BOOK OF ABSTRACTS

ORAL PRESENTATIONS

PARACRINE EFFECT OF MESOANGIOBLAST MEMBRANE VESICLES ON DIFFERENT CELL TYPES

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Introduction: Extracellular vesicles (EV), an important mediator of cell-to-cell communication, are involved in both autocrine and paracrine signaling. A6 mouse mesoangioblasts, vessel-associated multipotent progenitor stem cells, are able to release in the extracellular environment plasmamembrane derived vesicles. Today takes hold the idea that EV can replace stem cells, opening a new scenario in regenerative medicine. To this aim, we investigated the possible interaction of A6-EV with different cell types and their effects.

Materials and Methods: A6-EV were collected from conditioned media by ultracentrifugation. Endothelial cells (ECV304) were incubated with A6-EV and wound-healing assay was performed to study in vitro their migration, and their capability of capillary-like structures formation. Moreover, also Jurkat lymphocytes were cultured with A6-EV to investigate their effect on both cell activation and proliferation. Enzymatic removing of N-lynked glycans was performed by treating A6-EV with either PNGaseF or EndoH, and cell-EV interaction was evaluated by FACS.

Results: We have demonstrated that A6-EV interact with ECV304 cells inducing their differentiation versus capillary-like structures, and increasing their migration capability. Furthermore, A6-EV have a positive role on Jurkat cell proliferation but a negative one on their activation.

We have investigated the role of sugar residues on the membrane of A6-EV in their interaction with other cell types. In particular, PNGaseF induces a substantial reduction in EV-target cell interaction. On the contrary, EndoH increases this interaction.

Conclusions: We demonstrated that mesoangioblast-EV interact with Jurkat and ECV304 influencing their behaviour. Furthermore, we showed that EV sugar residues exert a role in EV-cell interplay.

CEREBROSPINAL FLUID ISOLATED EXTRACELLULAR VESICLES AS POSSIBLE BIOMARKERS OF MULTIPLE SCLEROSIS STATUS

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Introduction: Several studies identified Extracellular Vesicles (EVs) in the cerebrospinal fluid (CSF), having a role in both physiological and pathological conditions, such as inflammatory neurodegenerative diseases and multiple sclerosis (MS). In MS patients, EVs were also detected in blood samples, suggesting that they could be used as biomarkers enabling monitoring of disease onset and progression. Intensive research has focused on identifying and validating molecular biomarkers that could reflect its heterogeneous clinical course and determine the best treatment option for patients. This study compared the amount and surface marker expression of CSF-EVs of subjects affected by either MS or other neurological disorders correlating the EVs with indicators of MS disease severity.

Materials and Methods: EVs, isolated by ultracentrifugation of CSF samples, were characterized by flow cytometry. To identify EV origin a panel of fluorescent antibodies was used: CD4, CD193, CD195, CD19 and CD200, and the lectin IB4. Kruskall-Wallis and Mann-Whitney U test were used for statistical analyses.

Results: A higher EV concentration in progressive MS and in Clinically Isolated Syndrome was observed compared to all the other groups examined. In relapsing MS patients in the course of clinical relapse we observed an increase in CSF-EV level and a reduction of CD19+/CD200+ EVs. In contrast, in the presence of Gad+ lesions in MRI we observed an increase of CSF-EV CD193+/CD195+, CD4+/CD193+, and CD4+/CD195+.

Conclusions: Our results indicate that CSF-EVs could be a promising tool to investigate the immunopathology of MS and to identify biomarkers specific to the different phases of the disease.

Validation of a circulating miRNA detection assay to monitor therapy response in classical Hodgkin lymphoma

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Introduction: Previously, we showed in a pilot study that classical Hodgkin lymphoma (cHL) patients have increased levels of certain cell-free EV-bound circulating miRNAs that are functionally implicated in lymphomagenesis. The elevated EV-miRNA levels appear to reflect active metabolically disease as determined by PET-CT imaging. We postulate that EV-bound miRNAs are useful for monitoring of therapy-response, minimal residual disease and predict relapse. To test this hypothesis, we are optimizing an EV-miRNA detection assay in a cohort of longitudinally collected plasma samples of cHL-patients before, during and after treatment.

Materials and Methods: EV-associated extracellular RNA (exRNA) are isolated with size-exclusion chromatography (SEC) from patient plasma (n=25, 120 samples and healthy subjects (n=19). Comprehensive small RNA sequencing was performed on 11 patients pre- and post-treatment and compared to 10 healthy donors. qRT-PCR is used to quantify EV-miRNAs and sTARC was measured by ELISA. EV-miRNA and sTARC-levels were correlated with clinical parameters at presentation, platelet numbers and treatment outcome, determined by FDG-PET.

Results: Matched serial monitoring of EV-miRNA levels and sTARC in patients before, directly after treatment and during long-term follow-up revealed robust, stable association with treatment results observed by FDG-PET, both for responders (n=21) and non-responders (n=4). Platelet concentrations correlate more strongly with sTARC levels than EV-miRNAs during and after treatment.

Conclusions: Changes in cHL-related circulating EV-miRNA levels are suitable for therapy-response and relapse monitoring in individual cHL patients. Further optimization of the marker-panel for blood-stabilizing collection tubes will confirm whether our assay can be applied to monitor the efficacy of new treatment regiments in large clinical trials.

SINGLE-CELL RT-qPCR ANALYSIS BY AN INNOVATIVE CONTINUOUS-FLOW DROPLET MICROFLUIDIC DEVICE

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Introduction: RT-qPCR is a key method in cancer diagnostic which, however, presents important costs and throughput. Therefore, the constant increases of biomarkers and patients to be screened, requires the implementation of faster and cheaper approaches. Droplet microfluidics, allowing the manipulation of small amount of liquid, represents a good candidate to support these needs. We present here, a compact and reliable microfluidic device perfectly suitable for qPCR application.

Materials and Methods: 100nL droplets (aqueous phase in FC-40 oil) flow in a capillary, embedded in a combined 3D-printed structure for heating and fluorescent detection. During their path, droplets pass alternatively through two regions, kept at controlled temperatures, performing the concentional PCR cycles. Droplets excitation is given by LEDs array and fluorescence signal is captured by a camera. The microfluidic device was validated evaluating the expression level of two genes (β-actin and HER2) starting from: i) total-RNA extracted from cell lines (MCF7 and SKBR3) and ii) the direct analysis of entire cells.

Results: Employing this device, we succeeded in determining the expression of HER2 gene in SKBR3 and MCF7 cell lines at single cell level, obtaining Ct comparable with those obtained in conventional technology, but with important reductions in sample/reagent volumes and processing time. Additionally, the device showed PCR efficiency higher than 95% and single cell sensitivity.

Conclusions: We developed a fully automated droplet microfluidic RT-qPCR device, that shows higher parallelization capabilities than lab-scale technology and single cell sensitivity. Additionally, it was validated determining the expression level of genes relevant in breast cancer.

CHEMOTHERAPY AFFECTS EXTRACELLULAR VESICLES RELEASED BY GLIOBLASTOMA BRAIN TUMOUR CELLS

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Introduction: Glioblastoma multiforme (GBM) is the most aggressive primary tumour within the brain as well as the most common and lethal cerebral cancer, mainly because of treatment failure. Indeed, tumour recurrence is inevitable and fatal in a short period of time. Glioblastoma stem-like cells (GSCs) are thought to participate in tumour initiation, expansion, resistance to treatments, including to the chemotherapeutic agent temozolomide, and eventually, tumour relapse (Harford-Wright et al. *Brain* 2017). As extracellular vesicles (EVs) may contribute to Glioblastoma (André-Grégoire et al. *Cell Adh Migr* 2016), our objective was to assess whether EVs released by GSCs could disseminate factors involved in resistance mechanisms.

Materials and Methods: We characterized EVs either circulating in peripheral blood from newly diagnosed patients or released by patient-derived temozolomide-resistant GSCs in vitro.

Results: We found that EVs from both sources were mainly composed of particles homogeneous in size (50-100 nm), while they were more abundant in liquid biopsies from GBM patients as compared to healthy donors. Further, mass spectrometry analysis from GSC-derived EVs unveiled that particles from control and temozolomide-treated cells share core components of EVs, as well as ribosome- and proteasome-associated networks. More strikingly, temozolomide treatment led to the enrichment of EVs with cargoes dedicated to cell adhesion processes.

Conclusions: Thus, while relatively inefficient in killing GSCs in vitro, temozolomide could instead increase the release of pro-tumoral information (André-Grégoire et al. *Biochimie* 2018).

CONDITIONED MEDIUM FROM FAT-LADEN HYPOXIC HEPATOCYTES ACTIVATES PRO-FIBROGENIC SIGNALS IN STELLATE CELLS: POSSIBLE INVOLVEMENT OF EXTRACELLULAR VESICLES

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Background: Transition from isolated steatosis (IS) to non-alcoholic steatohepatitis (NASH) is a key issue in Non-alcoholic fatty liver disease (NAFLD). Recent observations in patients with obstructive sleep apnea syndrome (OSAS), suggest that hypoxia may contribute to disease progression mainly through induction of hypoxia inducible factor 1α (HIF-1α), but the mechanisms at play remain incompletely understood.

Aim: to explore whether hypoxia modulates the release of extracellular vesicles (EVs) from free fatty acid (FFA) exposed hepatocytes and determines cellular crosstalk between hepatocytes and fibrogenic liver cells.

Methods: HepG2 human hepatoma cells were treated with FFAs (250 μM palmitic acid + 500 μM oleic acid) and chemical hypoxia (CH) was induced with Cobalt (II) Chloride ($CoCl_2$), which is a chemical inducer of HIF-1α. Induction of CH was confirmed by Western blot of HIF-1α. EVs characterization and quantification was performed by electronic microscopy and nanoparticle tracking analysis respectively. LX-2 cells (human hepatic stellate cell line) were treated with conditioned medium (CM) from hepatocytes from different groups and markers of pro-fibrogenic signaling were determined by quantitative PCR.

Results: FFA and CH-treatment of HepG2 cells increased the release of EVs compared to non-treated HepG2 cells ($P=0.0033$). When LX-2 cells were cultured with CM from fat-loaded hypoxic (FFA + $CoCl_2$) hepatocytes, increased expression of IL-1β, TGF-β, CTGF and Collagen-1A1 was observed compared to LX-2 cells treated with CM from non-treated hepatocytes.

Conclusion: CH promotes EV release from hepatocytes and CM from hypoxic fat-laden hepatocytes evokes profibrotic responses in LX-2 cells. Further characterization of EVs released by steatotic cells in hypoxic conditions is needed to establish their role in the crosstalk between hepatocytes and stellate cells in the setting of NAFLD and OSAS.

MICROGLIA-DERIVED EXTRACELLULAR VESICLES REGULATE THE RECRUITMENT, PROLIFERATION AND DIFFERENTIATION OF OLIGODENDROCYTE PRECURSOR CELLS

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Introduction: Microglia have an enormous plasticity in response to CNS injury and acquire different activated phenotypes, participating in mechanisms of injury and repair. However, the mode(s) of action of microglia in exerting their functions is unclear. Here, we explored the action of Extracellular Vesicles (EVs) released from differently activated microglia on Oligodendrocyte Precursor Cells (OPCs), the glial cells which supports remyelination.

Materials and Methods: We first injected EVs produced by pro-inflammatory microglia (M1 EVs) or pro-regenerative microglia (M2 EVs) in the lysolecithin mouse model of focal demyelination. Brain sections were stained with specific antibodies to analyze OPC recruitment, proliferation, differentiation at the site of lesion. EV-effects on OPC proliferation was analysed *in vitro* by EdU incorporation in cultured OPCs while OPC migration was assessed using the transwell based migration assay. OPC differentiation and myelin deposition were determined by expression of the differentiation marker MBP and by quantification of linear MBP-positive segments extending along axons of DRG neurons.

Results: Immunofluorescence analysis revealed that M1 EVs tend to limit OPC proliferation at lesion site and block re-myelination, whereas M2 EVs promote OPC proliferation, recruitment and myelin repair. However, M2 EVs and, to a lesser extent, M1 EVs enhance the differentiation of cultured OPCs into mature oligodendrocytes and myelin deposition in an *in vitro* system of OPCs-DRG neurons co-cultures. Interestingly, all type of EVs are able to act as chemoattractants for OPCs.

Conclusions: Collectively, these results unveil EVs as key players in microglia-OPCs cross-talk and suggest that the phenotype acquired by microglia greatly influences the proliferative/differentiation potential of OPCs.

CIRCULATING EXOSOMES AS A PROMISING SOURCE OF BIOMARKERS FOR ALS

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Introduction: Amyotrophic Lateral Sclerosis (ALS) is a progressive, lethal neurodegenerative disease characterized by selective loss of upper and lower motor neurons. The symptoms and the time of progression of the disease are heterogeneous in patients and the clinical scale (ALSFRS-R) is not optimal to stratify patients, impairing for instance the evaluation of outcome of clinical trials or administration of effective therapies and should be paired to a complementary approach. Extracellular vesicles circulate in biological fluids and are involved in intercellular communication, carrying a variety of cargos such as RNA. We and others have shown that normal cells release exosomes constitutively, but their release is altered when mutant ALS proteins are expressed.

Materials and Methods: In this study we tested the feasibility to use circulating exosomes as a source of biomarkers for ALS. We first took advantage of a newly established protocol to purify EVs that preserve their morphology and physical and biochemical properties. Next, we analysed concentration and size of EVs from plasma of ALS patients and controls (healthy, neurological and muscular dystrophy) by tunable resistive pulse sensing-based technology.

Results: We found that ALS EVs are smaller and more abundant and their number increases with the progression of the disease. Finally, we analyzed the level of a priori-selected candidate biomarkers (TDP-43, cyclophilin A, heterogeneous nuclear ribonucleoprotein A2/B1 and SOD1).

Conclusions: Plasma concentration of EVs and their biochemical properties are accessible and measurable parameters that can help to stratify ALS patients according to molecular features and clinical assessment.

EPIGENETIC AND TRANSCRIPTOME CHANGES IN ENDOTHELIAL CELLS EXPOSED TO GLIOBLASTOMA-DERIVED EXTRACELLULAR VESICLES

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Introduction: Glioblastoma (GBM) is an extremely aggressive brain tumor characterized by a high mortality. Extracellular vesicles (EVs) from tumor cells convey information to other cells to create a microenvironment that favors tumor success. The aim of this study was to investigate phenotypic as well as DNA methylation and transcriptome changes in human brain microvascular endothelial cells (HBMVECs) exposed to GBM-derived EVs.

Materials and Methods: An *in vitro* tube formation assay has been performed to evaluate EC growth in matrigel under treatment with EVs isolated by ultracentrifugation from GBM8, a primary GBM cell line, and proper controls. After 16 hours of incubation, tube formation has been phenotypically evaluated, RNA and DNA have been isolated from ECs for total RNAseq and DNA methylation, respectively.

Results: Phenotypically, ECs treated with GBM-derived EVs showed a similar tube formation when compared to ECs treated with growth factors (positive control) in terms of tubule number, tubule length, branching point number, and mesh count. DNA methylation showed that GBM EVs stimulate tissue remodeling and recruitment of vasculature.

Discussion: GBM EVs support EC growth and could be therefore involved in the angiogenesis process, stimulating the recruitment of new blood vessels. Integrating DNA methylation and mRNAseq data will shine a light on the mechanisms used by GBM EVs to promote angiogenesis.

POSTER PRESENTATIONS

HYPOXIA AMELIORATES THE FUNCTION OF EXTRACELLULAR VESICLES (EVs) DERIVED FROM ADIPOSE TISSUE-MESENCHYMAL STEM CELLS (AT-MSCs) IN DELAYED WOUND HEALING

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Introduction: Mesenchymal stem cells (MSCs) as key role in wound healing via paracrine effects by secreting cytokines and small particles. Extracellular vesicles (EVs) are small particle containing nucleic acids and proteins which can be transferred to the target cells. Recently, MSC-derived EVs reported promotes the wound healing; therefore EVs-based therapy becomes attractive strategy in clinical application. Hypoxia reported enhance the MSCs wound healing ability. However, whether hypoxia affects to EVs biogenesis and how the mechanism EVs to improve the wound healing had not been elucidated yet.

Materials and Methods: AT-MSCs were cultured under normoxia (20%O_2) and hypoxia (1%O_2) condition. EVs were isolated from supernatant by ultracentrifugation. The effects of normoxic and hypoxic EVs to MSCs and endothelial progenitor cells (EPCs) in wound healing were identified by scratch assay and gene expression. Wounds in type-1 diabetic mice model were created and injected with EVs. Wound closure and histological analysis of wound tissues were conducted.

Results: Hypoxia upregulated the expression of wound healing-related genes in EVs. However, there was no affect on EVs biogenesis released. Of note, hypoxic EVs showed higher ability to support the wound healing function of target cells: elderly MSCs and EPCs by improvement of migration and upregulation of CXCR4, SDF1, bFGF and VEGF. Hypoxic EVs injection into diabetic mice significantly reduced wound area within 7 days. Hypoxic EVs also significantly recruit inflammatory cells and support angiogenesis in wound site.

Conclusions: Hypoxic EVs considered as promising candidate for delayed wound healing therapy in future.

RAMAN SPECTROSCOPY FOR THE MOLECULAR PROFILING OF EXTRACELLULAR VESCICLES IN PARKINSON'S DISEASE

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Introduction: Parkinson's Disease (PD) is an irreversible neurodegenerative disorder characterized by the progressive controlled movement loss and by the neuronal accumulation of misfolded proteins. The early diagnosis and therapy monitoring rely on complicated cognitive tests and clinical scaling. Circulating Extracellular Vescicles (EVs) deriving from cells residents in all the body tissues can provide an important biomarker to evaluate the pathology advent, progression and the therapy effects.

Materials and Methods: Serum EVs were isolated by a combination of size exclusion chromatography and ultracentrifugation. Label-free Raman spectroscopy was used to obtain a biochemical profile of EVs isolated from healthy subjects and PD patiens. A Raman spectroscope (Aramis, Horiba) operating with a 532nm laser was used in the spectral ranges between 600-1800 cm^{-1} and 2600-3200 cm^{-1}. Multivariate statistical analysis was applied for the comparison of the Raman fingerprins.

Results: Our preliminary data showed the different Raman signal from the two analyzed groups resulting in a different biochemical composition of the specific EVs. In particular we demonstrated the presence in the PD human serum of circulating EVs associated or loaded with specific biomarkers compared those of the healthy controls.

Conclusions: Our data provide support to the PD prion hypothesis confirming the possibility to analyze the whole EVs populations through a label-free and fast methodology. Moreover our results demonstrated the feasibility to use the Raman fingerprints of circulating EVs as potential tool for the fast diagnosis and therapy efficiency monitoring of PD patients.

IDENTIFICATION OF THE MOST EFFICIENT METHOD TO ISOLATE AND CHARACTERIZE URINARY EXTRACELLULAR VESICLES, NOVEL POSSIBLE KIDNEY DAMAGE BIOMARKERS

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Introduction: Extracellular vesicles are lipid membrane-bound nanoparticles released from different kind of cells. They may carry different types of proteins, lipids and miRNAs reflecting the physio pathological status of the cells they originated from. Particularly, urinary extracellular vesicles (UEVs) and their miRNA, could be useful biomarkers for kidney allograft injury discovery. Due to their low concentration, UEVs quantification and characterization remain a challenge. The aim of this study is to define the most efficient method to isolate UEVs and evaluate their miRNA content.

Materials and Methods: UEVs were isolated from urine samples using four different commercial kits. Urine and the UEVs isolated samples were purified by Izon qEVsingle SEC. Pre and Post purification samples were quantified showing different concentrations, size and protein contamination. UEVs miRNA fraction was extracted by commercial kit and evaluated by Bioanalyzer 2100.

Results: The UEVs isolated showed a high variability in concentration and size among the different methods considered. The raw concentration of pre purified samples were between 1.27 x 108 – 4.38 x 109 with a protein concentration between 0.42 – 8.17 mg/ml. Instead, the post purified samples showed lower raw concentrations (1.53 x 107 – 6.47 x 108), whereas their protein contamination was 0 mg/ml. The particle size of UEVs samples correspond to the range of the microvesicles. The miRNA concentration was between 37 – 231 pg/µl.

Conclusions: These results suggest that the most efficient method to isolate UEVs is the Izon qEVsingle SEC column, whereas the high concentration of miRNAs is detected by QIAGEN.

CANCER-DERIVED EXOSOMES INFLUENCE IMMUNE RESPONSE SIGNALS

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Introduction: Cancer-derived exosomes support the survival and progression of tumours in many ways and also contribute to the neutralization of the anti-cancer immune response. Exosomes contain miRNAs that can affect the function of target cells. Therefore, it is believed that exosomes secreted from cancer cells may participate in the development of immunosuppression as a form of defence against the body's immune system defence. The mechanisms underlying these processes are poorly understood. Therefore, we want to investigate the influence of tumour-derived exosomes on immune signalling pathways by analysing altered surface antigen expression on immune cells.

Materials and Methods: The commercially available Transwell plate was used. Inserts with a permeability diameter of 0.4μm = 400 nm were employed to let smaller particles such as exosomes (40-150 nm) pass through the pores while excluding the influence of cell-cell contacts. Two cell lines were used: acute monocytic leukemia (THP1) and melanoma (A375). Changes in monocyte surface receptors in response to the A375 cell line exosomes were analysed by FACSFlow cytometer.

Results: The obtained results show a decreased expression of the HLA-DR antigen receptor on the surface of monocytic cells.

Conclusions: Lowering the level of the HLA-DR receptor is a mechanism by which cancer cells may take control over the immune system. However, the influence of cytokines involved in the immune response cannot be excluded, therefore further research in this direction will be performed.

INVESTIGATING EXTRACELLULAR VESICLE-NEURON DYNAMICS THROUGH OPTICAL TWEEZERS

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Introduction: Extracellular Vesicles (EVs) are membrane vesicles released by most cells. By exposing adhesion receptors, EVs interact with cells to deliver proteins, lipids and RNAs. In pathology EVs become vehicle for the transfer of pathogens. However, almost nothing is known about EV-neuron dynamics.

Materials and Methods: We used optical manipulation to investigate the interaction of EVs released from cultured astrocytes with neurons. EVs, isolated by differential centrifugation, were added to the medium of cultured hippocampal neurons. To monitor EV-neuron interaction, single EVs were trapped and delivered to neuron surface by using an IR laser collimated into the optical path of the microscope.

Results and Conclusions: Analysis of time-lapse recordings revealed that EVs efficiently adhered to neuron processes and about 63% showed a displacement along the surface of neurites. Interestingly, the EVs velocity (143nm/sec) is in the same range of adenoviral particles binding to surface receptors and similar to retrograde actin flow, which regulates membrane diffusion of receptors linked to actin, suggesting that EV movement could be driven by the contact with neuronal receptors.

Accordingly, we found that EV movement is highly dependent on neuron energy metabolism. Indeed, only 33% of EVs were able to move on energy depleted neurons treated with 2μM rotenone.

Unexpectedly, we found by cryo-electron microscopy that a subpopulation of EVs contain actin filaments, suggesting an intrinsic capacity of EVs to move. We are addressing this hypothesis by inhibiting actin rearrangements in EVs with Cytochalasin D. Preliminary data indicate a significant decrease, from 71% to 45%, of EVs able to drift on neuron surface.

ISOLATING INTACT AND DISPERSE EXTRACELLULAR VESICLES FROM MOUSE SPINAL AND CORTICAL ASTROCYTES AND MOTOR NEURON-LIKE CELLS

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Introduction: In numerous neurodegenerative diseases, the interplay between neurons and glia modulates the outcome and progression of the pathology. A particularly intriguing way of interaction between neurons, astrocytes and microglia is characterized by the release of extracellular vesicles (EVs) able to transport proteins, lipids and nucleotides from one cell to the other. We have previously reported that astrocytes carrying a pathogenic mutation for Amyotrophic Lateral Sclerosis can selectively induce neuronal death through EVs and their content. In particular, we showed that exosomes derived from mutant SOD1-primary astrocyte cultures were sufficient to induce selective motoneuronal death.

Materials and Methods: In this context and with the purpose to analyze the cargo of polydisperse EVs derived from mouse spinal and cortical astrocytes, we are using a novel method allowing rapid and efficient purification of EVs. By this approach, we are able to preserve the integrity and the stability of vesicles and, importantly, to selectively analyze them according to their size, revealing a discrete enrichment of popular protein markers.

Results: Astrocyte-derived EVs cultured from mutant mice are different in number and size compared control mice, suggesting that in ALS, EV biogenesis is impaired. Interestingly, ALS-derived EVs impact on cortical and spinal neurons viability implying that EV cargo is important in mediating toxicity. EVs RNA-seq and proteomic analysis are undergoing in the Lab.

Conclusions: Intercellular miscommunication contributes to ALS by contributing to neuronal death.

ROLE OF MICROGLIAL EXTRACELLULAR VESICLES IN EARLY SYNAPTIC DYSFUNTION IN ALZHEIMER'S DISEASE

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Introduction: Alzheimer's disease (AD) is characterized by loss of synapses and neurons, extracellular amyloid-beta (Aβ) deposition, intraneuronal *tau* aggregation and microgliosis. Extensive literature implicates synaptic dysfunction as an early mechanism in AD. However, how synaptic dysfunction starts and propagates throughout the brain is largely unclear. This study aims at investigating the involvement of microglia-derived extracellular vesicles (EVs), carrying beta-amyloid (Aβ-EVs), in synaptic alterations.

Materials and Methods: We analyzed the morphology and the strength of synaptic transmission in basal conditions and in response to chemical long-term potentiation (LTP) in cultured hippocampal neurons exposed to Aβ-EVs. Using cortico-hippocampal slices we also measured LTP in the entorhinal cortex (EC) and in its main target region, the dentate gyrus (DG), after Aβ-EVs injection into mouse EC.

Results: Aβ-EVs cause early alteration of dendritic spine morphology and density and impair chemical LTP in culture. Furthermore, Aβ-EV injection in EC impairs LTP in EC 1h after the injection, while the effect is transferred to the DG after 24h.

Conclusions: Aβ-EVs are able to spread LTP impairment among connected brain regions. The underlying molecular mechanism will be discussed.

ADIPOCYTE DERIVED EXTRACELLULAR VESICLES ARE REGULATORS OF GLUCOSE HOMEOSTASIS

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Introduction: Extracellular vesicles (EVs) are important signaling entities with the ability to reprogram cellular function in a paracrine or endocrine fashion. Here, we aimed to assess if adipocyte derived EVs from epididymal white adipose tissue (eWAT) play an active role in conveying information between adipose tissue and metabolic target organs.

Materials and Methods: Adipocyte derived EVs from eWAT (eWAT EVs) were isolated from lean and obese mice using differential ultracentrifugation and subjected to proteomic and miRNA content analysis. For functional characterization, fluorescently labelled EVs were injected into mice and traced after 4 and 24 hours using cryo-imaging. Subsequently, in vivo glucose tolerance test and insulin secretion test (GTT-IST) was performed in mice pre-treated with eWAT EVs from obese and lean mice for 4 hours. Results obtained in vivo were confirmed in vitro by glucose stimulated insulin secretion test on primary murine islets.

Results: eWAT EVs of lean and obese mice displayed profound compositional differences in their proteome and miRNA profiles that suggest differences in lipid metabolism and endocrine effects on pancreatic insulin secretion. Whole mouse cryo-imaging revealed an enrichment of injected eWAT EVs to the pancreas, which was reflected by increased insulin secretion from isolated murine pancreatic islets exposed to eWAT EVs ex vivo, and increased insulin secretion and significant improvements of glucose tolerance in mice injected with eWAT EVs.

Conclusion: eWAT EVs from obese mice seem to decrease the threshold for insulin secretion from pancreatic β-cells, thereby compensating for increased blood-sugar levels and insulin needs under obese conditions.

AN INFLUENCE OF STORAGE PROCEDURES ON THE STABILITY AND PHENOTYPIC PROPERTIES OF EXTRACELLULAR VESICLES RELEASED BY HUMAN CELLS IN VITRO- PRELIMINARY REPORT

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Introduction: Extracellular vesicles (EVs) are heterogeneous group of small vesicles released by the cells. Growing data demonstrate that EVs may enclose bioactive content and transfer it into the target cells. Thus, recent focus is placed on EVs utilization in the field of biomedical applications, which typically requires their prolonged storage. However, there is limited data considering the stability of freeze-thawed EV samples. Thus, in recent study, we evaluated an impact of long-term storage on the selected morphological and phenotype features of EVs.

Materials and Methods: EV samples were obtained ultracentrifuged from conditioned media from human iPS, fibroblast and mesenchymal cells via ultracentrifugation. oncentration and size distribution of freshly isolated and freeze-thawed EVs were analyzed with nanoparticle tracking analysis (NTA). Simultaneously, high resolution flow cytometry was utilized to examine an influence of long-term storage on EVs phenotype, including expression of tetraspanin and surface markers as well as the content of RNA.

Results: Preliminary results revealed that storage of EVs has differential effect on their global size distribution and can affect their concentration. Moreover, obtained data indicate, that freeze-thaw cycles may have a significant effect on the stability of EVs, as RNA content, which also corresponds to the presence of intact vesicles, changes in selected timepoints. Additionally, as CD81 expression remains stable, the presence of CD90 antigen can be influenced by the storage conditions.

Conclusions: In conclusion, our findings suggest that storage conditions of EVs may influence their stability, which should be considered as an important factor determining their therapeutic effect.

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HOW NOT TO PURIFY EXOSOMES?

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Introduction: Exosomes are endosomal origin vesicles, transporting proteins and nucleic acids between cells. They seem to be responsible for cell to cell communication, and even more interestingly also to cancer cells. On their membrane many proteins are localized which allow to penetrate the cellular membrane. They also do not elicit immunogenic reactions. From those reasons they could be an ideal carrier for therapeutic agents. Application of exosomes in medicine is promising but for that their purity has to be excellent. One step purification is not sufficient for this purpose.

Materials and Methods: HEK293T cell line was cultured and medium was collected. Exosomes were obtained in different purification methods: series of ultracentrifugation, precipitation, size exclusion chromatography, proteolytic digestion and concentrators. Purity was determined by Flow Cytometry, Western Blotting, RT-PCR and electron microscopy.

Results: The yield and purity levels differ between the used methods. The precipitation method provide the highest numbers of vesicles, but only part of them were proved to be exosomes. This method also seems to increase aggregation of exosomes, but has no influence of miRNA content and could be availed in diagnostics. The best purity was obtained by series of ultracentrifugation but with very low yield.

Conclusions: The purification of exosomes forces you to go to compromise - either great yield and low purity or struggle with loss of a great percentage of exosomes on each additional step of purification.

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IMPACT OF EVs FROM GENETICALLY MODIFIED HUMAN iPS CELLS ON FUNCTIONAL PROPERTIES OF CARDIAC CELLS *IN VITRO*

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Introduction: Extracellular vesicles (EVs) are population of small (100-1000nm) circular membrane vesicles secreted by most cell types including stem cells (SCs). It has been recently reported that EVs may carry bioactive cargo including proteins, microRNAs and mRNAs. Recent studies conducted by our group reveled that native human induced pluripotent SC (iPS)-derived extracellular vesicles (hiPS-EVs) may enhance several cardiac cells (CCs) function in vitro including their survival in cytotoxic environment**.** The aim of this study was to verify if EVs derived from hiPS cells overexpressing procardiomyogenic miR1 and miR199a, might have impact on various properties of human CCs including proliferation, migration, differentiation and survival.

Materials and Methods: EVs were isolated from conditioned hiPS culture media using differential centrifugation followed by ultracentifugation. NHCF-V cells (Lonza) were used as a model of target CCs. In each experimental setup cells were treated with 20ng of EVs per 1000 cells.

Results: Our preliminary data indicate that hiPS-EVs may protect cardiac cells from apoptosis and inhibit the progress of this process. Antiapoptotic effect was stronger after miR1-hiPS-EVs or miR199a-hiPS-EVs treatment compared to control (EVs from unmodified hiPS cells). hiPS-EVs had also impact on NHCF-V cells proliferation, migration and differentiation toward cardiomyocytes.

Conclusions: These results may suggest positive impact of EVs from hiPS cells overexpressing miR1 and miR199a on cardiac cell properties in myocardium after ischemia, which needs to be tested in further experiments in vivo.

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DEVELOPMENT AND VALIDATION OF ANTIBODIES AGAINST ADIPOSE TISSUE DERIVED EXTRACELLULAR VESICLES

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Introduction: Extracellular vesicles (EVs) are secreted from a variety of tissues into body fluids and are stable carriers of genetic material, proteins and lipids from their tissue of origin. Circulating EVs thereby provide a rich source for molecular diagnostic analysis. Here we aimed to develop capturing antibodies for the extraction of adipocyte derived EVs from blood samples.

Material and Methods: EVs from cell lines and serum samples were isolated via differential ultracentrifugation and characterized via transmission electron microscopy, marker expression and nanoparticle tracking analysis. For antibody development mice were immunized with EVs isolated from differentiated human adipocytes. Immunized mice are euthanized and spleen cells were fused with myeloma cells.Resulting antibodies were characterized by dot blot and flow cytometry analyses.

Results: Immunization of mice with EVs resulted in 2009 antibody producing hybridoma cell clones. In dot blot analyses, three out of 2009 antibodies displayed selective binding to adipocyte derived EVs, but not to EVs from other cell lines originating from liver, colon, pancreas, bone and breast. Addition FACS analysis confirmed antibody specificity against adipocyte derived EVs. The antibodies allowed for the immunoprecipitation of intact EVs for miRNA and proteome analyses.

Conclusions: Our selected antibodies allow for the extraction of tissue specific EVs for subsequent molecular characterization. The selective extraction and analysis of tissue specific EVs from the blood stream increases the current diagnostic power of serum EVs.

HIGH THROUGHPUT SCREENING (HTS) TO IDENTIFY EXOSOME BIOGENESIS AND RELEASE INHIBITORS

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Introduction: Exosomes are extracellular vesicles (EVs) of 30–150 nm that are generated by the inward budding of multivesicular bodies (MVBs), fusion with the plasma membrane, and extracellular secretion[1]. Exosomes have emerged as important mediators of intercellular communication[2] that help to form pre-metastatic niches[3] and influence cancer therapy resistance[4]. Exosomes markers include tetraspanins (e.g., CD9, CD63, CD81) and specific lipids[5]; however, current methodologies employed to purify exosomes are time consuming and difficult to translate to clinical practice. We have begun an HTS for drugs that inhibit tumor-derived exosome biogenesis/release by combining AlphaScreen technology and immunocytochemistry.

Materials and Methods: For AlphaScreen, we used CD9 antibody conjugated to acceptor beads, and CD63 biotinylated antibody that binds to streptavidin donor beads. A signal appears if the distance between the beads is less than 200nm. For lipid immunocytochemistry, cells are labelled with Hoechst, MitoTracker, and an antibody specific for an exosomal lipid[6] that allows the quantification of exosome amount normalized to a single cell. We employed two cells lines representing two breast cancer subtypes (Luminal A and HER2+).

Results: Results confirm five and three drugs as exosome inhibitors in the luminal A and HER2+ cell lines, respectively.

Conclusions: A combination of AlphaScreen and immunocytochemistry in an HTS format can provide more information about the mechanism of action of candidate drugs. The antibodies used allowed the visualization of the CD9+/CD63+ population of exosomes. Thanks to the flexibility of this technique, we can personalize the assay to discriminate other population, including CD9+/CD81+ or CD63+/CD81+.

TGFB3 PRESENT IN EXTRACELLULAR VESICLES MODULATES RESPONSE TO CYTOTOXIC THERAPY IN HEAD AND NECK SQUAMOUS CELL CARCINOMA

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Approximately 30% of patients with locally advanced head and neck squamous cell carcinoma (stage III-IV HNSCC) treated with cisplatin-based chemoradiotherapy (CRT) present incomplete response to the treatment. Unfortunately, no biomarker is able to prospectively segregate nonresponder (NR) patients from those that respond to CRT. Since extracellular vesicles (EVs) are source of markers and was shown the role of TGFβ to CRT response in several carcinomas, the main goal of this study was correlate the presence of TGFβ in HNSCC EVs to cytotoxic therapies resistance and its useful as marker. Firstly, it was noted the TGFβ isoform-3 upregulation in the cisplatin-resistant cells (CisR). The TGFβ3 knockout sensitizes HNSCC cells to cisplatin and taxol treatment and when CisR were co-treated with exogenous TGFβ3 the drug-resistant phenotype was re-established. Besides, these drugs co-treatment with the TGFβRi (LY2109761) increased the cells sensitivity. Furthermore, EVs shed by CisR carried high amounts of TGFβ3 in comparison to those released by sensitive cells and these EVs presented a paracrine ability to turn cisplatinsensitive cells in more resistant ones, activating the TGFβ signaling pathway. Moreover, TGFβ3 was significantly higher in plasma EVs from HNSCC NR patients to CRT. Additionally, the TGFβ3 higher expression in a subpopulation of plasma EVs was associated with progression-free survival and remains as an independent prognostic factor for CRT final response. Therefore, TGFβ3 could be a target for therapies that aim to improve cisplatin response in HNSCC patients and its expression in plasma circulating EVs could be useful as prognostic marker for CRT response.

TIOTROPIUM INHIBITS THE RELEASE OF PROINFLAMMATORY EXTRACELLULAR VESICLES BY ACETYLCHOLINE-STIMULATED ENDOTHELIAL CELLS

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Introduction: Tiotropium is a long-acting muscarinic antagonist used as a bronchodilator in chronic obstructive pulmonary disease (COPD). Based on its role in preventing acute exacerbations of COPD, it has been speculated that besides its known bronchodilator properties tiotropium also exerts anti-inflammatory effects. We have shown that extracellular vesicles (EV) generated by mononuclear cells induce a pro-inflammatory phenotype in human lung epithelial cells. The aim of this study was to investigate whether muscarinic stimulation induces the generation of proinflammatory EV by human umbilical vein endothelial cells (HUVEC) and whether tiotropium modulates such effect.

Materials and Methods: The generation of HUVEC-derived EV induced by acetylcholine (Ach; 1mM; 1h) was investigated through a prothrombinase assay. Ach-induced HUVEC-EV were incubated overnight with HUVEC cells and the concentrations of IL-8 and MCP-1 assessed by ELISA.

Results: Ach stimulation of HUVEC cells caused an increase in EV from 0.40±0.04 to 1.07±0.49mM PS ($p<0.05$; paired t-test). EV generated by Ach-stimulated A549 cells caused an autocrine stimulation of the synthesis of IL-8 [(118±33 pg/mL vs 240±75 pg/mL) and MCP-1 (503±702 pg/mL vs 1422±1874 pg/mL), for unstimulated and EV---stimulated HUVEC cells]; $p<0.05$ for both comparisons; paired t-test. Preincubation of cells with tiotropium prior to Ach stimulation caused a dose-dependent inhibition of EV generation that reached maximum at 50 pg/mL (0.49±0.17 nM PS).

Conclusions: Muscarinic stimulation causes the generation of proinflammatory EV by human lung epithelial cells that is inhibited by tiotropium. This observation could contribute to explain the effect of tiotropium in the reduction of acute exacerbations of COPD.

DEVELOPMENT OF A NOVEL METHOD TO ISOLATE EXTRACELLULAR VESICLES IN CLINICAL ASSETS

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Introduction: Extracellular vesicles (EVs) are membranous particles released by cells into body fluids. They play a significant role in cell-cell communication in numerous physiological processes as well as in pathological conditions, through the transfer of functional proteins and nucleic acids. We developed a novel procedure that allows to purify heterogeneous EVs in an efficient, fast, cost-effective and reproducible way. We isolated EVs from glioblastoma and neuroblastoma cancer stem cells derived from patients, extracted RNA and performed a 3'RNA-seq analysis.

Materials and Methods: This method has been tested in protein-enriched systems using cutting-edge technologies in the field (transmission electron microscopy, tunable resistive pulse sensing, flow cytometry). Total RNA was extracted from EVs using Trizol reagent and we estimated the concentration and quality by Agilent 2100 Bioanalyzer. Furthermore, we performed a 3'RNA-Seq analysis using QuantSeq 3' mRNA-Seq Library Prep Kit (Lexogen).

Results: EVs isolated with our method are more intact and stable compared to other isolation techniques. A preliminary RNA-seq analysis shows the presence of enriched RNA species in EVs.

Conclusions: Our method can be easily applied to clinical practice and EVs exploited as an innovative tool for biomarkers detection in liquid biopsies. Thus, it opens to a new perspective for biomarker identification mediated by extracellular vesicles.

CHARACTERIZATION AND BIOGENESIS OF EXOSOMES SECRETED BY MELANOMA CELLS

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Introduction: Melanoma derives from melanocytes and is characterized by its high capacity to metastasize. Recently, two secreted entities, exosomes and Apolipoprotein E (ApoE), have been involved in the regulation of melanoma metastasis. We recently showed that melanoma cells secrete exosomes harbouring ApoE providing a major and unconventional pathway for the secretion of this apolipoprotein. In this study we aimed at characterizing melanoma derived exosomes with a particular focus on the biogenesis of ApoE exosomes and the trafficking of ApoE toward exosomes.

Materials and Methods: EVs released by melanoma cells were isolated by differential ultracentrifugation and floatation on density gradient and characterized using biochemical methods and electron microscopy. The trafficking of ApoE towards exosomes and the role of CD63 in this pathway were investigated after siRNA depletion by immunofluorescence and electron microscopy after high-pressure freezing.

Results: Our results show that melanoma cell lines secrete subpopulations of exosomes that can be distinguished by their density and composition. Contrary to metastatic melanoma, non-metastatic melanoma cells secrete a subpopulation of exosomes enriched in ApoE. Depletion of the tetraspanin CD63 decreases the secretion of ApoE on exosomes and induces an accumulation of ApoE in the cell, suggesting that CD63 is a regulator of the trafficking of ApoE toward exosomes.

Conclusions: Altogether this study shows that melanoma cells secrete exosome subpopulations with distinct compositions that may differently modulate melanoma progression. Our data shed new light on the role of CD63 in intracellular trafficking and in exosomes biogenesis that highlight its potential relevance during melanoma development.

DIFFUSE INTRINSIC PONTINE GLIOMA AND PEDIATRIC GLIOBLASTOMA DERIVED-EXOSOMES HAVE SPECIFIC ONCOGENIC SIGNATURES

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Introduction: Diffuse intrinsic pontine glioma (DIPG) and pediatric glioblastoma (pGBM) are heterogeneous brain tumors characterized by different anatomical and molecular subgroups and the presence of genetically and phenotypically distinct subclonal populations. It is recognized that exosomes mediate cross-talk among tumor cells. We hypothesize that there are different exosome-mediated paracrine signaling promoting tumour progression in DIPG and pGBM. Our aim was to determine the specific DIPG and pGBM-derived exosome oncogenic signatures.

Materials and Methods: We used a panel of fifteen primary patient-derived cell lines: nine DIPG (seven H3.3 K27M, one H3.3 K27M/ACVR1 and one H3.1 K27M/ACVR1), one diffuse midline glioma H3.3 K27M and three GBM (one H3.3 G34R and two histone WT). We collected conditioned medium from cells maintained

under stem-cell culture condition and exosomes were harvested through serial centrifugations. Exosomal protein cargo was analyzed by proteomic analysis and miRNA profile by qPCR.

Results: Electron microscopy showed microvesicles sized between 50-80 nm. Proteomic analysis revealed that proteins associated with vesicle docking, exocytosis and synaptic transmission were exclusively enriched in pontine-derived exosomes, while cell-cell and cell-matrix interaction proteins were exclusive to hemispheric ones. Proteins in common to the two locations were involved in metabolism and energy pathways. Exosomal miRNA profile appeared to be driven by the two main histone mutated subgroups H3.3 K27M and H3.1 K27M with the latter overexpressing hypoxia and angiogenic-associated miRNAs.

Conclusions: DIPG and pGBM-derived exosomes showed specific oncogenic signatures. Ongoing further investigations will enable the development of new diagnostic/prognostic tools and therapeutic strategies for DIPG and pGBM patients.

DETECTION AND CHARACTERIZATION OF BRAIN-DERIVED PLASMA EXOSOMES BY SURFACE PLASMON RESONANCE IMAGING

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Introduction: The use of exosomes for diagnostic and disease monitoring purposes is becoming particularly appealing, considering that the pathological status greatly affects the exosomes content. Moreover, brain-derived exosomes present in blood plasma could be studied as peripheral biomarkers of neurological disorders. Inspired by remarkable development of plasmonic biosensors having the ability to detect exosomes, we have designed an antibody array using surface plasmon resonance imaging (SPRi) with the aims to detect and characterize CNS-derived plasma exosomes according to the presence and the relative amount of membrane molecules.

Materials and Methods: Exosomes were isolated from plasma of healthy volunteers by size-exclusion chromatography and analyzed by nanoparticles tracking analysis, transmission electron microscopy, western blot and a nanoplasmonic assay to check the sample purity. The SPRi array was optimized for the detection of exosomes subpopulations, by using a suitable surface chemistry and specific antibodies for each class of vesicle to be detected.

Results: Exosomes were adsorbed on the SPRi chip, demonstrating the possibility to simultaneously distinguish exosomes derived specifically from neurons, microglia, astrocytes and oligodendrocytes using the multiplexing SPRi approach. Moreover, the presence and relative amount of other membrane constituents were evaluated using a sandwich approach, showing a different composition of exosomes according to their cellular origin.

Conclusions: SPRi can be used to discriminate different neural populations of circulating exosomes and to perform their concomitant characterization. The optimized SPRi biosensor represents a promising platform for the characterization of exosomes involved in neurological diseases and for their possible use as clinical biomarkers.

EXTRACELLULAR VESICLES DERIVED FROM PRO-REGENERATIVE MICROGLIA REGULATE OLIGODENDROGLIAL PROGENITORS' RESPONSE FOLLOWING CEREBRAL ISCHEMIA

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Introduction: Oligodendrocytes are severely affected by ischemia contributing to stroke-associated deficits. Recent data showed that the subpopulation of oligodendrocyte progenitors (OPCs) expressing the receptor GPR17 actively responds to the ischemic injury by increasing both proliferation rate and migratory ability but, at late stages, only some of these cells undergo maturation. This limited repair is likely due to the local unfavorable inflammatory environment regulated by blood-borne macrophages and resident microglia. Here, we aimed at understanding the time-dependent role of microglia/macrophages following cerebral ischemia and how these cells contribute to OPC response after middle cerebral artery occlusion (MCAo).

Materials and Methods: MCAo was executed in GPR17-iCreERT2:CAG-eGFP mice, allowing to visualize GPR17-expressing OPCs thanks to GFP production. The effect of partial microglia/macrophages depletion, achieved by Gadolinium chloride administration, on GFP+-OPC behavior was investigated by immunohistochemistry at early and late stages after ischemia. Moreover, infusion of extracellular vesicles (EVs) derived from pro-regenerative microglia was performed in the ipsilateral corpus callosum of ischemic mice to evaluate the impact on GFP+-OPC differentiation.

Results: Microglia/macrophages depletion led to a decrease in the number of GFP+-OPCs at early phase after ischemia while it induced GFP+-OPC increase at late stages. Furthermore, infusion of EVs released by pro-regenerative microglia enhanced GFP+-OPC maturation.

Conclusions: Data suggest that microglia/macrophages impact differently on OPC response during early or late stages after ischemia. Moreover, our results pointed out that EVs produced by pro-regenerative microglia positively affect OPC behavior, revealing EVs as important players in microglia-OPC cross-talk. *Supported by Fondazione Cariplo grant 2015-0910 to MF.*

DEVELOPING MULTIPLEX EXOSOMAL BIOMARKER ASSAY FOR AIDING EARLY DIAGNOSIS OF LEWY BODY DEMENTIAS

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Introduction: Prevalence of Lewy body dementias (LBD) is second only to Alzheimer's dementia (AD), and LBD cause earlier mortality, more morbidity, and higher costs than AD. Failure to diagnose LBD early risks potentially fatal neuroleptic sensitivity, and hinders appropriate management. Circulating exosomes help detecting molecular changes in living human brain, and hold promise for biomarker discovery.

Materials and Methods: We have completed three interlinked studies, and extensive bioinformatic analyses: 1) Next-generation RNA-sequencing (RNA-Seq) of post-mortem LBD anterior cingulate (ACC) and dorsolateral prefrontal (DLPFC) cortices to identify differentially expressed genes (DEG); 2) RNA-Seq of serum exosomes of people living with LBD to identify potential RNA markers; 3) Mass-spectrometry of LBD serum exosomes.

Results: We have identified 12 genome-wide significant DEG, distinct from genetic markers of AD, in LBD brains. Differential expression of 67 DEG ($p<0.05$), including cytokine genes such as ILIB and CXCL8, in post-mortem LBD brains could be detected in serum exosomes of people living with LBD. Reduced expression of cytokine and associated RNA in serum exosomes may help early diagnosis of LBD, and we have developed a 10-plex serum exosomal RNA assay using Luminex xMAP® technology.

Conclusions: Being the first study investigating LBD serum exosomal transcriptomics and proteomics, we identified potential RNA biomarkers, and developed the first bespoke serum exosomal multiplex assay for LBD. The identified DEG, and their dysfunctional molecular networks advanced molecular level mechanistic understanding of neurodegeneration in LBD. They do not corroborate chronic neuroinflammation, but support immune system dysfunction and mitochondrial dysfunction in LBD.

DO ASTROCYTES COMMUNICATE METABOLIC CHANGES VIA EXOSOMES?

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Introduction: Obesity is associated with both systemic and central inflammatory processes. In addition, specific nutrients for example, palmitic acid (PA), can directly induce hypothalamic inflammation. Hypothalamic astrocytes participate in this response, with PA altering their function and morphology both *in vivo* and *in vitro*. However, much remains unknown regarding how this astrogliosis affects the function of metabolic circuits, as well as the mechanisms involved. We hypothesized that astrocytes communicate changes in the nutritional environment to other cells through the release of exosomes.

Materials and Methods: Primary astrocyte cultures were prepared from male Wistar rats (2 days of age) and treated with vehicle, OA, PA or both (OA+PA). After 24 hours, exosomes were isolated from the culture media using a commercial extraction protocol. These exosomes were then added to new primary astrocytes cultures for 24 hours. The effect of these exosomes on the expression levels of cytokines [interleukin (IL6 and IL1β)] and markers of inflammatory pathways (NFKbia) and endoplasmic reticulum stress (CHOP) were determined by RT-PCR. Protein levels of the astrocyte markers glial fibrillary acidic protein (GFAP) and vimentin were analyzed by western blotting.

Results: Exosomes from OA-treated, but not PA-treated, astrocytes increased IL6 mRNA levels in another population of astrocytes, with no changes in any of the other genes studied. Vimentin and GFAP protein levels were not affected by any of the exosome treatments.

Conclusions: Astrocytes may modulate the response of neighboring cells by modifying the messages contained in the exosomes that they release; however, further studies are necessary.

EVS: A PROMISING TOOL TO DELIVER MIRNAS AGAINST MACHADO-JOSEPH DISEASE

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Introduction: Extracellular vesicles (EVs) are membrane-contained vesicles that are produced by the majority of cells. These EVs can communicate between cells, by carrying specific proteins, lipids and genetic material, particularly small nucleic acids. Among the different species of nucleic acids, miRNAs are small non-coding RNAs with around 22 nucleotides in size that are able to regulate gene expression at the post-transcriptional level.

Our aim was to evaluate the enrichment of specific miRNAs into EVs, aiming at their use as vehicles to treat neurodegenerative disorders, namely Machado-Joseph Disease – a neurodegenerative disorder associated with an abnormal over-repetition of the CAG tract within the ataxin 3 (*ATXN3*) gene, conferring toxic properties to the corresponding ATXN3 protein.

Materials and Methods: We first designed 2 artificial miRNAs targeting mutant ataxin 3 (mutATXN3) mRNA. The silencing efficiency was evaluated by Western Blotting in Neuro2A and HEK293T cells, previously infected with lentivirus encoding mutATXN3.

In the second part, in order to evaluate the efficacy of artificial miRNAs packaging into EVs, HEK293T cells were infected with lentivirus encoding each artificial miRNA. Medium and cells were collected and EVs were isolated by differential ultracentrifugation. All EVs were characterized by Nanoparticle Tracking Analysis (NTA) and Western Blotting and a RT-PCR analysis was performed to evaluate the levels of miRNAs in EVs and cells.

Results and Conclusions: Regarding the first task, we observed that both artificial miRNAs were able to silence mutATXN3 in Neuro2A and HEK293T cells. Moreover, these artificial miRNAs were found to be enriched into EVs, suggesting a promising tool to carry artificial miRNAs against MJD.

NEUTROPHIL EXTRACELLULAR VESICLES AS WEAPONS AGAINST *ASPERGILLUS FUMIGATUS*

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Introduction: Human polymorphonuclear granulocytes (PMNs) are essential determinants in the control and clearance of fungal infections caused by the opportunistic filamentous saprophyte *Aspergillus fumigatus*. An underexplored extracellular strategy of PMNs is the biogenesis and release of neutrophil-derived extracellular vesicles (PMNDEVs). Here we report for the first time an *ex vivo* host cell-fungus interaction that leads to a production of PMNDEVs that we refer as antifungal EVs (afEVs).

Materials and Methods: We used filtration and ultracentrifugation as EV enrichment techniques, flow cytometry for EV immunophenotyping, CLSM and EM imaging coupled to 2D and 3D-image-based analysis for EV functional studies and TMT-based nLC-M/MS proteomics for quantitative profiling of EV payload.

Results: We show that afEVs are produced by dose-dependent fungal PMN activation accompanied by induction of multivesicular bodies (MVBs) in PMNs. We reveal a novel role of the well-known fungal virulence determinant dihydroxynaphtalene (DNH)-melanin in modulation of EV kinetics, EV surface repertoire and EV proteome cargo. We showed that AfEVs have synergistic fungistatic and fungicidal effects related to their antimicrobial payload. These effects proceed from afEV binding and internalization into fungal elements, which is dependent on EV's intact membrane and cytoskeleton. AfEV-hyphae interactions led to induction of fungal cell death and alterations in cell-wall morphology related to cell-wall damage.

Conclusions: We describe novel immunomodulatory properties of DHN-melanin- a virulence factor with multifaceted properties in immune evasion. Our work shows the new potential of PMNDEVs as fungistatic and fungicidal agents and extends on the understanding of extracellular killing of fungi by PMNs.

NOVEL APPROACH OF UTILISING SERUM/PLASMA EV FOR TREATMENT MONITORING IN GLIOBLASTOMA PATIENTS

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Introduction: Glioblastoma (GBM) is the most aggressive primary brain tumour in adults. Treatment monitoring based on brain imaging is challenging due to the unreliable differentiation between true progression and treatment-associated changes. In this project, we evaluate different methods of extracellular vesicles (EV) purification, in order to specifically isolate GBM-EVs from human serum/plasma and introduce EVs as possible biomarkers for treatment monitoring in GBM patients.

Materials/Methods: EVs from primary GBM cells and the Gaussia luciferase expressing Gli36-GLuc cells were isolated via size-exclusion chromatography (SEC) and ultracentrifugation. EV-surface markers were evaluated by flow cytometry. Gli36-GLuc EVs containing GLuc mRNA were spiked in healthy plasma. Thereafter, plasma EVs were isolated via ultracentrifugation, SEC and immunoprecipitation. Subsequently, RNA was isolated from vesicles and evaluated for GLuc levels via qRT-PCR. These results were compared to total cell-free RNA isolated from the above-mentioned plasma.

Results: EVs from GBM cells expressed high levels of CD29 and CD44, when compared to EVs from healthy donor plasma. Gli36-GLuc EVs spiked in healthy plasma were more effectively isolated with CD44-based immunoprecipitation than with ultracentrifugation or SEC, as shown by higher GLuc RNA levels in the corresponding vesicles. When compared to total cell-free RNA extracted from this plasma, RNA from EVs exhibited a higher GLuc yield.

Conclusions:

1. CD44 could serve as a novel, promising target for GBM-EV and be utilised for immunoprecipitation-based EV capturing.
2. Using the appropriate EV purification method possibly affects their potential as biomarkers for GBM.