan population. Moreover, 16 of 35 (46%) IE patients resulted carriers of SNVs in the HFE gene.
Conclusions: In addition to previously known PHD2 gene alterations, we report the occurrence in HE patients of a novel PHD2 mutation with an autosomal-dominant inheritance likely involved in disease pathogenesis. The milder phenotypic features of this patient's daughter and sister in terms of erythocytosis, may be explained by the childbearing age and the absence of H63D SNV, respectively. Moreover, further studies are warranted to establish the role of the oxygen sensing PHD2p.C127S variant in non-highlanders Europeans and its effect on erythropoiesis. The increased prevalence of HFE SNVs in patients with IE may indicate an effect of impaired iron metabolism on erythropoiesis. Our data show that the inclusion of HFE SNVs and oxygen pathway mutational analysis in the diagnostic algorithm of erythrocytosis may help to better define the genetic basis of erythrocytosis. Further studies – including the analysis of molecules involved in iron storage pathway- in larger IE patient cohorts are warranted in order to clarify the link between HFE genes and IE.

THE IMPACT OF ACCIDENTAL FALLS ON HEALTH-RELATED OUTCOMES IN COMMUNITY-DWELLING OLDER ADULTS

Ph.D. Student: Dr. Caterina TREVISAN - TUTOR: Proff. Enzo MANZATO, Giuseppe SERGI
Ph.D. Course: Clinical and Experimental Sciences
Curriculum "Hematological and Geriatric Sciences"

Background. Falls are a major concern in the older population, with one out of three individuals aged 65 years or older experiencing at least one fall every year. Although previous research focused mainly on the risk factors for falls, the impact of such detrimental events on the individuals' general health needs to be further elucidated.
Material and Methods. Data come from 2976 community-dwelling individuals aged ≥65 years, enrolled in the Progetto Veneto Anziani (Pro.V.A.) study. At baseline and at the 4- and 7-year follow-ups, participants underwent assessments of: cognitive status, through Mini-Mental State Examination (MMSE); depressive symptoms, through the Geriatric Depression Scale (GDS); functional status, through Activities of Daily Living (ADL) and instrumental ADL (IADL); and gait speed at usual pace. Data on the number of accidental falls in the previous year, and on hospitalizations were also collected at each assessment. Changes in the above mentioned outcomes as a function of accidental falls were evaluated through linear mixed models. Multiplicative interactions between accidental falls and time were tested.
Results. The number of participants who reported at least one accidental fall in the previous year was 735 (24.7%) at baseline, 616 (20.7% of the 2152 who participated to the first follow-up) at the 4-year follow-up, and 135 (4.5% of the 647 who participated to the second follow-up assessment) at the 7-year follow-up. After adjusting for potential confounders, having experienced accidental falls was associated with higher depressive symptoms (β=0.50, SE=0.16, p=0.002) and worse functional status in ADL (β=-0.06, SE=0.03, p=0.07) during the study period, and with a steeper decline in self-sufficiency in ADL over time ($p_{interaction}$<0.05). No significant results were observed for MMSE, gait speed and hospitalizations in the sample as a whole.
Conclusions. Accidental falls can negatively affect older adults' health by influencing depressive symptoms and functional status over time.

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PhD COURSE "CLINICAL AND EXPERIMENTAL SCIENCES"

COORDINATOR: Prof. Paolo ANGELI

CURRICULUM "HEPATOLOGICAL AND TRANSPLANTATION SCIENCES"

A SERPINB3 INHIBITOR AS A NOVEL DRUG FOR NON-ALCOHOLIC STEATOHEPATITIS

Ph.D. Student: Dr. Andrea MARTINI - TUTOR: Prof. Massimo BOLOGNESI
Ph.D. Course: Clinical and Experimental Sciences
Curriculum "Hepatological and Transplantation Sciences"

Background: Nonalcoholic fatty liver disease (NAFLD) and Non-Alcoholic Steatohepatitis (NASH) are increasingly relevant public health issues and their prevalence is predicted to increase in parallel with the increase of obesity and diabetes, but pharmacological treatments are still lacking. SerpinB3 is a serine protease inhibitor that progressively increases in the liver in relation to the extent of hepatic damage. In animal models of NASH, SerpinB3 transgenic mice display increased lipid accumulation, fibrosis and inflammation, while these features were significantly reduced in SerpinB3-KO mice. 1-Piperidine Propionic Acid (PPA) has been recently identified as SerpinB3 inhibitor. The aim of this study was to evaluate the effect of PPA on NASH experimental models.

Material and Methods: Cell lines with different SerpinB3 expression have been incubated with PPA to assess its inhibitory activity. Real time cell proliferation was assessed using the xCELLigence instrument. Recombinant SerpinB3 (100ng/ml) was also added to primary monocytes in the presence or absence of PPA. SerpinB3-transgenic and SerpinB3-KO mice and their control littermates were fed on MCD and CDAA diets to induce experimental NASH. Starting from the second month, mice were injected daily with PPA and were sacrificed at week 8. Liver pathology, IHC for F4/80, Sirius red staining, fibrosis and inflammation gene expression was carried out at sacrifice.

Results: In cell lines PPA was found to inhibit SerpinB3 mRNA expression in a dose dependent manner and these features were associated to a corresponding reduction of cell proliferation. In monocytes SerpinB3 induced overexpression of sCD163, a recognized marker of NASH, that was markedly inhibited by PPA. SerpinB3-KO mice showed significantly lower steatosis, inflammation and fibrosis after both dietary regimens, while opposite findings were observed in SerpinB3 transgenic mice. Treatment with PPA reverted these features, leading to liver profiles similar to controls.

onclusions: SerpinB3 has been identified as a new druggable target for NASH and PPA proved to be an efficient compound that markedly reduces NASH through the inhibition of SerpinB3.

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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"

TARGETED NGS FOR THE DIAGNOSIS OF CHILDHOOD MOVEMENT DISORDERS AND CEREBRAL PALSY

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Ph.D. Course: Developmental Medicine and Health Planning Sciences
Curriculum "Emato-Oncology, Genetics, Rare Diseases and Predictive Medicine"

Background Childhood Movement Disorders (MD) constitute a heterogeneous group of neurological conditions characterized by abnormal voluntary or involuntary movements also recurring in Cerebral Palsy (CP), a permanent non-progressive neurodevelopmental condition involving movement and posture alterations. Commonly reported, co-morbidity includes intellectual disability (ID), autism spectrum disorders (ASD), seizures disorders, speech, visual and hearing problems. Despite largely considered acquired or multifactorial, the role of a genetic basis for these disorders is increasingly evident. The pathophysiology is still not entirely understood and many patients remain without a precise genetic diagnosis. The aim of this work was to develop a genetic diagnostic strategy for a paediatric population affected by MD and the associated comorbidity, to better define these phenotypic spectra. The identification of *novel* mutations may increase the evidence for the role of disease-genes and support the association of these diseases with putative candidate genes.

Material and Methods We used a high-throughput strategy to sequence the coding regions of genes selected from literature and public databases with the platform Ion PGM™ System. DNA samples is extracted from peripheral blood. Data-analysis was performed by an in-house automated pipeline and a combination of integrated bioinformatics tools. Variant segregation and validation was made with Sanger sequencing.

Results In the first step DNA samples of 32 paediatric patients with a diagnosis of neurodevelopmental disorders were subjected to NGS analysis with the use of a targeted gene panel including 74 genes prioritized for specific for ID/ASD phenotypes; all of these subjects were referred for ID and/or ASD but were also affected with MDs. In three out of 32 of these cases we were able to detect: a *de novo* pathogenic missense mutation in *DYRK1A,* a likely pathogenic (LP) rare variant in *SCN2A* and, in a girl with non-progressive ataxia, a missense single nucleotide variant (SNV) in association with an intronic rare variant, predicted *in silico* as possibly altering the splicing mechanisms, in the *GAD1* gene. It is worth mentioning that for all of the above reported genes, there is literature evidence for their association with movement disorders in childhood.

As part of the project we had developed a 59 gene panel specifically designed for childhood onset MDs that we applied to the NGS analysis of samples of patients with ID/ASD and MDs, previously tested for ID/ASD, or directly referred for etiological evaluation of MDs phenotypes.

From November 2018 we analysed with the MD/CP panel 14 patients: 6 of these previously resulted negative to the ID/ASD panel, and 8 with prevalent early onset MD. This latter analysis has allowed to identify a total of 22 SNVs, new or with low frequency in public databases; we have found causative and possibly causative mutations in 5 patients. In 2/14 patients we detected SNVs in genes associated with recessive phenotypes (*AP4S1, MCPH1*). One child affected by a progressive neurological condition with spasticity of the lower limbs associated with ID, epilepsy and absent speech, was found to carry a homozygous known truncating mutation in *AP4S1;* the same genotype was also found in her newborn early symptomatic sister. A child with severe microcephaly was found to harbor a homozygous missense mutation in *MCPH1*, inherited from heterozygous unaffected parents. Interestingly, 2 brothers with similar phenotypes, were found to harbor a maternally inherited LP variant in *COL4A2*. We also identified one novel variant p.Ile1682Met in *SCN8A* (a gene well known to be cause of epilepticencephalopathy), predicted to be deleterious by several computational methods in a boy with ataxia and tremor but no epilepsy.

Conclusions Despite still preliminary, these results reinforce the utility of a targeted NGS approach in cohorts of clinically well characterized patients, and contribute to the recently highlighted evidence of a strong genetic base for these rare disorders.

ANALYSIS OF ANTIBIOTIC PRESCRIPTION PATTERNS FOR PAEDIATRIC PATIENTS IN HOSPITAL AND COMMUNITY SETTINGS AND IMPACT OF AN ANTIMICROBIAL STEWARDSHIP PROGRAM

Ph.D. Student: Dr. Elisa BARBIERI - TUTOR: Prof. Carlo GIAQUINTO
Ph.D. Course: Developmental Medicine and Health Planning Sciences
Curriculum "Emato-Oncology, Genetics, Rare Diseases and Predictive Medicine"

Background: The World Health Organization and the United Nations at the General Assembly of 2016 identified in the development of a country-level and institutional antimicrobial stewardship program (ASP) one of the key factor to tackle this antibiotic misuse and selection of resistant pathogens. The primary aim of my thesis is to improve the antibiotic prescription patterns both at community (WP1) and hospital (WP2) levels; I hypothesize that ASPs implementation will reduce the risk of inappropriate prescriptions and will results in a better healthcare for pediatric patients.

Material and Methods:

WP1: Data from family paediatricians prescriptions collected using Pedianet (Italian database), regarding AOM and GABHS were analyzed. Then a systematic review was performed on ASP implementation in pediatric settings to identify the best ASP to implement after WP1 data collection, if needed.

WP2: In order to evaluate the long term impact of the Clinical Pathways implemented in 2015 as ASP tool at hospital level the following aspects of antibiotic prescriptions were assessed: proportion of prescriptions following 'wait and see' approach (AOM only); rate of antimicrobial prescriptions by specific disease and active agent; proportion of prescriptions in accordance with CPs

Results:

WP1: 81.5% of AOM diagnoses were treated with an antibiotic within 48 hours (mainly amoxicillin and amoxicillin/clavulanate) and the "wait and see" approach was adopted only in 18.5% of cases. 79620 (63%) cases of pharyngitis were treated and among GABHS pharyngitis confirmed by rapid test 56% were treated with amoxicillin. The ones not test confirmed were treated mainly with broad spectrum antibiotics. As regarding the systematic review it was found that twenty papers described ASP in an outpatient setting, among which five were focused on education combined with audit and feedback, four were based on clinical decision support integrated in the electronic health record, two described only guideline distribution, one discussed an audit-and-feedback approach alone, and the others adopted a combination of two or more approaches previously mentioned.

WP2: During the 2 year post-intervention period 316 patients evaluated for pharyngitis were included in the study (Figures 5), accounting for the 2.40% (316/13,141) of total PedsED visits. One hundred and thirty-one children were diagnosed with GAS pharyngitis (42,7%; 135/316). Antimicrobial prescription rate for pharyngitis and complete statistical analysis for AOM and CAP will be completed during the end of the second year.

Conclusions:

Despite guidance to use the 'wait and see' approach in the age group analyzed, this strategy is not often used for AOM, as previously noted in other studies in hospital settings. Broad-spectrum antibiotic prescription was more frequent when pharyngitis was not confirmed by rapid test, in keeping with evidence from other studies that diagnostic uncertainty leads to overuse of antibiotics.

Paediatric ASPs have a significant impact on antimicrobial use, healthcare costs, and antimicrobial resistance in both inpatient and outpatient settings. Because of this significant impact, paediatric ASPs are spreading rapidly in the USA. Their implementation in Europe is still limited, possibly due to the fact that guidelines published so far (IDSA/SHEA) are designed for the USA healthcare system and easily adopted in this setting, while the diversity of healthcare systems throughout Europe and Asia implies a wide range of approaches to the same problem.

THE MOLECULAR LANDSCAPE OF PEDIATRIC ACUTE MYELOID LEUKEMIA: FROM GENES TO THERAPY

Ph.D. Student: Dr. Maddalena BENETTON - TUTOR: Dr. Martina PIGAZZI
Ph.D. Course: Developmental Medicine and Health Planning Sciences
Curriculum "Emato-Oncology, Genetics, Rare Diseases and Predictive Medicine"

Background
The mutational landscape of pediatric acute myeloid leukemia (AML) increased in the last ten years and nowadays the ongoing trials include a variety of somatic chromosomal rearrangements and genetic mutations screened at diagnosis as prognostic factors predictive of treatment strategies and outcome. The achievement of complete remission (CR) is the most important endpoint during treatment, and many efforts have been spent for the development of better tools to assess a precise response to treatment by measuring minimal residual disease (MRD). MRD after therapy may reflect the sum of all diagnosis and post-diagnosis resistance mechanisms being instrumental in reducing relapse and increasing children survival. The role of molecular MRD (MMRD) assessment is gaining relevance in pediatric AML and its integration in clinical trials and treatment decisions is under evaluation. This study would explore if AML MMRD testing can be included in a standard clinical practice to preventively identify disease occurrence and its role in identifying the dynamic changes in leukemic clonal heterogeneity during disease progression.

Material and Methods
We set up ten multiplex RT-PCR panels to detect up to 40 AML-specific genetic abnormalities with a prognostic value at diagnosis, included in the risk stratification of the ongoing AIEOP AML2013-01 protocol. For each rearrangement we set up a real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) to quantify mRNA transcripts in the 130 out of the 208 AML (62%) *de novo* pediatric cases with a molecular marker at diagnosis and monitor their MMRD after the I and II induction therapy courses. Retrospectively, we measured MMRD in 105 bone marrow samples collected before hematopoietic stem cell transplantation (HSCT). Moreover, three new mutations in *ASXL1*, *WT1* and *CEBPA* genes are currently screened by Sanger sequencing and GeneScan fragment analysis to increase the number of patients for whom MMRD can be monitored. Kaplan-Mayer curves have been created to analyze MMRD and new mutations impact in these patients.

Results
MMRD after the II induction course showed a worse overall survival (OS) for patients who reduced MMRD less than 3 logs with respect to diagnosis (75% vs 94%, p=0.05). MMRD identified among the Intermediate Risk patients with a higher risk to relapse and that could shift to the High Risk group after induction therapy as a better treatment option (48% vs 100%, p=0.04). Within the genetic subtypes, MMRD was significantly revealing those patients with t(8;21)*RUNX1-RUNX1T1* (CIR=53% vs 0%, p=0.04) and *FLT3ITD* with higher risk to relapse, playing a role in re-stratifying patients with the same genetic lesion. The role of MMRD on clinical outcome (OS 60% vs 82%, p=0.02) was confirmed also for the patients who underwent HSCT with higher levels of MMRD (>0.002 as cut off defined by ROC curve). The new screening of *ASXL1*, *WT1* and *CEBPA* mutations identified 37 new AML positive cases: at diagnosis, 19 were negative and 18 harbored a concomitant mutation, and its biological role is still under evaluation.

Conclusions
In this study, we found that MMRD monitoring is an independent factor to predict the effectiveness of therapy and relapse after induction therapy and before HSCT and its inclusion in trials must be pursued. New genetic screenings may refine AML pediatric patients risk stratification, open for new treatment tailored strategies and shed light into myeloid transformation process.

OSTEONECROSIS IN CHILDREN AND ADOLESCENTS WITH ACUTE LYMPHOBLASTIC LEUKEMIA: EARLY DIAGNOSIS AND NEW TREATMENT STRATEGIES

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Background/Aim:
In the last few decades, treatment strategies for acute lymphoblastic leukemia (ALL) have been associated not only with improvement of prognosis, but also with an increasing rate of late complication as osteonecrosis (ON). Herein, the cumulative incidence, risk factors, new conservative therapeutic strategies as hyperbaric oxygen therapy (HBO), and outcome of symptomatic ON were studied in pediatric patients with ALL.
Patients and Methods:
Between 2000 and 2017, 495 children and young adolescents with a diagnosis of ALL were evaluated. All the symptomatic patients underwent magnetic resonance imaging (MRI) to detect bone vascularization and structure.
Results:
Twenty-three out of 495 patients presented ON (4.6%). ON was associated with an older age ($p<0.0001$) and a higher steroid dose ($p=0.0013$). All the patients underwent standard therapies and HBO was performed in 8 of 23 patients. During the follow-up, 15 patients were stable: 6 were totally asymptomatic, 5 complained of pain during activity, and 4 presented mild function limitation.
Conclusion:
Our data highlight the importance of early diagnosis of ON by screening MRI in asymptomatic patients, in order to start conservative treatment strategies. Moreover, HBO could have beneficial effects on ON patients.

INTRATRACHEAL ADMINISTRATION OF MESENCHYMAL STROMAL-CELL-DERIVED EXTRACELLULAR VESICLES REDUCES LONG TERM LUNG INJURY IN A RAT MODEL OF BRONCHOPULMONARY DYSPLASIA

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Background
Bronchopulmonary dysplasia (BPD) is now the most common respiratory disorder among infants born extremely preterm, the degree of prematurity being the main predisposing factor. Its incidence, approximately 40% of infants born at <28 weeks of gestation, has remained the same for twenty years due to survival improving for the smallest newborn. The pathogenesis of BPD includes developmental immaturity with prenatal and postnatal mechanisms that cause inflammation and injury, consequently disrupting lung growth and triggering aberrant repair mechanisms. Current approaches to BPD (antenatal corticosteroids, surfactant therapy, protective non-invasive ventilation) are of limited efficacy in its prevention, but new areas of research are suggesting novel preventive and therapeutic options.
Both others and we have shown that extracellular vesicles secreted by mesenchymal stromal cells (MSC-EVs) can reduce hyperoxia-induced lung damage in a murine model of bronchopulmonary dysplasia (BPD). Since preterm newborns surviving with BPD tend to develop chronic obstructive lung disease later in life, we explored the long-term outcome of MSC-EV treatment in the BPD animal model following a recovery period until adulthood. In order to increase the translational significance of our work, we used human clinical grade EVs. We also opted for the intratracheal (IT) way of administration, since in clinical practice it should be less invasive than IV administration in preterm intubated newborns.
Material and Methods
At birth, rats were distributed in 5 groups (n=10): a (room air), animals raised in room air for 6 wk; b (sham-treated hyperoxia), animals exposed to 60% hyperoxia for 2 weeks and to room air for additional 4 weeks and treated with IT-administered saline solution; c (EV-hyperoxia), animals exposed to 60% hyperoxia for 2 weeks and to room air for additional 4 weeks and treated with IT-administered MSC-EVs. IT administration was given on postnatal days 3, 7, 10 and 21 in 70 uL volume for a total of 0.9x10E10 EVs. The animals were sacrificed at 6 weeks of age, the lungs were harvested and stereological assessment of alveolarization was performed by acquiring bright-field images of the preparations using a Leica DMR microscope and a high-resolution digital camera. Images were processed with the computer-based software tool STEPanizer.
Results
The sham-treated hyperoxia animals showed reductions in total surface area (Sair) and total volume (Vair) of alveolar air spaces and an increased mean linear intercept (a measure of the entire acinar space complex) with respect to the room air group. MSC-EVs administration resulted in a significant increase in Sair and Vair and in a significant decrease in mean linear intercept in comparison to sham-treated hyperoxia animals. In particular, mean linear intercept values in the EV- hyperoxia group were similar to those of animals raised in room air.
Conclusions
The present results demonstrate that IT treatment with human clinical grade MSC-EVs during the development of BPD results in a long-term improvement of lung alveolarization in a murine model of BPD, confirming the potential therapeutic value of these nanoparticles in preterm newborns.

STUDY OF ALVEOLAR RHABDOMYOSARCOMA MICROENVIRONMENT: ROLE OF GLYCOSAMINOGLYCANS IN TUMOR PROGRESSION

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Background: Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma among children and adolescents. It develops from immature mesenchymal stromal cells committed to a muscle lineage. It presents metastases in about the 25% of cases that are often associated with a malignant cellular phenotype but also with an altered extracellular matrix (ECM). In all organs, ECM is known to exert a pivotal role in cell proliferation and crosstalk. However, so far very low attention has been drawn toward RMS ECM. Like other cancer cells, also RMS cells possess the ability to produce the ECM proteins they need to create the optimal microenvironment for their growth. Indeed, ECM is composed not only of fibrillar proteins that provide structural support to cells, but also of a mesh of proteoglycans that regulate cytokines and growth factors availability in the proximity of their membrane receptor and are thus involved in tumor progression. Among all the glypicans, glypican 3 (GPC3) seem to be of interest for RMS biology. This heparan sulphate proteoglycan is known to be differentially expressed in tissues and at different moments of their development. Thus the study of the signals involved in GPC3 pathway may shed light on RMS development and metastasis formation.
Material and Methods: We started analysing GPC3 expression in different tissues at different stages of development using qRT-PCR: in particular, we compared GPC3 expression in different embryonal (RD, RH36...) and alveolar RMS cell lines (RH30, RH4…), xenogenic RMS samples, spheroids, healthy adult and pediatric muscle, neuroblastoma and colorectal cancer. We observed the complete GPC3 protein production using western blot, and we started studying the downstream signalling in two ways: (1) we analysed FGFR activity adding an FGFR inhibitor to tumor spheroids colture medium and (2) we observed metalloproteases expression using qRT-PCR and zymography. For the study of GPC3 function in RMS cells we proceeded with GPC3 silencing using two different approaches: (a) a transient silencing using a pool of siRNAs; (b) a stable silencing using CRISPR/CAS9 lentiviral vectors.
Results: We proved that RMS cells secrete their own ECM components of the structural moiety (laminin, fibronectin and collagen) and also of glycocalyx, such as GPC3. This proteoglycan is overexpressed in RMS cell lines of alveolar and, to a less extent, of embryonal origin. On the other hand, GPC3 expression is significatively reduced in fetal and paediatric healthy muscle as well as it is absent in adult healthy muscle cells and tissue respectively. In a 3D structure like tumor spheroids, GPC3 gene expression increased. As negative control we used the pediatric tumor neuroblastoma and the adult colorectal carcinoma. FGF signalling, that exerts different functions such as proliferation enhancement, strongly correlates with GPC3 expression in alveolar RMS cell lines, while a milder GPC3 detection was evident in embryonal RMS cell lines. All the tested cell lines were characterized by metalloproteases expression that was clearly enhanced in the xenogenic samples. Finally, the transient silencing technique allowed us to obtain a five-fold decrease of the initial amount of GPC3 in rhabdomyosarcoma cells. The procedure for CRISPR/CAS9 genome editing is ongoing.
Conclusions: The different behaviour of cells between 2D (petri culture) and 3D (spheroids and xenogenic samples, enriched in proteoglycans) encourages the study of ECM as a source of potential drug targets; in particular GPC3 may be a valid candidate due to its specific expression in RMS and absence in healthy and non-RMS samples (such as neuroblastoma and colon carcinoma). The final goal of the project foresees to modulate the composition of ECM, reducing tumor growth and, in the future, ameliorating patients' response to chemotherapy.

CHARACTERIZATION OF EXOSOME SMALL RNA CONTENT IN ANAPLASTIC LARGE CELL LYMPHOMA OF CHILDHOOD

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Background. Although modern therapies for Anaplastic Large Cell Lymphoma (ALCL) can treat more than 70% of patients, those who relapse have often a poor prognosis. Emerging evidence is indicating that circulating vesicles in plasma, particularly exosomes, carrying proteins, transcripts and other small non-coding RNAs (sRNA) play a role in several biological processes and actively contribute to the development of cancer *[Balaj et al. Nat Commun 2011, Skong et al. Nat Cell Biol 2008]*. The main aim of this study is to identify exosome-associated transcriptional biomarkers with clinical significance, with focus on relapse.
Material and Methods. Exosomes were obtained from plasma samples at diagnosis of 20 ALCL pediatric patients. RNA from exosomes and paired tumor biopsies was extracted and processed for small RNA-seq on Illumina platform. In addition, exosomes from plasma of 5 healthy donors were used as controls. RNA-seq data were analyzed using miR&moRe computational pipeline to obtain a comprehensive characterization of sRNAs produced by miRNA precursors *[Bortoluzzi et al. Blood 2012, Agnelli et al. Blood Cancer J 2019]*. Next, custom Python and R scripts were implemented to identify non miRNA-derived sRNAs by processing the sequenced RNA fragments not characterized by miR&moRe, such as reads not aligned on miRNA precursors or exceeding miRNA length. Small RNA differential expression was tested using DESeq2 *[Love et al. Genome Biology 2014]*.
Results. MiRNAs, miRNA-like RNAs and other sRNAs were detected and quantified in plasmatic exosomes. Of note, sRNA expression profile discriminated biopsies from exosomes and exosomes of cancer and healthy donors. miR-122-5p was the most significantly up-regulated miRNA in exosomes of ALCL patients both in comparison with matched primary tumours (LFC 10.3, adj p-value<0.001) and with exosomes of healthy donors (LFC 1.55, adj p-value<0.1). Most reads of exosome samples uncharacterized by miR&moRe were slightly longer than miRNAs (31 nt in average) and did not map to miRNA precursors. A bioinformatics pipeline was developed for non miRNA-derived sRNAs analysis extending miR&moRe software potential. Annotations showed that specific Y-RNA fragments were very abundant in exosomes, identifying as well putative Y-RNA-derived sRNAs significantly more abundant in exosomes of ALCL patients compared to healthy donors, which are undergoing experimental validation.
Conclusions. Exosomal sRNA species composition and expression profiles were different among ALCL patients and controls. Current investigation aims at clarifying the possible role of miRNAs and non miRNA-derived sRNAs in ALCL progression.

AIRWAY-ARTERY QUANTITATIVE ASSESSMENT IN CHILDREN WITH PRIMARY CILIARY DYSKINESIA USING CHEST COMPUTED TOMOGRAPHY

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Background
Chest computed tomography (CT) is the gold standard for detecting structural abnormalities in patients with Primary ciliary dyskinesia (PCD), such as bronchiectasis, bronchial wall thickness and mucus plugging. There are no studies on quantitative assessment of airways abnormalities in children with PCD.
The aim of our study is to quantify airway and artery dimensions on chest CT in a cohort of PCD patients compared to controls.
Material and Methods
14 spirometer-controlled chest CTs of 13 PCD patients and 12 CTs of control patients were collected retrospectively. The bronchial tree was segmented semi-automatically and reconstructed in a 3D-view. All visible airway-artery-pairs were measured perpendicular to the airway centre-line, annotating per branch inner and outer airway and adjacent artery diameter and computing inner airway diameter-artery ratio (AinA-ratio), outer airway diameter–artery ratio (AoutA-ratio), wall thickness (WT), WT-outer airway diameter ratio (Awt-ratio) and WT–artery ratio (AwtA-ratio).
Results
In PCD patients (38,5% male, mean age 13,5 y, range 9,8-15,3) 1526 AA-pairs were measured vs 1516 in controls (58,3% male, mean age 13,5 y, range 8-14,8). AinA-ratio and AoutA-ratio were significantly higher in PCD patients than in control patients (both $p<0,001$). Awt-ratio was significantly higher in controls than in PCD patients ($p<0,001$), but AwtA-ratio was not significantly different between the two groups ($p=0,193$). Furthermore, in PCD patients AoutA-ratio is significantly higher in distal segmental generations than in proximal ones ($p<0,001$).
Conclusions
Our study showed that in PCD patients airways are more dilated and do not show thickening compared to controls. Peripheral airways were more involved than proximal ones.

STUDY OF EXTRACELLULAR MATRIX IN HEALTHY AND PATHOLOGICAL CONDITION

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Congenital diaphragmatic hernia (CDH) is a birth defect characterized by the failure in diaphragm development that leads to abdominal organs herniation into the thoracic cavity and partial failure in lung development. CDH requires early surgical intervention, with the application of artificial patches to repair large defects. Unfortunately, these materials face major issues such as the lack of growth and integration with the surrounding muscle tissue, leading to an associated high rate of hernia recurrence.

The aim of my project is to recreate *in vitro* a biological functional skeletal muscle tissue using tissue engineering methods specifically developed for the diaphragmatic muscle. This engineered tissue can be used primarily to respond more efficiently to the clinical and surgical needs of diaphragmatic hernia replacing the current synthetic patches. It will also allow me to study the interactions between cells and extracellular matrix (ECM), the two main components that form and determine the characteristics of the tissue.

Recently we characterized the ECM obtained from decellularized mouse diaphragm. Decellularized ECM (dECM) demonstrated to be a suitable scaffold for the growth and differentiation of human muscle precursor cells (hMPCs) *in vitro*, allowing the generation of a functioning three-dimensional skeletal muscle structure containing both muscle stem cells and differentiated myotubes.

One of the major challenges in the skeletal muscle *in vitro* regeneration is the degree of fibres organization and alignment inside the dECM. In a static setting, part of seeded cells randomly aligned because not influenced by the pre-existing scaffold fibre direction. Combining an engineering approach with biological knowledge, we successfully developed an in-house bioreactor in which diaphragmatic constructs could be cultivated also exploiting a radial mechanical stimulus that helps all the cells to align and fuse in the correct direction and then mature in a functional construct. We designed the bioreactor platform exploiting numerical modelling based on the Finite Element Method (FEM) approach to estimate the correct structural behaviour of each single component. After pilot experiments, in which the mechanical radial strain was applied individually to the dECM and hMPCs, recellularized diaphragmatic-like constructs were cultivated in dynamic condition for 21 days, demonstrating a maintenance of proliferating cell pool but also an increased myotubes alignment and maturation.

In order to generate clinical-scaled dECM, we adapted our decellularization protocol to the piglet diaphragm. We combined perfusion via Inferior Vena Cava (IVC) and detergent/enzymatic treatment (3 cycle) using a specifically designed bioreactor to ensure sterility. Our histological and biochemical validation confirmed successful cell and DNA removal, as well as the preservation of native ECM components and structural architecture. Then we performed pilot experiments of recellularization using the piglet-derived dECM as scaffold and human muscle precursor cells.

ROLE OF THE NRF2 SIGNALING PATHWAY IN SUSTAINING CHEMORESISTANCE IN MEDULLOBLASTOMA

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Background: Nuclear factor erythroid 2 (NF-E2)-related factor 2 (NRF2) is a master regulator of redox homeostasis and cell detoxification. In various tumors it has been described that the cytoprotective effects of NRF2 enhance resistance of cancer cells to chemotherapeutic drugs. It remains unknown the role NRF2 has in sustaining chemoresistance in medulloblastoma, a highly aggressive pediatric brain tumor and the deadliest brain tumor of childhood.
Material and Methods: We recapitulated *in vitro* the chemotherapy-induced evolution of medulloblatoma (MB) by applying conventional therapy pressure to cells. Our work involved 3 primary MB cell lines and 1 primary patient derived cell line. Chemosensitivity, Nrf2 activation/expression and redox stress was assayed at various stages of the chemotherapy-induced selection. EdU incorporation assay was used to test cell cycling ability of resistant cells. Protein extracts have been collected for proteomic analysis.
Results: We were able to obtain chemoresistant cells showing a 6-fold increase in IC50 while at the same time maintaining cell cycling ability close to the therapy-naïve (untreated) cells. This chemoresistant phenotype is maintained also in "3D conditions" as proved by sphere formation assays. Spheres derived from resistant MB cells unlike therapy-naïve spheres maintain their diameter and architecture even after chemotherapy exposure.
NRF2 expression and activation as demonstrated by western blot and ARE-Luc (antioxidant response element) reporter assay, occurs after 2 rounds of chemotherapy and its levels rise significantly after each chemotherapy cycle. Furthermore, silencing of NRF2 on resistant cells restores chemosensitivity to its original levels (naïve-like response).
We also found that NRF2 activation triggered the expression of enzymes involved in glycolysis (glucose-6-phophate dehydrogenase, G6PD) and pentose phosphate pathway (transketolase, TKT) suggesting that also from a metabolic point of view, the resistant cells are different from the naïve cells.
Considering the role of NRF2 as a regulator of oxidative stress we compared therapy-naïve cells and resistant cells in their ability to counteract ROS production after hydrogen peroxide exposure. As measured through the DCFDA probe, resistant cells have a higher ability than naïve-therapy cells at scavenging ROS production.
Conclusions: We were able to prove that NRF2 has an important role in the emergence of resistance to chemotherapy in medulloblastoma tumors. Silencing of this transcription factor in resistant cells restored chemosensitivity. Resistant cells were characterized by a higher ROS scavenging ability and a different metabolic state than the therapy-naïve cells.
The protein extracts collected throughout the study will be analyzed in mass-spec proteomics to further characterize the NRF2-driven changes that occur during acquisition of the resistant phenotype.

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PhD COURSE "DEVELOPMENTAL MEDICINE AND HEALTH PLANNING SCIENCES"

COORDINATOR: Prof. Carlo GIAQUINTO

CURRICULUM "HEALTH PLANNING SCIENCES"

DIFFERENTIATED THYROID CARCINOMA IN PEDIATRIC AGE: GENETIC AND CLINICAL SCENARIO

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Curriculum "Health Planning Sciences"

Background Follicular-derived differentiated thyroid carcinoma (DTC) is the most common endocrine and epithelial malignancy in children. The differences in the clinical and pathological features of pediatric versus adult DTC could relate to a different genetic profile. Few studies are currently available in this issue, however, and most of them involved a limited number of patients and focused mainly on radiation-exposed populations.

Material and Methods We considered 59 pediatric patients who underwent surgery for DTC between 2000 and 2017. *RET/PTC* rearrangement was investigated with fluorescent in situ hybridization and real-time polymerase chain reaction. Sequencing was used to analyze mutations in the *BRAF*, *NRAS*, *PTEN*, *PIK3CA* genes, and the *TERT* promoter. The pediatric patients' clinical and molecular features were compared with those of 178 adult patients.

Results In our pediatric sample, male gender and age <15 years coincided with more extensive disease and more frequent lymph node and distant metastases. Compared with adults, the pediatric patients were more likely to have lymph node and distant metastasis, and to need second treatments ($p<0.01$). In all, 44% of the pediatric patients were found to carry molecular alterations. *RET/PTC* rearrangement was confirmed as the most frequent genetic alteration in childhood DTC (24.6%), and correlated with aggressive features. *BRAFV600E* was only identified in 16% of the pediatric DTCs, while NRASQ61R, NRASQ61K and TERTC250T mutations were very rare.

Conclusions Pediatric DTC is more aggressive at diagnosis and more likely to recur than its adult counterpart. Unlike the adult disease, point mutations have no key genetic role.

TAILORED NEURAL NETWORK AND SUPPORT VECTOR CLASSIFIER IN ORIENTING THE DIAGNOSIS OF RARE DISEASES

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Curriculum "Health Planning Sciences"

Background
The diagnosis of a rare disease is often a hard problem. First, the physician is unlikely to be familiar with rare diseases. Consequently, rare disease patients frequently experience delay in the diagnosis. The rareness of such diseases also makes them hard to study. Therefore, the scientific knowledge could be inadequate for proper diagnosis and treatments, resulting in a frequent problem of undiagnosed patients. Machine learning strategies have been widely employed in various medical applications, such as diseases diagnosis, image reconstruction and predictive analysis. As well known, such techniques need to be chosen and adapted with respect to the considered task, in order to obtain satisfactory results.

Material and Methods
In this study, we consider a group of 156 patients enrolled in an Italian ministerial project; they have been diagnosed of one out of eight rare pathologies and their distribution is not homogenous over the eight classes. Each patient is described by 277 binary features, obtained from a larger set of items by restricting to phenotypical information and by grouping items sharing properties related to features that are relevant for the considered syndromes. Moreover, in order to build a large dataset for the construction of the algorithms, for each considered pathology 10000 patients have been simulated, consulting the more recent scientific literature and information in Orphanet, OMIM and MeSh. We consider three different machine learning techniques: a feed-forward neural network, a support vector classifier and a random forest. For the first two methods, we adopt solutions devoted to the construction of tailored algorithms with respect to the considered task.
For the support vector classifier, we adopt a Boolean kernel, which is particularly suitable for our binary data with respect to other widely used kernels. Moreover, we adapt the neural network to our task by considering a weighted loss function, which is constructed using information about the prevalences of the considered features in the pathologies; the network weights different types of misclassifications in different manners.

Results
The neural network and the support vector classifier presented better results with respect to the random forest. In particular, the neural network with customized loss function outperformed the other proposed methods, presenting high scores in terms of precision and recall. We observed some difficulties related to the prediction of the less represented classes.

Conclusions
We tested and compared three different methods, addressing a problem of classification in the diagnosis for a group of patients affected by rare diseases. The considered diseases presented different reciprocal degrees of similarity concerning phenotypical and genotypical features. In our case, as well as in other settings, tailored constructed machine learning algorithms proved to represent an important resource in the diagnosis of rare diseases, being able to exploit the available information in an effective way.

PhD COURSE "PHARMACOLOGICAL SCIENCES"

COORDINATOR: Prof. Nicola FERRI

CURRICULUM "PHARMACOLOGY, TOXICOLOGY AND THERAPY"

PCSK9 INDUCES SMOOTH MUSCLE CELL-MEDIATED CALCIFICATION: *IN VITRO* AND *IN VIVO* EVIDENCES

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Ph.D. Course: Pharmacological Sciences
Curriculum "Pharmacology, Toxicology and Therapy"

Background Vascular calcification represents a main risk factor of cardiovascular events in patients with chronic kidney disease (CKD). Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9) is one of the major players into cholesterol homeostasis process. PCSK9 plasma levels correlate with the presence of calcific aortic valve stenosis and carriers of the PCSK9 R46L loss-of-function variant have a low calcific aortic valve stenosis.
We investigated a possible role of PCSK9 on aortic calcification by using a uremic rat model of vascular calcification and *in vitro* cultured human smooth muscle cells (hSMCs) overexpressing PCSK9.
Material and Methods Sprague-Dawley rats were fed a standard diet (n=10) or uremic diet containing 0.5% adenine (n=10) for 6 weeks. Urine volumes have been measured every two weeks by leaving rats in metabolic cages for 24h. At sacrifice, abdominal aortas, plasma, livers and kidneys have been collected. Hydroxyapatite deposition into the media has been measured by a calcium colorimetric assay and visualized by von Kossa staining. Plasma creatinine and phosphate levels have been evaluated by clinical standardized methods. PCSK9 expression in kidneys and liver has been visualized by Western Blotting. The overexpression of PCSK9 in hSMCs has been realized through retroviral infection. Both control and PCSK9-overexpressing hSMCs have been cultured with low-FCS/high-phosphate media (0.4% FCS *plus* 2.0mM or 2.4mM of NaH_2PO_4) for 7 days, changing media every two days. Hydroxyapatite deposition by cells has been measured by a calcium colorimetric assay.
Results The uremic condition was documented by increased urine volume (26 ml/day vs 58 ml/day), plasma creatinine (25.7 μM vs 208 μM) and phosphate levels (2.64 μM vs 6.11 μM). High phosphate concentration was associated to aortic calcification determined by measuring aorta Ca^{2+} concentrations (0.34 mg/g tissue vs 2.48 mg/g tissue) and by Von Kossa staining. This pathological condition was associated to a significant increase of total cholesterol (from 75.3 mg/dL to 107.6 mg/dL) and PCSK9 levels (from 40.1 ng/ml to 109.7 ng/ml). Higher expression of PCSK9 was also observed in kidney (+4.8 fold) and liver (+1.5 fold). The overexpression of PCSK9 in hSMCs (from 0.02 ng/ml to 11.3 ng/ml) induced a significant increase of extracellular calcification in response to 5 days exposure to 2.4 mM NaH_2PO_4 (+39% compared to control hSMCs), while NaH_2PO_4 reduced the release of PCSK9 from hSMCs (-33.6%) and the mRNA expression levels (-43%).
Conclusions The present study indicates a direct role of PCSK9 on vascular calcification associated to a CKD condition. Further analysis will attempt to identify the molecular mechanism of this action and to study the effect of monoclonal antibodies anti PCSK9.

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PhD COURSE "MOLECULAR MEDICINE"

COORDINATOR: Prof. Stefano PICCOLO

CURRICULUM "BIOMEDICINE"

MICROBIAL QUORUM SENSING SYSTEM: A TARGET FOR TREATMENT OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* INFECTIONS

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

Background

Staphylococcus aureus is involved in a large variety of diseases. Due to the invasive nature of infections and the spreading of antibiotic resistance, the development of new interventions without the selective pressure of antibiotics is mandatory. Quorum Sensing (QS) is a bacterial signalling mechanism that regulates virulence in clinically relevant bacterial pathogens. Our approach is to investigate and discover new inhibitors of the *S.aureus* QS acting on the *agr* operon. A recent report highlighted that Diflunisal reduces the expression of MRSA virulence factors. However, Diflunisal is a very insoluble drug and causes severe adverse effects. In this study we investigated the anti-virulence properties of some newly synthesized aza-analogs of Diflunisal.

Methods

Sixteen analogs of Diflunisal were screened. The cytotoxic activity of the analogs were tested by MTT assay on different human cell lines. Then, we evaluated the bacterial proliferation in presence of compounds. To identify the most active compound on QS, RNA was extracted from MRSA, incubated with or without analogues, and the expression of RNAIII was measured by qRT-PCR. Regulation of genes involved on MRSA virulence was investigated by RNAseq using the most promising compound, 2797. We tested compound's specificity to measure *agrA* expression on treated or non treated *S. epidermidis*. The mechanism of action of compound 2797 was assessed by EMSA assay evaluating the ability of compound 2797 to inhibit the binding of AgrA protein to promoter P2 or P3. Functional actions were investigated through haemolysis assay, macrophage killing assay, low pH assay, biofilm formation assay. Resistance or tolerance to 2797 inhibition of *agr* was evaluated measuring by qRT-PCR the expression of RNAIII on MRSA treated with compound for 10 days vs MRSA treated for 16h.

Results

We observed that on human cells our compounds are less cytotoxic than Diflunisal and that they didn't inhibit bacterial proliferation. Our data demonstrated that 2797 was the most active compound and partially inhibit the binding of AgrA to P3. These data were confirmed with all the functional assay: it inhibited hemolysis, it increased macrophages killing capability, the ability of MRSA to survive at low pH broth was reduced and at the end it increased the biofilm susceptibility to antibiotic. We can observe that 2797 down-regulated genes involved in MRSA virulence. Our analogue is active against MRSA but not against *S. epidermidis,* even if the homology of AgrA is about 80% between the two strains. Resistance or tolerance to 2797 inhibition of *agr* was not observed after multiple passages in culture, under the same conditions that instead induced resistance to conventional antibiotic.

Conclusions

We demonstrated the antivirulence activity of 2797 and its molecular mechanism through which this small molecule exerts its inhibitory activity on the production of MRSA toxins through quorum sensing. In the future, after molecular docking we will try to modify molecular structure of our compound to improve its activity.

DISRUPTION OF PROTEIN-PROTEIN INTERACTIONS AS A NEW ANTIVIRAL STRATEGY AGAINST INFLUENZA VIRUS AND HUMAN PAPILLOMAVIRUS

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

Background Influenza viruses cause a highly contagious respiratory disease in humans. In particular, influenza A virus is responsible for periodic epidemics that can have high mortality rates. Currently available drugs against influenza viruses possess several drawbacks, including the development of drug resistance. On the other hand, vaccines provide good protection against the infection, but they must be reformulated every year according to the circulating strains. Thus, there is still an urgent need for new anti-influenza compounds. Recently, the viral RNA polymerase complex emerged as an attractive target for drug development. Indeed, disruption of the correct assembly of the polymerase complex causes the inhibition of viral replication.
Human papillomaviruses (HPVs) are small double-stranded DNA viruses with epithelial tropism. Low-risk genotypes mainly cause cutaneous warts, while high-risk HPVs are responsible for the large majority of cervical carcinoma cases worldwide, and several other types of cancers, including ano-genital and head-and-neck cancers. The carcinogenic potential of HR-HPVs is related to two major viral oncoproteins, E6 and E7. In particular, E7 oncoprotein is able to interact with and inhibit a plethora of cellular partners, causing alterations in cellular pathways that contribute to cancer initiation and progression. Among these, E7 causes the degradation of the cellular phosphatase PTPN14, that behaves as an oncosuppressor by causing YAP retention in the cytoplasm, inhibiting its co-trascriptional activity.
Material and Methods The disruption of the interaction between the PA and PB1 subunits of the viral RNA polymerase by test compounds was assessed through ELISA-based assays. The antiviral activity in influenza virus-infected cells was tested by plaque reduction assays. Compounds cytotoxicity was assessed through MTT assay.
Protein levels were assessed through Western Blot experiments. Intracellular shuttling of proteins was visualized by means of immunofluorescence experiments and western blotting, following subcellular fractionation of protein lysates.
Results Some of the anti-PA-PB1 compounds were able to inhibit viral replication at low micromolar concentrations, although some compounds also resulted to be toxic. Some of the active compounds were also able to inhibit the PA-PB1 interaction in the ELISA-based assay.
Preliminary data confirmed the role of E7 in PTPN14 degradation, and the importance of some crucial residues in the C-terminal domain of E7 for the interaction between the two proteins.
Conclusions For what concerns PA-PB1 inhibitors, these data will be useful for Structure-Activity Relationship studies, which will allow the development of better analogs. Few compounds were active in both plaque reduction and ELISA-based assays, although some were also toxic. Further characterization of the mechanism of action of these compounds through *minireplicon assay* is ongoing.
Since we confirmed the role of few crucial residues driving E7-PTPN14 interaction, we will proceed with an *in silico* screening of small molecule compounds in order to find some inhibitors of this interaction, to be tested first in *in vitro* experiments and then in cellular context. The aim is to find one/more compound(s) able to restore the intracellular levels of PTPN14 protein.

GENERATION AND CHARACTERIZATION OF NEW HSV1-BASED ONCOLYTIC VIRUSES TOWARDS AN INNOVATIVE THERAPEUTIC APPROACH FOR TRIPLE NEGATIVE BREAST CANCER (TNBC)

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Curriculum "Biomedicine"

Background

Many tumors surrounded by an immunosuppressive tumor microenviroment (TME), like triple negative breast carcinoma (TNBC), are resistant to immunotherapy. Oncolytic viruses (OVs) selectively replicate in cancerous rather than healthy cells. OVs are promising immunotherapeutic agents for cancer treatment, as they provide immunogenic stimuli in the TME. Simultaneously, they can be gene therapy vectors. The HSV-1 based talimogene laherparepvec (Imlygic®, Amgen) was approved for clinical use in the EU and US.

Based on these considerations, the aim of my PhD project is the generation of an array of oncolytic viruses optimized to target the complex immunosuppressive features of the TME.

Material and Methods

Herpes simplex virus type 1 (HSV-1) genome was modified by bacterial artificial chromosome (BAC) mutagenesis in a strain of *Escherichia coli* allowing lambda-red recomineering. Viral stocks were reconstituted by lipofectamine transfection of BAC DNA into 29T cells. We measured the replicative capacity of the recombinant viruses in mammary tumoral cell lines and in tumoral and non- tumoral murine mammary organoids. The expression of transgene was evaluated.

Results

The Us_{12} gene was delete from the parental BAC. This shifts the expression kinetics of Us_{11} gene from late to immediate early, enhancing viral replication, without affecting safety. Recombinant viral genome were generated by insertion within the UL55-UL56

Intergenic region of therapeutic genes,including one enconding a single chain antibody targerting CCR4, expressed on regulatory T cells. Another HSV-1 genome carrying the enhanced green fluorescent protein gene was produced. Recombinant viruses efficiently replicated in breast cancer cell lines *in vitro* and in tumoral murine mammary organoids, but not in non-tumoral organoid. Exogenous genes were also expressed.

Conclusions

We are inserting further therapeutic genes including soluble Programmed cell Death–1 (sPD-1), human IL-12, and FMS-like tyrosine kinase 3 ligand (FLT3L) in a $\Delta\gamma34.5/\Delta Us_{12}$ BAC.

Next step will include *in vitro* characterization of the new viruses and testing viral therapeutic efficacy on an *in vivo* model.

VESICULAR STOMATITIS VIRUS PSEUDOTIPIZATION WITH CRIMEAN-CONGO HEMORRHAGIC FEVER VIRUS GLYCOPROTEINS AND ITS POTENTIAL APPLICATION

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

Background

Crimean-Congo hemorrhagic fever (CCHF) is considered one of the major emerging disease threats spreading to and also within the European Region following an increasing circulation of its main vector, ticks of the genus *Hyalomma*. CCHF is caused by Crimean-Congo hemorrhagic fever virus (CCHFV) that belongs to the *Orthonairovirus* genus of the *Nairoviridae* family . The spherical virions are enveloped and the RNA genome consists of three segments, small (S), medium (M) and large (L). The M segment encodes for a polyprotein that is processed to form the two envelope glycoproteins, Gn and Gc, that play a key role in the interaction with target cells and the subsequent viral entry. The characterization of CCHFV biology and the anti-infective drug discovery present significant logistical and safety challenges due to the requirement for BSL4 containment and procedure. With the aim to have some insight of the viral entry mechanism, we combined the use of the Vesicular Stomatitis Virus (VSV) pseudotyping approach with a reverse genetics approach at genome-wide scale on a mutagenized haploid murine embryonic stem cells library (haploid mESC).

Material and Methods

Viral Pseudotyping, Viral Serum Neutralization, Screening of mutagenized haploid stem cells

Results

We successfully obtained a pseudotyped virus bearing the CCHFV glycoproteins on its envelope (VSV-CCHFVg), which can be used in a BLS2 environment. The serum neutralization assay showed that the VSV-CCHFVg maintain the same antigenicity of the CCHFV. After that, we used the VSV-CCHFVg to infect the haploid mESC library in order to select resistant clones that should be mutagenized in genes essential for viral entry. The DNA extracted by the selected cells, is under analyses by NGS sequencing to identify the mutated genes conferring the resistant to viral infection.

Conclusions

With the present research project, we intend to develop a tool - that can be used in a BSL2 laboratory – to identify:

i) genes essential for CCHFV entry into target cells;
ii) molecules inhibiting the viral entry, as potential antivirals,
iii) the humoral immunity in people and/or animal models used for the development of innovative vaccines.

Our preliminary data showed that VSV-CCHFVg can be used to detect neutralizing antibodies in sera and that this virus can be used as a promising platform for the discovery of viral receptors and other cellular entry factors.

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PhD Courses
Medical and Biomedical Sciences

PhD COURSE "MOLECULAR MEDICINE"

COORDINATOR: Prof. Stefano PICCOLO

CURRICULUM "REGENERATIVE MEDICINE"

A COMPUTATIONAL APPROACH FOR RECONSTRUCTION OF THE HUMAN PLURIPOTENCY GENE REGULATORY NETWORK

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

Background
Pluripotent Stem cells (PSCs) are characterized by the ability to form all the differentiated cell types found in the adult organism and by the capacity to self-renew in vitro virtually indefinitely.
The choice between differentiation and self-renewal is affected by external cues including TGFb and FGF signals. Moreover, several pluripotency regulators have been identified such as the three master regulators NANOG, OCT4 and SOX2 but little is known about their interplay with external signals and their role during differentiation. This represents a complex biological problem, whose study requires a computational approach. The aim of this project is to understand how human PSCs compute external signals in order to choose between self-renewal and differentiation.
Material and Methods
Human Embryonic Stem Cell Culture, Microarray data analysis, RNA sequencing data analysis, RT-qPCR analysis, Cloning, Overexpression experiments, Reasoning Engine for Interaction Networks (RE:IN).
Results
In first instance, I exploited the Reasoning Engine for Interaction Networks (RE:IN), a Microsoft Research online tool, to investigate the role of TGFb in the maintenance of Human pluripotency.
This tool allowed me to generate a Boolean model consisting of functionally validated components and interactions inferred by co-expression data and Gain-of-Function (GOF) and Loss-of-Function (LOF) experiments. Such model was then challenged with experimental constraints proving to be compatible with observed human PSCs behaviour opportunely translated into a Boolean formalism. Experimental challenging refines the model empowering its predictive ability and generating a constrained Boolean Network. Such refined model was then used to formulate predictions on the effect of inactivation of two novel pluripotency regulators identified in our laboratory with remarkable results.
Conclusions
In this work we developed a computational model of the gene regulatory network controlling pluripotency in human Pluripotent Stem cells. The model generated is consistent with a total number of 94 constraints over 10 experimental observations. Furthermore, this model was used to perform *In-Silico* experiments of combined inactivation of different gene regulatory components whose results indicate remarkable predictive power and accuracy with 14 out of 15 correct predictions. To date, this ABN represents a promising starting point for a more comprehensive model of the human pluripotency network that will include also FGF signal. I will use this model for the study of the reprogramming process, with the aim of understanding the critical steps and enhancing the efficiency of induced PSCs generation. Moreover, this model will allow to elucidate how PSCs differentiate toward different germ layers.

CHARACTERIZATION OF THE BIOLOGICAL AND STRUCTURAL PROPERTIES OF CHEMICALLY MODIFIED POLYVINIL ALCOHOL AS A SCAFFOLD FOR TISSUE REGENERATION: AN *IN VITRO* AND *IN VIVO* STUDY

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

Background: Tissue engineering investigation is constantly in search of an ideal customizable scaffold to better fit the specific features of the tissue to be repaired. In this context, we recently developed a chemical modification of polyvinyl alcohol (PVA) by controlled oxidation with potassium permanganate ($KMnO_4$), replacing the 1% or 2% of hydroxyl groups with carbonyl groups in the polymeric chain. This resulted in a novel PVA-based scaffold with improved biodegradation rate and capacity to be loaded with biological molecules. The present work investigated for the first time the use of halogens [bromine (Br_2), chlorine (Cl_2) and iodine (I_2)] as less aggressive chemical agents to perform controlled PVA oxidation, with the aim of finding the reaction conditions that allow for the minor degradation of polymer molecular size.
Material and Methods: Solutions of PVA oxidized with a) potassium permanganate (OxPVA_$KMnO_4$), b) bromine (OxPVA_Br_2) c) chlorine (OxPVA_Cl_2) and d) iodine (OxPVA_I_2) were chemically characterized (i.e., viscosity measurements, moisture content, dinitrophenylhydrazine assay) before preparing hydrogels by physical cross-linking through freezing/thawing procedure. Finally, neat and oxidized PVA scaffolds were characterized for their specific ultrastructural morphology, mechanical properties, swelling index, ability of absorption/release of a model protein (bovine serum albumin), propensity to sustain cell adhesion and proliferation and capacity of *in vivo* biodegradation.
Results: The effect of chemical oxidation on PVA molecular weight (MW) preservation was assessed for the four oxidized hydrogels, verifying that bromine and iodine allow for minor alteration of polymer MW. According to Scanning Electron Microscopy (SEM), the surface of both neat and oxidized PVA exhibited a certain microporosity, which seemed to increase after chemical modification. Uniaxial tensile tests demonstrated that PVA mechanical properties can be customized through the polymer oxidation. After assessing that chlorine did not favour polymer MW preservation compared to $KMnO_4$-based oxidation, we decided to perform the following biological characterization only on OxPVA_Br_2 and OxPVA_I_2, considering PVA and OxPVA_$KMnO_4$ as references. Analysing polymer absorption capacity, both neat and oxidized PVA scaffolds reached the maximum swelling rate after 48 hours of incubation in PBS, with superior values for OxPVA_Br_2 and OxPVA_I_2 with respect to neat PVA, but inferior in comparison to OxPVA_$KMnO_4$. Given the necessity to develop controlled administration systems with the best pharmacokinetic and pharmacodynamic properties, we assessed the impact of halogen-based oxidation on PVA capacity to absorb/release a model protein (bovine serum albumin). The scaffolds demonstrated an exponential release kinetic up to 24 hours, reaching a plateau at 144 hours. Remarkably, the protein-loading study highlighted the possibility to modulate the absorption and release capacity of PVA not only through cross-linking, but also using different oxidizing agents. Interestingly, oxidized PVA biodegradation profile was investigated by subcutaneous implantation of hydrogel disks into BALB/c mice for 4 weeks. Explanted samples showed no serious inflammatory reactions and mild lympho-monocytic infiltration of the connective tissue surrounding the implanted material, confirming the low immunogenicity of the hydrogels. Macroscopic and histological study did not highlight meaningful changes of explanted scaffolds in comparison to the pre-implantation samples. Nevertheless, microscopic structural analysis seemed to demonstrate a superficial morphology modification, suggesting the beginning of a reabsorption process that will lead to the partial or total degradation of the polymer. Anyway, further tests at longer end-points will be necessary for a more complete analysis of biodegradation parameters.
Conclusions: Collected data showed that PVA oxidation with less aggressive agents like Br_2 and I_2 was as efficient as $KMnO_4$-mediated modification to implement the biological and structural properties of the polymer. Thus, it could be possible to customize the oxidative reaction in order to produce optimized PVA scaffolds for specific tissue engineering applications.

THE ROLE OF AUTOPHAGIC-RELATED PROTEINS AMBRA1 AND EPG5 IN THE GONADAL DEVELOPMENT, GAMETOGENESIS AND REPRODUCTION OF ZEBRAFISH

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

Background Autophagy, a conserved and ubiquitous cell recycling pathway essential to maintain cellular homeostasis, has been implicated in several physiological processes, including reproduction. Primordial germ cell (PGC) survival, gametes development, degradation of maternal material during the first stages of embryogenesis and cell differentiation are among key reproductive processes in which autophagy is involved. This project aims at analysing the interplay between autophagy and reproduction using three zebrafish knockout lines for autophagy-related proteins recently prepared. Our targets are: 1) AMBRA1, a positive regulator of the Beclin1-dependent autophagic pathway and also a regulator of apoptosis, cell proliferation and development and 2) EPG5, a Rab7 effector involved in the fusion specificity between autophagosomes and late endosomes/lysosomes during autophagy.
Materials and methods PCR-based genotyping of larval or adult tail biopsies. Microinjection with ATG and splicing-morpholino (MO) and *hAMBRA1* mRNA. Autophagy markers Western blotting. qPCR. Histological analyses. Immunohistochemistry against Vasa protein. Reproductive capabilities analysis. Sperm velocity, viability and count analyses. Electron microscopy of sperm samples.
Results Ambra1: The zebrafish genome contains two *ambra1* paralogous genes, *ambra1a* and *1b*. Differently from knockdown results, *ambra1a*$^{-/-}$ and *1b*$^{-/-}$ mutant embryos do not display overt developmental defects, due to compensatory effects of the paralogous gene remaining active. Preliminary western blot analysis shows that autophagy is reduced in mutant larvae. Silencing of the *ambra1b* gene leads to all-male individuals as demonstrated by histological analysis of 35 days post fertilization (dpf) larvae as well as visual investigation of secondary sexual features and reproductive behaviour of adults. However, KO *ambra1b* males do not present reproductive impairment nor alteration in the testis structure. Immunohistochemistry, performed at very early developmental stages against the germ cells marker Vasa, revealed a statistically significant reduction in PGCs number, confirmed by knockdown experiments with specific ATG and splicing MOs. A standard number of PGCs was rescued by injection of human *AMBRA1* mRNA. Presently, *ambra1b*$^{-/-}$ gonads of larvae deriving from *AMBRA1* mRNA microinjected embryos are being histologically analysed for ovary rescue.
Epg5: The *epg5* KO line is viable and fertile but present a reproductive impairment in both sexes starting at 8-10 months post fertilization. Histological analysis of female gonads revealed morphological alterations leading to the block of folliculogenesis at pre-vitellogenic stage. In males ultrastructural morphology of sperm samples was analysed by electron microscopy together with quality sperm features such as mobility, viability and velocity. No clear and statistically significant differences were found between mutant and WT sperm samples.
Conclusions The results obtained suggest a significant and paralogous-specific role of Ambra1b in protecting from PGCs over-loss during their development and migration. This underlines the sub-functionalization of the two paralogous genes, favouring their maintenance in the zebrafish genome. Moreover, the rescue of the phenotype with human *AMBRA1* mRNA implies that this function is conserved during evolution and suggests a potential role for hAMBRA1 in mammalian reproductive processes, a role that should be further investigated. A clear reproductive impairment in the *epg5* KO line was found in both male and female, with variable severity of phenotypes. To this date, a clear understanding of the pathways involved is still to be found.

ROLE OF MIRNAS IN GASTRIC ATROPHY, AUTOIMMUNITY AND DISEASE PROGRESSION

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Curriculum "Regenerative Medicine"

Background: MicroRNAs (miRNAs) are small non coding RNAs able playing a critical role in the regulation of gene expression at post-transcriptional level and acting thanks to an imperfect sequence complementarity between miRNA and target mRNA. MiRNA work as key regulators in many biological processes including mantainance of immune homeostasis and normal cell function. Indeed, a miRNA dysregulation seems to be involved in autoimmunity and in inflammatory and neoplastic diseases. We selected as a model two different types of chronic atrophic gastritis, the autoimmune gastritis (AIG) and the multifocal H.pylori-related gastritis (MAG), both representing gastric preneoplastic conditions, and eventually evolving into gastric adenocarcinoma with a different risk of neoplastic transformation, greater for MAG compared to AIG. Furthermore, in AIG the enterochromaffin cells hyperplasia, can further develop into gastric carcinoid, a neuroendocrine tumor (NET).
Material and Methods: we selected five specific miRNAs (miR-21, miR-155, miR-142-3p, miR-146, miR-223), involved into autoimmunity and cancer and compared their tissue expression in 30 patients, 10 AIG, 10 MAG and 10 patients without atrophic changes (controls). We performed an upper GI endoscopy in all patients with biopsy sampling, with staging by experts pathologists according to the OLGA staging system. Clinical and anamnestic data were collected. The expression of miRNAs on the collected gastric samples was determined by using qRT-PCR technique (subsequently quantified by the Livak method). Kolmogorov-Smirnov test was perform in order to determinate if the levels were normally distributed and parametric and non parametric, tests were used for statistics.
Results. Between AIG and MAG there was no difference in OLGA stage (p=ns), but a higher stage of atrophy in the antrum for MAG (p=0.0007) and in the corpus for AIG (p=0.0001). Dysplasia was diagnosed only in MAG (p=0.0032). Analyzing the expression of each single miRNA among AIG, MAG and controls, a significant difference (Kruskal-Wallis) was obtained for all miRNAs ($p< 0.05$). In the analysis between AIG and controls (Mann-Whitney) all miRNAs were characterized by a significant overexpression ($p <0.008$), as well as in the comparison between MAG and controls ($p < 0.05$). In the analysis between AIG and MAG, only miR-155, -142, -223 were significant overexpressed in the former ($p <0.05$). Analyzing miR-21 in controls and in OLGA I-II and III, a significant difference in miRNA in controls, OLGA I-II and III was observed ($p<0.0001$).
Conclusion: We observed a miRNA overexpression in the gastric mucosa with atrophy and chronic inflammation, suggesting a role for miRNA in the immunity-cancer axis. All miRNAs, excepted miR-21, were overexpressed in AIG as compared to MAG. We can therefore hypotesize a pathogenetic role especially for miR-155, -142, -223 in AIG, as they are involved in the control of T_{reg}-Th_{17}.
Homeostasis loss has already been postulated as a possible pathogenetic event in AIG. The overexpression of the carcinogenetic miR-21, in MAG vs AIG can be explained by the greater risk of gastric cancer in MAG. Furthermore, the higher expression of mirR-21 in advanced OLGA stages compared to early ones and non atrophic conditions, could make it a marker for progression of preneoplastic lesions towards cancer.

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

COORDINATOR: Prof. Annalisa ANGELINI

CURRICULUM "*BIOSTATISTICS AND CLINIC EPIDEMIOLOGY*"

DEVELOPMENT OF PREDICTIVE MODELS IN MULTICENTER STUDIES: A MACHINE LEARNING APPROACH

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Ph.D. Course: Translational Specialistic Medicine "G.B. Morgagni"
Curriculum "Biostatistics and Clinical Epidemiology"

Background
Risk prediction is a crucial topic in clinical research. Indeed, it is important to understand how the probability of a clinical endpoint changes given the characteristics of the subject to properly manage medical and economic resources for patient's care.
Logistic Regression (LR) and Cox Proportional Hazards (CPH) model are two examples of traditional statistical methods used so far to build predictive tools. Despite being extremely useful from a clinical perspective, these models may present some limitations: interactions among predictors and non-linear relationships must be pre-specified and it is often not clear how this procedure should be implemented. Machine Learning Techniques (MLTs) represent a valuable alternative to classical statistical methods, given their modelling flexibility which is potentially able to describe complex relationship between predictors and clinical outcomes.
Multicenter studies are often conducted to build predictive tools. In such studies, patients are clustered into units of higher hierarchy, such as hospitals, and it is likely that belonging to a given center may impact the risk of the outcome's occurrence. Hence, modelling the heterogeneity between centers may be important to include the influence of other factors related to the site that cannot easily be retrieved.
The aim of this study is to evaluate if the implementation of predictive models in multicenter studies with MLTs can be enhanced including center-specific effects. The approach will be applied to a multicenter prospective non-randomized study for the treatment of unprotected left main coronary artery disease.
Material and Methods
In this study we used the preliminary data of the Revascularization Of LEft main with resolute onyx (ROLEX) Registry, a multicenter prospective registry whose aim is to assess the safety and the efficacy of the new-generation zotarolimus-eluting stent Resolute Onyx in the treatment of unprotected left main coronary artery disease (ULMCAD), both isolated or in association with two or three-vessel coronary artery disease.
We employed a Bayesian Additive Regression Trees (BART) algorithm to build a predictive model for the risk any type of myocardial infarction at discharge. Two models were compared, both without and with center-specific effect through additive random intercepts. The comparison was carried out using Brier score.
Results
Overall, 270 patients were included in the preliminary analysis recruited October 1th, 2016 and June 4th, 2019. Models were built using information from 35 patient's features, i.e. demographic, clinical and procedural characteristics.
A moderate heterogeneity between centers was observed, with probability of the standard deviation of the random intercepts posterior distribution being higher than 0.15 equal to 0.68. Both models showed similar posterior distribution of Brier score. However, the model with center-specific effect showed a probability of almost 0.60 of having lower Brier score values than the model without center-effect.
Conclusions
The inclusion of heterogeneity between hospitals in multicenter studies can enhance the implementation of predictive models for risk of clinical outcomes with MLTs.

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

COORDINATOR: Prof. Annalisa ANGELINI

CURRICULUM "CARDIOTHORACIC AND VASCULAR SCIENCES"

SURGICAL TREATMENT OF ADVANCED HEART FAILUR

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Curriculum "Cardiothoracic and Vascular Sciences"

Background
Heart transplant (HTx) is the gold standard therapy for end-stage HF, however its application is restricted by the limited number of donor organs available and the mortality on waiting list remains high. Implantable left ventricular assist devices (LVADs) have been increasingly used to support patients before HTx, yet whether their use favorably impacts on pre- and post-HTx survival is still unclear.

Material and Methods
We retrospectively reviewed our institutional experience and included all patients diagnosed with end-stage heart failure, who were listed for HTx between January 2012 and December 2017. Exclusion criteria were: LVAD use as bridge to candidacy, technical/anatomical contraindications to LVAD, delisting due to causes other than clinical worsening. Data were analysed by a Machine Learning Analysis (MLA).

Results
Among 251 patients listed for HTx during the study period, we included 194 cases. Among these, 43 patients were directly implanted with an LVAD as BTT (LVAD group). Of the remaining 151 patients, 26 received a delayed LVAD implant (No-LVAD to LVAD patients) and 125 were never assisted with an implantable device (No-LVAD group). Baseline patient's characteristics were comparable among patients. MLA analysis showed that overall (sum of pre and post-HTx) survival was significantly higher in LVAD group.
Patients who underwent HTx were 138: 46 LVAD (including No-LVAD to LVAD patients) and 92 No-LVAD. LVAD patients were younger and more frequently male. Cardiac risk factors were similarly distributed amongst the two groups. No-LVAD patients showed higher pre-HTx pulmonary artery pressure and bilirubin level, with a lower glomerular filtration rate. Clinical status before transplant was worse in this group, with a significantly higher proportion of patients hospitalized in intensive care unit, on inotropic support and on temporary mechanical circulatory support. Donor characteristics were similar in the two group. Mean time of follow-up was 26±22 months. Mortality after HTx was similar between LVAD and No-LVAD. Rates of adverse events after HTx were comparable as well. LVAD patients showed a higher rate of donor specific antibody development.

Conclusions
Post-HTx survival is comparable between patients previously assisted with LVAD or not. Nevertheless, when considering also the pre-HTx period, the strategy of supporting patients with LVADs as BTT confers a significant survival benefit.

LEFT VENTRICULAR PHENOTYPE IN ARRHYTHMOGENIC CARDIOMYOPATHY: PREVALENCE AND DIFFERENTIAL DIAGNOSIS WITH DILATED CARDIOMYOPATHY

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Curriculum "Cardiothoracic and Vascular Sciences"

Background: The prevalence and morpho-functional features of left ventricular (LV) involvement in Arrhythmogenic cardiomyopathy (ACM) in living patients has not been deeply investigated. Furthermore, the left ventricular phenotypes (LVP) in biventricular and left-dominant variants of ACM may overlap with dilated cardiomyopathy (DCM), making differential diagnosis challenging.

We aimed to assess the prevalence and clinical features of LV involvement in a large cohort of patients with ACM and to identify the clinical, electrocardiographic (ECG) and cardiovascular magnetic resonance (CMR) features, which could help in differential diagnosis between the LV phenotype in ACM and DCM.

Methods: 87 patients with ACM (56% men, median age 34 years) diagnosed according to 2010 International Task Force Criteria and 153 patients with idiopathic DCM (69% men, median age 51 years) were studied. All patients underwent a comprehensive cardiovascular evaluation including medical history, 12-lead ECG and CMR. LV involvement in ACM was defined by the CMR evidence of LV dysfunction, non-ischemic LV late gadolinium enhancement (LGE) or both.

Results: 58 (67%) ACM patients showed LV involvement, consisting of LV LGE with LV dysfunction in 41 subjects (47%) and LV LGE without LV dysfunction in 17 (20%); all 41 patients with LV dysfunction had LV LGE (ACM-related LV phenotype). LV LGE was found in 69 (45%) patients with DCM (DCM-related LV phenotype). Compared to DCM, patients with ACM LV phenotype showed significantly more often low QRS voltages in limb leads (42% vs 4%, $p<0.001$), infero-lateral T-wave inversion (32% vs 6%, $p=0.001$) and major ventricular arrhythmias, while they rarely had a left bundle branch block (0 vs 28%, $p<0.001$) and the ECG pattern of LV hypertrophy. At post-contrast CMR images, LV LGE amount was significantly greater in ACM patients (25% vs 13%, $p<0.001$), and more commonly localized in the subepicardial layers (98% vs 16%, $p<0.001$). LV LGE amount $\geq$ 20% (the best cut-off at receiver operating characteristics analysis) had a 68% sensitivity and 100% specificity in diagnosis of ACM. LV EF inversely correlated with the extent of LV LGE in ACM patients ($r= -0.63$, $p<0.001$), but no in DCM patients ($r= -0.01$, $p=0.94$).

Conclusions: LV involvement in ACM is common and is characterised by clinical and CMR features which allow differential diagnosis with DCM. Distinctive clinical findings included low QRS amplitude in ECG limb leads, inferolateral repolarization abnormalities, propensity to major ventricular arrhythmias and the correlation between the LV systolic dysfunction and the extent of LV LGE.

COMBINED PERCUTANEOUS EDGE-TO-EDGE MITRAL VALVE REPAIR AND LEFT ATRIAL APPENDAGE OCCLUSION

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Curriculum "Cardiothoracic and Vascular Sciences"

Background. Percutaneous edge-to-edge mitral valve repair with the MitraClip system has emerged as a safe and efficacious treatment for selected patients with severe mitral regurgitation (MR) deemed at high surgical risk or inoperable. Similarly, percutaneous left atrial appendage (LAA) occlusion has proven its safety and efficacy for the prevention of cardioembolic stroke in patients with non-valvular atrial fibrillation (AF) and contraindications to oral anticoagulant therapy or at high risk for major bleedings. Patients undergoing percutaneous mitral valve repair are frequently affected by AF and are at high risk for major bleedings due to comorbidities or concomitant indications for antiplatelet therapy. Since both the procedures are performed through a femoral venous route and require trans-septal puncture, it seems reasonable, from an interventional cardiologist's perspective, to combine them. To date, however, only few case reports and small case series have been reported on this topic.

Material and Methods. We performed a retrospective multi-center study of patients with significant MR and AF undergoing MitraClip device implantation plus LAA occlusion in the same procedure. Demographic and clinical characteristics, including risk of stroke and major bleeding (CHA_2DS_2-VASc score, HAS-BLED score), echocardiographic and procedural data were captured. Follow-up were carried out as per each institution's standard practice. Endpoints were defined according to the Mitral Valve Academic Research Consortium classification, the Bleeding Academic Research Consortium classification, and the Munich consensus.

Results. Thirty patients underwent combined MitraClip implantation and LAA occlusion. Median patient age was 74.5 years (interquartile range [IQR] 68.1-79.8 years) and the left ventricular ejection fraction was 41.5% (IQR 31-56%). Median CHA_2DS_2-VASc and HAS-BLED scores were 4 and 3.5, respectively. Median predicted mortality according to EuroScore II was 6.57% (IQR 3.51-15.16%). Fifty-three percent of patients had a history of myocardial infarction. The etiology of MR was functional in 40%, degenerative in 30%, and mixed in 30% of the population. In 90% of patients mitral valve repair was performed as first procedure. Median procedural time was 133 (IQR 103-172) min, and radiation time 39 (IQR 33-50.5) min. Technical success was 97% in the overall study population. Procedural success was achieved in 28 patients. Follow-up was available in 90% of patients (27/30). Four non-cardiovascular deaths were reported at follow-up. No thromboembolic events occurred. There was one major bleeding. One patient underwent additional MitraClip procedure 14 months after the index procedure. Sixty-three percent of patients showed persistent clinical improvement (reduction of at least one NYHA functional class) during follow-up.

Conclusions. Combined percutaneous edge-to-edge mitral valve repair and LAA occlusion appears to be feasible and safe, and associated with acceptable procedural success and a favorable outcome in terms of prevention of AF-related thromboembolism and reduction of bleeding. These preliminary results have to be validated in large randomized studies, with longer follow-up.

AORTIC VALVE REPLACEMENT: A PATHWAY TOWARD EXCELLENCE

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Background: Pathologies of ventricle outflow tracts (LVOT and RVOT), are a large spectrum of diseases that involves people from childhood to senescence. Although repair techniques are preferred especially for younger patients, replacement of aortic or pulmonary valve (AVR and PVR) represents nowadays the definitive treatment in grown-up and aging patients. Biological prostheses (or bioprostheses) offer advantages compared to mechanical ones in terms of low rate of thromboembolism and no long-term anticoagulation. However, they are related to limited durability, especially regarding structural valve degeneration (SVD), and other complications. Preclinical research and clinical experience allowed advancements in techniques and technologies for cardiac valve replacement.
Aims of this research are to evaluate innovations in commercial and bioengineered prostheses in terms of valve manufacturing, implantation methodology, and biomaterial evolution, using advanced experimental models too.
Materials and Methods: This research is divided into 3 preclinical and clinical projects:

1) A systematic literature review of all studies focused on the performance of current biological prostheses implanted in aortic position, which represents the main clinical scenario for these substitutes. Endpoints are early- and long-term clinical and hemodynamic outcomes.
2) A retrospective study of the most recent bioprostheses, i.e. rapid-deployment valves (RDV). Early- and mid-term clinical and hemodynamic outcomes from a National Registry were analysed.
3) Experimental surgery with tissue engineered heart valves in allogenic (pig-to-pig and human-to-human decellularized valves) models and in a novel xenogenic model represented by a genetically engineered pig to mimic the human immune response toward xenoantigens.

Results:

- The metanalysis is still ongoing.
- 1402 patients from 21 Italian centres underwent RDV implantation for aortic valve stenosis treatment (March 2012-March 2019). Minimally invasive approaches occurred in 614/946 isolated AVR (65%). The Valve Academic Research Consortium success was 98%. Postoperative pacemaker implantation occurred in 86 patients (6.1%). Early overall mortality was 2% (28 patients), and survival at 3 years was 94±1%. Postoperative transvalvular mean gradient was good (10.8±4.6 mmHg) and it remained stable at follow-up.
- The well-established TRICOL protocol will be applied in porcine aortic and pulmonary valve conduits. These roots will be implanted in orthotopic position in an allogenic model to evaluate their hemodynamic performances, immunologic compatibility and potential of tissue regeneration. The same protocol will be applied in a xenogenic model in order to assess possible immunologic response to decellularized porcine graft. This novel model is under validation. TRICOL protocol was also applied for aortic homografts for RVOT replacement in 3 human patients. At 3-month follow-up, transthoracic echocardiography showed a good hemodynamic performance of homografts without stenosis or regurgitation; at 2-year follow-up (1 patient) the hemodynamics was stable (peak gradient 12 mmHg, mild regurgitation).

Conclusions: Rapid-deployment valves provide good early and mid-term clinical and hemodynamic outcomes. These devices may be a suitable alternative to conventional bioprostheses especially in minimally invasive and combined operations. A word of caution should be mentioned for conduction disorders sequelae. In vivo preclinical research will verify whether allogeneic and/or xenogeneic decellularized valve scaffolds can be proposed as the ultimate therapeutic solution for SVD.

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

COORDINATOR: Prof. Annalisa ANGELINI

CURRICULUM "ENDOCRINE AND METABOLIC SCIENCES"

EFFECTS OF FENOFIBRATE ON CIRCULATING STEM AND ENDOTHELIAL PROGENITOR CELLS IN PATIENTS WITH DIABETES AND RETINOPATHY - A RANDOMIZED PLACEBO-CONTROLLED TRIAL

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Curriculum "Endocrine and Metabolic Sciences"

Background. Long-standing diabetes is often complicated by retinopathy. The mechanisms that induce the development of diabetic retinopathy are incompletely understood and include alterations in bone marrow derived circulating stem cells (CSCs) and endothelial progenitor cells (EPCs).
Fenofibrate is a PPAR-alpha agonist used for the treatment of mixed dislipidemia and hypertriglyceridemia. In addition to lowering triglyceride-rich lipoproteins, fenofibrate exerts several additional benefits on the vessel wall, including reduction of inflammation. In a trial conducted in type 2 diabetic patients, fenofibrate reduced retinopathy-related endpoints, suggesting a direct effect of the drug on the mechanisms that drive the development of this complication.
Preliminary data show that fenofibrate has the potential to improve CSC survival in vitro and, consequently, may benefit patients with retinopathy. Herein, we tested whether treatment with fenofibrate increases CSC and EPC levels in diabetic patients with retinopathy, compared to placebo.
Material and Methods. Patients with type 1 and type 2 diabetes were randomized to receive fenofibrate 145 mg or matching placebo for 12 weeks. CSC ($CD34^+$, $CD133^+$, $CD34^+CD133^+$) and EPC ($CD34^+KDR^+$, $CD133^+KDR^+$ and $CD34^+CD133^+KDR^+$) were measured by flow citometry at baseline and at study end. The primary end-point was the change in CSC and EPC levels in fenofibrate-treated versus placebo-treated patients over 12 weeks.
Results. Forty patients were enrolled in the study, n=20 in the fenofibrate group and n=20 in the placebo group. One patient in the placebo group was lost to follow-up, leaving 39 completers for the analysis. Patients were on average 57.1 year old, with a known diabetes duration of 18.3 years and a baseline HbA1c of 7.6% (59 mmol/mol). After 12 weeks, CSCs $CD34^+CD133^+$ increased significantly (from $134.3 \pm 17.3/10^6$ to $185.1 \pm 26.1/10^6$; $p = 0.012$), other phenotypes increased non-significantly in the fenofibrate group. In the placebo group, CSCs and EPCs decreased non-significantly or remained stable. The change from baseline of all CSC phenotypes was significantly different between the two groups ($CD34^+$ $50.6 \pm 33.4/10^6$ in the fenofibrate group vs $-44.2 \pm 34.2/10^6$ in the placebo group, $p = 0.049$; $CD133^+$ $48.0 \pm 24.8/10^6$ in the fenofibrate group vs $-46.3 \pm 22.7/10^6$ in the placebo group, $p = 0.007$; $CD34^+CD133^+$ $50.8 \pm 18.8/10^6$ in the fenofibrate group vs $-22.4 \pm 17.0/10^6$ in the placebo group, $p = 0.005$).
Conclusions. A treatment of 12 weeks with fenofibrate significantly increased CSCs in diabetic patients with retinopathy. Thus, the beneficial effect of fenofibrate on diabetic retinopathy could involve the modulation of stem / progenitor cells.

DIABETES MELLITUS REDUCES THE LEVELS OF CIRCULATING PROANGIOGENIC NEUTROPHILS

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Background
Diabetes mellitus impairs the ability of ischemic tissue to restore perfusion, which contributes to cardiovascular risk. One of the defective angiogenic pathways in diabetic patients is the reduction of different subsets of blood-derived proangiogenic cells. Among these, we evaluated if any alteration in proangiogenic neutrophils (PANs) occurs in diabetic patients.

Material and Methods
PANs were identified as $CD49d^{+}CXCR4^{high}VEGFR^{high}$ neutrophils and quantified by flow cytometry in fresh peripheral blood samples of diabetic patients (type 1 and type 2 diabetes) and non-diabetic control individuals.
To characterize pro angiogenic properties of PANs *in vitro*, neutrophils were isolated from healthy donors by immunomagnetic depletion, CD49d+ and CD49d- neutrophils were sorted from the granulocyte morphologic gate and coincubated with HUVECs to perform 2D (tubules formation) assay and 3D (spheroid sprouting) assay.

Results
In healthy individuals, 7.6±0.8% of the granulocytes were positive for CD49d expression. $CD49d^{+}$ neutrophils had a markedly increased percentage of $CXCR4^{+}$ cells and mean fluorescence intensity (MFI) of CXCR4 than $CD49d^{-}$ neutrophils. Moreover, most of the $VEGF^{+}$ neutrophils also expressed CD49d.
Their ability to support angiogenesis during 2D assay was tested, and length and number of tubules generated were significantly higher when HUVEC were cocultured with $CD49d^{+}$ than $CD49d^{-}$ neutrophils. Furthermore, $CD49d^{-}$ neutrophils interspersed in the Matrigel, while $CD49d^{+}$ neutrophils mainly located within tubules. Similarly, when performing the 3D assay, presence of $CD49d^{+}$ versus CD49d- neutrophils significantly increased the sprouting area of HUVEC spheroids. Furthermore, neutrophils from diabetic patients were less able to promote in vitro tubules formation, if compared to neutrophils from healthy individuals. In fact, the percentage of $CD49d^{+}$ neutrophils was significantly lower in diabetic patients (5.5±0.5% of the granulocytes versus 7.6±0.8% in non-diabetic individuals). Particularly, an inverse correlation was found between blood glucose level and PANs number.

Conclusions
PANs were shown to support in vitro angiogenesis by physically interacting with HUVECs. Neutrophils from diabetic patients showed a defective support of angiogenesis and a significantly reduced percentage of PANs, providing evidence for a direct role of hyperglycemia.

CIRCULATING miRNAs AS CARDIOVASCULAR BIOMARKERS IN TYPE 2 DIABETES

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Curriculum "Endocrine and Metabolic Sciences"

Background
Atherosclerosis is the leading chronic complication of type 2 diabetes (TDM2), in terms of both morbidity and mortality. However, pathogenetic mechanisms underlying atherosclerotic process in T2DM are not completely understood. Recently, microRNA (miRNAs), either circulating in plasma or associated to circulating microparticles (MPs) seem to participate in atherosclerosis. MPs are a heterogeneous population of vesicles, released from various cell types (monocytes, platelets, endothelial cells, etc.) by different stimuli, such as inflammation, oxidative stress, or shear stress. MPs contribute to the transfer of RNAs from the parent to target cells, to modulate the expression of specific genes; miRNAs contained in MPs may influence vascular homeostasis. We observed a significant reduction of miR-30c-5p in circulating MPs of non-diabetic subjects who, followed up for 11 years, developed atherosclerotic carotid plaque (CP). Therefore, the aim of this study is to identify miRNAs associated with atherosclerosis, in patients with T2DM.

Material and Methods
First, a wide panel of miRNAs will be tested in MPs of a discovery cohort of selected TDM2 patients (n 44), to identify miRNAs associated with atherosclerosis. Second, miRNA differently expressed with atherosclerosis will be tested in a wide sample of T2DM subjects, for validation. The 44 T2DM patients (age 59±7 yrs) of the discovery cohort were identified from the database of our Outpatient diabetic clinic, 22 of them developed a carotid plaque (CP+), while 22 did not (CP-), after a mean follow-up of 6±2 yrs. CP+ and CP- T2DM were matched for age, diabetes duration and glucose control (HbA1c). Mean stenosis degree of CP was 30±7 % in CP+. All patients had plasma samples collected at baseline. As a first step, we characterized plasma MPs in baseline specimens. MPs were centrifuged at 3000g for 10 minutes and at 16000g for 40 minutes to isolate MP from plasma samples. MPs were identified by a Cytoflex flow cytometer (Beckman Coulter, Miami Florida). The MPs gate was established using a blend of mono-dispersed fluorescent beads of three diameters (0.5, 0.9, and 3 μm). MPs were directly incubated with 2 μl of fluorescent conjugated monoclonal antibodies against cell-type specific antigens and 2 μl of annexinV-FITC. Endothelial-derived MPs (CD62E-phycoerythrin (PE), platelet-derived MPs (CD62P-allophycocyanin (APC)), and leukocyte-derived MPs (CD45-PE), were determined.

Results
Endothelial-derived, platelet-derived, and leukocyte-derived MPs were significant higher (all $p<0.05$) in CP+ T2DM subjects.

Conclusions
In T2DM patients prone to atherosclerosis, MPs appear to be higher. The clinical significance of this finding has to be confirmed in future studies. Next research step will be the characterization of miRNAs in MPs isolated from T2DM with or without carotid atherosclerosis, and then the validation of these miRNAs in a wide T2DM population sample with and without atherosclerotic cardiovascular disease.

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

COORDINATOR: Prof. Annalisa ANGELINI

CURRICULUM "NEUROSCIENCE"

WILD-TYPE TRANSTHYRETIN AMYLOIDOSIS: CLINICAL, NEUROPHYSIOLOGICAL, IMAGING AND PATHOLOGICAL PROFILE

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Curriculum "Neuroscience"

Background Wild-type transthyretin-related amyloidosis (wt-TTR amyloidosis) is an under-recognized cause of congestive heart failure with preserved systolic function in the elderly population. Only a subgroup has peripheral neuropathy, but bilateral carpal tunnel syndrome (CTS) is a common feature.

Material and Methods All patients underwent family and personal history, physical examination, and complete cardiological evaluation including EKG (rhythm, QRS voltages, atrio-ventricular or intraventricular delay, repolarization changes) and echocardiography [ventricular wall thickness, systolic function, regional motion abnormalities, deformation (strain) indexes, diastolic filling pattern, presence of valvular abnormalities].

A selected subgroup underwent also:

- cardiac MRI (to quantify T1 and T2 weighed-images, presence and amount of late-gadolinium enhancement)
- 99mTc-DPD bone scintigraphy (to look for cardiac uptake, using the following classification: score 0 absent cardiac uptake with normal bone uptake; score 1 mild cardiac uptake, inferior to bone uptake; score 2 moderate cardiac uptake with attenuated bone uptake; score 3 strong cardiac uptake with mild/absent bone uptake)
- tissue biopsies (periumbilical fat, endomyocardial and salivary glands).

Moreover, all patients underwent complete neurological examination, with a selected cohort undergoing also neurophysiology and nerve ultrasound.

TTR gene molecular analysis was scheduled to define the possible presence of mutated TTR.

Results To date 16 patients (14 men, mean age 77.1±8.6 yrs, range 56-86) diagnosed with wt-TTR amyloidosis were considered. All were admitted to the hospital for cardiac symptoms; 7/16 had atrial fibrillation, 4/16 underwent pacemaker implantation, 2/16 aortic valve replacement. In 11/16, serum NT-proBNP and troponin levels were stably increased. Low voltages and repolarization abnormalities were observed at the EKG in 7/16 patients. Echocardiography disclosed increased thickness of ventricular walls in all patients, with median ejection fraction 53% (range 37-66), and reduction in 6/16 patients. Cardiac MRI and bone scintigraphy, available in 10/16 patients, supported the diagnosis. Endomyocardial and salivary glands biopsies (performed in 5 and one patient, respectively) showed wt-TTR deposits while periumbilical fat biopsies (4 patients) resulted negative. Nine patients had bilateral CTS, with 2 diagnosed also with cubital tunnel syndrome and one had lumbar stenosis; 2 had significant weight loss. Neurological examination showed no abnormalities in 11/16 patients and 3/16 had CTS. Neurophysiology, available in 8/16 patients disclosed mild axonal sensory-motor polyneuropathy in 2 patients, bilateral CTS in 3 patients and right ulnar nerve axonal neuropathy in one. Nerve ultrasound, performed in 7 patients, showed increased cross-sectional area of the median nerves in 2 patients and of both sciatic nerves in one of the patients with axonal neuropathy.

Conclusions Despite the slower progression and longer survival compared with other types of amyloidosis, cardiac involvement in wt-TTR still constitutes a leading cause of mortality. Neurological involvement other than CTS has been reported in 18.7% of our patients, both clinically and neurophysiologically. With increasing availability of treatments for TTR amyloidosis (both mutant and wild-type), a prompt diagnosis is crucial in order to guarantee early access to disease-modifying therapies.

EFFECT OF AGE ON THE MUSCLE SPINDLE IN TRICEPS SURAE AND THORACOLUMBAR FASCIA IN MOUSE

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Background: The muscle spindle (MS) is a specialized stretch receptor located within the skeletal muscle, which plays an important role in proprioception and coordination. MS is surrounded by a strong capsule proper of connective tissue that is composed of collagen fibers and fibroblasts and is continuous with the perimysium. A gelatinous fluid rich in glycosaminoglycans fills the space between this inner capsule and the capsule proper, probably mainly hyaluronan (HA). Age-related proprioceptive and coordinative deficits, as the position, kinesthesia, "dynamic position" sense, are unclear, although some hypothesises have been proposed to explain those neuro-physiological mechanisms both on the peripheral and CNS changes. The thoracolumbar fascia (TLF) could be considered fascial continuity areas of the lumbar, abdominal and pelvic region, which could perceive the state of contraction of different muscles, could play a major role in coordinating movement and in the activation of different muscles. The TLF has been proposed to represent a possible source of low back pain (LBP). However, it is unclear how these characteristics relate to the age. The aim of this study was to investigate the age-related changes of MS and the posterior layer of TLF in mouse under microscopic point of view to evaluate a possible role of age-related fascia changes of MS in the peripheral neuro-physiological mechanisms of age-related proprioceptive and coordinative deficits and to evaluate a possible role of age-related TLF changes and their relationship with the pathophysiological mechanisms, especially its role in pain. **Material and Methods:** Age-related changes of MS were compared in unmature (1month, M); young (4M); middle age (10M) and old (27 M) C57BL/6J male mice. Hematoxylin Eosin (HE), Sirius-red, Van Gieson staining were used to evaluate age-related changes the morphology of MS and TLF. The collagen type I, III, Hyaluronic acid binding protein and anti-elastin antibody immunostaining were used to monitor age-related changes in collagen type I, III, elastin and HA of MS and TLF. The Purple-Jelley HA assay (Biocolor Ltd.) was used to measure age-related changes HA contents. RT-PCR analysis were used to evaluated the age-related mRNA expression changes of link protein and enzymes/proteins associated with HA metabolism. S100, Calcitonin Gene Related Peptide (CGRP), Tyrosine hydroxylase (TH) antibody were used to measure age-related innervation changes of the posterior layer of TLF. **Results: MS**: MS is surrounded by a strong capsule and hyaluronan (HA) filled the capsule proper. The spindle diameter is 18.72 ± 4.05 μm at the equator. The capsule of the MS and its continuous with the perimysium, epimysium primarily consisted collagen type I and a small amount of collagen type III using polarized light. Van Gieson showed that the presence of the elastin in the capsule. The amount of HA in the Triceps Surae muscle is 27.74 μg/g. **TLF:** The posterior layer of TLF is mainly a three sub-layers structure. The outer sub-layer is undulating collagen fibers and with abundant elastic fibers. The middle sub-layer is made of collagen bundles and with few elastin fibers. The inner sub-layer has a similar manner with the outer sub-layer but has more loose connective tissue. The posterior layer of the TLF primarily consisted collagen type I and a small amount of collagen type III using polarized light.S100 showed that the outer sub-layer has more innervation than the others. The innervation of the posterior layer of the TLF showed a network manner using S100 straining. **Conclusions:** The presence of the collagen type I, III, elastin and HA in the capsule of the MS and its continuous with the perimysium, epimysium suggested that they may effect the MS function on the proprioception and coordination . If their proportion changed, they will influence the function of the MS, which properly explain part of the peripheral Neuro-physiological mechanisms of Age-related proprioceptive and coordinative deficits. The presence of the collagen type I, III, elastin and nerve elements in the TLF suggested that they may play a role on the mechanical and sensory stimulation, which proplaly explain part of the physiological mechanisms of age-related TLF function and its role on pain. (Future work: I will obtain the other groups data and compare the age-related changes in MS and TLF in the next year)

EXECUTIVE FUNCTIONS IN ANOREXIA NERVOSA: ALTERATIONS IN MATURATION TRAJECTORIES

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Ph.D. Course: Traslational Specialistic Medicine "G.B. Morgagni"
Curriculum "Neuroscience"

Background
Anorexia Nervosa (AN) is characterized by an early onset and by the presence of specific types of cognitive dysfunctions, especially cognitive inflexibility and visual-spatial difficulties. The role of these dysfunctions in the pathogenesis of the disorder is still unclear and they may represent a risk factor, an endophenotype, or a consequence of the illness itself.
The present study aims to investigate various types of executive functions, in order to assess the impact of the presence of AN on the trajectories of maturation of cognitive functions.
Material and Methods
We recruited 269 female patients with a lifetime diagnosis of AN and a control group consisting of 247 healthy women. All participants were administered a series of neuropsychological tests: Wisconsin Card Sorting Test (WCST), Iowa Gambling Task (IGT), Stop Signal Task (SST), Rey-Osterrieth Complex Figure Test (ROCF), and Reading-the-Mind-in-the-eyes task (RMET).
Results
Cognitive functions were found to be impaired in AN patients compared to the control group but adolescent patients performed as well as adolescent control (or better even if it was not significant), with the exception of decision making. By studying the relationship between cognitive performance and age, we identified specific directions of development, especially for WCST, SST, ROCF and RMET. SST and RMET showed a strong relationship with weight. A unique profile emerges for decision making, that was found impaired both during adolescence than adulthood.
Conclusions
Our data showed the alteration of maturation trajectories in AN subjects for some executive functions. It seems more likely that there is a crucial role for starvation in mentalization and in motor inhibition performances. More studies are needed, instead, to clarify the relationship between early risk factor, genetic profile or the role of a stabile trait, for decision making, set-shifting and central coherence. Our results should be associated also with neuroimaging data for more information about maturation trajectories.

MITOCHONDRIAL IMPLICATIONS IN CORE MYOPATHIES

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Background: Core myopathies are a spectrum of non-dystrophic congenital neuromuscular disorders that are caused in most of the cases by mutations in the skeletal muscle isoform of the ryanodine receptor (RyR1). RyR1 is an ion channel which releases sarcoplasmic calcium (Ca^{2+}) stores into the cytosol upon depolarization of the neuromuscular junction, enabling excitation-contraction coupling. The clinical manifestations of this group of diseases are heterogeneous, even if the histopathological features are characterized by ultrastructural derangement of myofibres resulting in the focal loss of mitochondria in a single longitudinal central core or multiple smaller cores. In this study, we aimed to elucidate correlations between genotype, protein structure and clinical phenotype in order to identify molecular pathways linked to mitochondrial dysfunctions and loss.

Material and Methods: This study includes a population of 13 patients diagnosed with central core disease (CCD) aged 6 to 53 years, 5 patients with multi-mini core disease (MmD) aged 6 to 41 years, 3 patients with malignant hyperthermia (MH) aged 33 to 55 years and 6 healthy controls aged 22 to 57 years. In order to test the hypothesis that RyR1 dysfunction leads to a redistribution of mitochondria network, RNA was extracted from muscle biopsies and was reverse-transcribed. Quantitative analysis of gene expression involved in mitochondrial biogenesis and respiratory chain was performed with Real Time PCR experiments. Protein expression level of RyR1, inositol trisphosphate receptor (IP3R) and several mitochondrial complex subunits, measured on patient muscle biopsies with Western Blot experiments, were also investigated in collaboration with Prof. Gyorgy Szabadkai's Laboratory.

Results: Genes involved in mitochondrial fusion (*OPA1*, *MFN1*, *MFN2)* and fission (*DRP1*) were up-regulated in both MmD (2 patients) and CCD (6 patients) muscle biopsies. Cluster analysis of these genes identified two subsets of patients, highlighting mitochondrial rearrangement as a possible molecular pathogenic mechanism in a group of patients. Expression levels of genes encoding for components of the respiratory chain (*NDUFV2*, *SDHA*, *SDHD*, *COX4I*, *CYTC*) were increased in both MmD (3 patients) and CCD (6 patients) muscle biopsies. Cluster analysis of these genes identified similar groups of patients as the ones obtained from the previous analysis. Protein expression levels of mitochondrial complexes subunits are also increased in both MmD and CCD patients. Moreover, a negative correlation between reduction of RyR1 expression and increase of IP3R expression was observed in 4 MmD patients and in 9 CCD patients. So, these results provide preliminary evidence of mitochondrial biogenesis at the transcriptional and protein levels which follows the loss of functional RyR1 in patient muscles.

Conclusions: The study results showed that *RYR1* mutations are associated to up-regulation of genes encoding complexes of the respiratory chain and proteins involved in mitochondrial fusion and fission, suggesting redistribution of mitochondria network as a possible adaptive mechanism. These mutations predominantly consist of missense substitutions and affect highly evolutionarily conserved positions, nevertheless they are associated with different degrees of clinical severity. Genotype-phenotype correlation is still a central question in core myopathies, since clinical manifestations are notoriously variable. In fact, mode of inheritance, variant position and physico-chemical amino acid changes contribute to determining disease phenotype. Given these preliminary data, we aim to perform functional studies of different *RYR1* mutations in order to investigate the role of mitochondrial biogenesis in the disease pathophysiology and to evaluate aberrant protein structure-phenotype correlations.

CORTICAL GAMMA-SYNCHRONY MEASURED WITH MAGNETOENCEPHALOGRAPHY IS A MARKER OF CLINICAL STATUS AND PREDICTS CLINICAL OUTCOME IN STROKE SURVIVORS

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Curriculum "Neuroscience"

Background: The long-term outcome of stroke survivors is difficult to anticipate. While the extent of the anatomical brain lesion is only poorly correlated with the prognosis, functional measures of cortical synchrony, brain networks and cortical plasticity seem to be good predictors of clinical recovery. In this field, gamma (> 30 Hz) cortical synchrony is an ideal marker of brain function, as it plays a crucial role for the integration of information flow, is an indirect marker of Glutamate/GABA balance and directly estimates the reserve of parvalbulin-positive neurons, key players in synaptic plasticity. The study of cortical gamma synchrony is not trivial. Although it could be achieved non-invasively with Magnetoencephalography (MEG) -a technique that measures brain rhythms with high spatio-temporal resolution-, to date there is lack of bed-side and standardized procedures and the relationship between this measure and stroke outcome is largely unknown. Here we focused on the entrainment of cortical gamma activity by auditory stimulation and developed the procedures and analysis pipeline for its investigation with MEG. The ultimate goal was to test whether auditory-entrained gamma synchrony is predictive of the clinical outcome in stroke survivors undergoing intensive rehabilitation in a tertiary health centre.
Material and Methods: Fifty healthy subjects and a total of 110 MEG scans were recorded to develop the procedures to map cortical synchrony. MEG was recorded both at rest and while delivering bi-aural 40Hz amplitude-modulated tones. MEG data was reconstructed over the individual cortical surface extracted from high-resolution anatomical MRI by applying a MEG-MRI co-registration procedure and a source imaging technique. Two measures of gamma synchrony were considered: a) gamma power activity and b) inter-trial phase consistency. A total of 15 stroke survivors undergoing rehabilitation at the Fondazione S. Camillo IRCCS were prospectively recruited. Within two weeks from admissions, they underwent a high-resolution anatomical 3T MRI -for extraction of cortical surface and lesion features- and a MEG scan (both resting state and 40Hz entrainment). Barthel index and Functional Independence Measure (FIM) scales were measured at the beginning and at the end of the rehabilitation.
Results: Resting state and entrained gamma-synchrony were consistently detected in all healthy participants. Spontaneous gamma synchrony mapped over the frontal cortex. Entrained gamma synchrony was right-hemisphere dominant and mapped over a large cortical region, covering the entire temporal lobe, insula, inferior part of primary sensory- motor regions, up to the inferior frontal regions and with maximal amplitude - as expected- in bilateral auditory cortices. The entire cortical surface displayed some degree of gamma synchronization at the time of 40Hz entrainment, suggesting that our procedure, rather than exploring the integrity of temporal lobe circuits only, allows a reliable and non-invasive test of the entire neocortex. In patients with stroke, the spatial distribution of cortical gamma synchrony was altered and right hemispheric dominance was attenuated or lost. Entrained gamma synchronization (but not resting state gamma synchrony) showed a significant correlation with both the clinical status at discharge (both Barthel and FIM, $R>0.9$, $p<0.001$ consistently). No significant correlation was found between lesion volume and clinical status.
Conclusions: cortical gamma synchrony related to auditory entrainment can be reliably measured in healthy individuals and stroke patients. Gamma synchrony significantly predicts the clinical outcome of stroke survivors undergoing rehabilitation.

PHARMACOGENETIC TESTING IN A REAL-WORLD SAMPLE OF INPATIENTS WITH DEPRESSION: PRELIMINARY RESULTS

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Background. In every-day psychiatric practice psychotropic medications are selected according to clinical data and managed using a trial-and-error approach, contributing to high costs and poor outcomes. Type of treatment is chosen mainly in relation to symptomatic profile and mood-lifecharting, while dose titration is empirically modulated. There are many psychotropic agents that target specific symptoms and there is considerable inter-individual variability in therapeutic drug response, required dosage and adverse effects for the same medication. The empiric approach often results in prolonged times to remission, overmedication and substantial increased risk for morbidity or mortality. Psychiatric pharmacotherapy is particularly likely to benefit from the introduction of pharmacogenetic testing, an innovative and emerging option that integrates documented pharmacodynamic and pharmacokynetic profiles in the selection and management of medications. However the translation of pharmacogenomics to clinical decision-making is evolving quite slowly and studies have produced inconsistent findings because of tecnical and methodological limitations. The aim of this study is to assess the utility of a pharmacogenetic-based decision support tool in a real-world inpatient psychiatric setting.
Material and Methods. Patients admitted to a Psychiatric Unit for a moderate-severe unipolar or bipolar depression and not responsive to at least 1 treatment (adequate for time of exposure and dose) were recruited since January 2018. Demographical and clinical variables were recorded. A farmacogenetic test was administered to all subjects and patients were randomly divided into a test-guided group and a non-guided group. Clinical rating scales (CGI-S, HAM-D17 and GAF) were administered at baseline and 4 weeks after treatment modification and side effects were recorded.
Results. To date, 30 inpatients with a depressive episode (63.3% unipolar depression) have been recruited. The sample was characterized by mean age of 56.6 (±12.6) years, mean duration of current episode 7.1 (±5.1) months and median number of failed treatments in the current episode 2.8 (±1.9). Median CGI-S at baseline was 5, mean HAM-D17 was 23.7 (±3.7) and mean GAF was 48.2 (±5.5). Patients randomly assigned to guided-treatment group were 17. There were no differences in clinical, pharmacokinetic and pharmacodynamic features between the guided and the unguided group (p=0.12). Both groups improved significantly after 1 month (p=.000), but the improvement was more pronounced in the guided group when analysis was restricted to patients with duration of current episode >=3 months. No significant differences were found in side effects (p=0.23).
Conclusions. A rapidly available pharmacogenetic interpretive report provided useful informations for a more tailored pharmacological treatment in inpatients with moderate-severe depressive episode longer than 3 months. More data are necessary to validate this preliminary result and to better stratify the sample according to the clinical staging of the disease.

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