

Profiling and characterization of embryo and seminal fluid extracellular vesicle-associated microRNAs in *Sus scrofa*: a possible role in differentiation and developmental communication processes

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ISEV

INTERNATIONAL SOCIETY FOR
EXTRACELLULAR VESICLES

The Fifth International Meeting of ISEV ISEV2016

Rotterdam, The Netherlands, 4 – 7 May, 2016

Abstracts

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Wednesday, 4 May 2016			
8:00 – 9:00 a.m.	Registration		
8:30 – 9:00 a.m.	Welcome Coffee		
9:00 – 9:20 a.m.	Opening Sessions		
9:20 – 10:30 a.m.	Plenary Session 1 – EVs in Cancer Speakers: K. Pantel (Germany) and D. Lyden (USA) Room: Willem Burger		
10:30 – 11:00 a.m.	Networking Coffee		
11:00 – 12:30 p.m.	Symposium Session 1 EVs and Implications to Cancer Therapy Room: Willem Burger	Symposium Session 2 EV Proteomics and Lipidomics Room: van Weelde	Symposium Session 3 Cell Biology and EVs I Room: Jurriaanse
12:30 – 1:30 p.m.	Lunch		
1:30 – 2:15 p.m.	Oral with Poster Session 1 EV Isolation, Characterization and Stem Cells Room: Willem Burger	Oral with Poster Session 2 EVs, Immunity and More Room: van Weelde	Oral with Poster Session 3 EVs in Cancer and Other Diseases Room: Jurriaanse
2:15 – 3:00 p.m.	Symposium Session 4 EVs of the CNS Room: van Weelde	Symposium Session 5 EV Isolation Room: Jurriaanse	Symposium Session 6 EVs in Cancer Transcriptomics Room: Willem Burger
3:00 – 3:30 p.m.	Networking Coffee		
3:30 – 5:00 p.m.	Biotech Sponsored session: CARIS Life Sciences (3:30–3:45 p.m.)	Symposium Session 8 EVs and Tumor Environment I Room: Willem Burger	Biotech Sponsored session IZON (3:30–4:00 p.m.)
	Symposium Session 7 (3:45–5:00 p.m.) EVs in Metabolic Syndrome, Fatty Liver Disease and Cancer		Symposium Session 9 (4:00–5:00 p.m.) EV Biomarkers for Cancer I Room: Jurriaanse
5:00 – 8:00 p.m.	Poster Session 1 - Cell Biology of EVs: Biogenesis and Transfer Poster Session 2 - EVs in Tumor Metastasis Poster Session 3 - Analysis of EVs in Body Fluids Poster Session 4 - EVs as Drug Delivery System for Antitumoral Therapies & Vaccination Poster Session 5 - EVs in Acute and Chronic Inflammation Poster Session 6 - EV Proteomics & Lipidomics Poster Session 7 - EV Isolation Poster Session 8 - EVs in Prostate Cancer		
6:30 – 8:30 p.m.	Satellite Event Meet the National and International Societies Room: Jurriaanse		
Thursday, 5 May 2016			
8:00 – 8:45 a.m.	Meet the Experts Session 1 EV Heterogeneity: Can we Overcome the Confusion? Room: Willem Burger	Meet the Experts Session 2 EVs in Regenerative Medicine Room: Jurriaanse	Meet the Experts Session 3 EVs in Infectious Disease Room: van Weelde
9:00 – 10:15 a.m.	Symposium Session 10 EVs in Cross-species Communication Room: Jurriaanse	Symposium Session 11 EV Characterization: Flow Cytometry Room: Willem Burger	Symposium Session 12 EVs in Cardiovascular Disease Room: van Weelde
10:15 – 10:45 a.m.	Networking Coffee		
10:45 – 12:30 p.m.	Plenary Session 2 - Exosomes and viruses Speakers: R. C. Gallo (USA), L. Margolis (USA), Shilpa Buch (USA) Room: Willem Burger		
12:30 – 1:30 p.m.	Lunch		
1:30 – 3:00 p.m.	Symposium Session 13 EVs in Remodelling and Repair Room: van Weelde	Symposium Session 14 Engineering EVs Room: Jurriaanse	Symposium Session 15 EVs and Tumor Environment II Room: Willem Burger
3:00 – 3:30 p.m.	Networking Coffee		

3:30 – 4:30 p.m.	Symposium Session 16 New Techniques for EV Characterization Room: van Weelde	Symposium Session 17 EV Biomarkers for Cancer II Room: Jurriaanse	Symposium Session 18 EVs in Neuropathies Room: Willem Burger
5:00 – 8:00 p.m.	Poster Session 1 - EVs, Microbes & Cross-species Communication Poster Session 2 - EVs in Cardiovascular Disease Poster Session 3 - EVs in Cardiovascular Disease and Coagulation Poster Session 4 - EVs in Stem Cells Poster Session 5 - EVs as Cancer Biomarkers Poster Session 6 - EVs in the CNS Poster Session 7 - EV Characterization from Different Sources or Different Subtypes Poster Session 8 - Late Breaking Poster Session 1		
6:30 – 8:30 p.m.	Satellite Event ISEV-ISAC-ISTH Workshop Room: Jurriaanse		
Friday, 6 May 2016			
8:00 – 8:45 a.m.	Meet the Experts Session 4 EVs as Modulators of Drug Resistance and Tumor Metastasis Room: Willem Burger	Meet the Experts Session 5 EV-Associated Versus Soluble Functional Molecules Room: Jurriaanse	Meet the Experts Session 6 Extracellular RNAs: EVs or not EVs Room: van Weelde
9:00 – 10:15 a.m.	Symposium Session 19 EVs in the Immune System Room: Jurriaanse	Symposium Session 20 EVs in Cancer Metastasis I Room: Willem Burger	Symposium Session 21 EVs in Reproduction and Pregnancy Room: van Weelde
10:15 – 10:45 a.m.	Networking Coffee		
10:45 – 11:25 a.m.	Plenary Session 3 – EVs in Cell Biology Speaker: F. Sanchez-Madrid (Spain) Room: Willem Burger		
11:25 – 12:30 p.m.	ISEV General Assembly		
12:30 – 1:30 p.m.	Lunch		JEV Editorial Board Meeting
1:30 – 2:45 p.m.	Symposium Session 22 EVs in Acute and Chronic Inflammatory Disease Room: van Weelde	Symposium Session 23 EVs in Cancer Metastasis II Room: Jurriaanse	Symposium Session 24 EVs in Immune Modulation in Bacterial and Parasitic Infections Room: Willem Burger
2:45 – 3:15 p.m.	Networking Coffee		
3:15 – 4:15 p.m.	Diamond Sponsor Session Caris Life Sciences Room: Willem Burger		
4:15 – 5:00 p.m.	Symposium Session 25 EVs Viruses and Viral Infections Room: Jurriaanse	Symposium Session 26 EVs in Cellular Differentiation and Development Room: van Weelde	Symposium Session 27 Cell Biology of EVs II Room: Willem Burger
5:00 – 7:30 p.m.	Poster Session 1 - Non-cancer Biomarkers Poster Session 2 - EVs and Cancer Poster Session 3 - EVs and the Immune System Poster Session 4 - EVs in the Tumor Microenvironment Poster Session 5 - MSCs & Tissue Regeneration and Morphogenesis Poster Session 6 - Novel Technical Developments in EV Characterization Poster Session 7 - Late Breaking Posters Session 2		
7:30 p.m.	Networking Event (registration required) Location: Laurens Church		
Saturday, 7 May 2016			
8:00 – 8:45 a.m.	Experts Meet Session 1 Biofluids: Blood	Experts Meet Session 2 Biofluids: Milk	Experts Meet Session 3 Biofluids: Urine
9:00 – 10:00 a.m.	Late Breaking Oral Session 1 Room: Willem Burger	Late Breaking Oral Session 2 Room: van Weelde	Late Breaking Oral Session 3 Room: Ruys, van Ruckevorsel, Plate Zaal
10:00 – 10:30 a.m.	Networking Coffee		
10:30 – 11:50 a.m.	Plenary Session 4 with Featured Abstracts ISEV2016 Room: Willem Burger		
11:50 – 12:35 p.m.	Special Achievement Award, Wrap-up Sessions, Oral and Poster Awards		
12:35 – 12:45 p.m.	Closing Remarks and Announcement of ISEV2017		

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We sincerely apologize for the inconvenience to the authors and will do our utmost to ensure this problem does not recur in the future.

The ISEV2016 organizers.

Scientific Program ISEV 2016 meeting Wednesday May 4, 2016

Room: Willem Burger

Plenary Session 1 - EVs in cancer

9:20-10:30 a.m.

Chairs: *Clotilde Théry and Pascale Zimmermann*

Speakers:

Klaus Pantel (University Medical Center Hamburg-Eppendorf, Germany)

Liquid Biopsy in Cancer Patients

David C. Lyden (Weill Cornell Medical College, USA)

The Systemic Effects of Exosome-Mediated Metastasis

Oral Presentations

Room: Willem Burger

Symposium Session 1 - EVs and implications to cancer therapy

Chairs: *Takahiro Ochiya* and *Hans van Leeuwen*

11:00-12:30 p.m.

OW1.1

To catch a ride: packaging and delivery of retrotransposons through extracellular vesicles

Kristina P. Friis¹, Leonora Balaj¹, Romy Verschoor¹, Rafael Contreras-Galindo², Shilpa Prabhakar¹, David M. Markovitz² and Xandra O. Breakefield¹

¹Department of Neurology and Radiology and Program in Neuroscience, Massachusetts General Hospital and Harvard Medical School, Boston, Maine, USA; ²Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan, USA

Introduction: Retrotransposons are remnants of ancient viral infections that infected the genome of our primate lineage through infection of germ cells. Scattered throughout the genome, these elements are capable of amplifying themselves as well as changing their position through RNA intermediates. Interestingly, retrotransposon elements are highly enriched in tumour extracellular vesicles (EVs) compared with their cells of origin and have been shown to be transferred in vitro from medulloblastoma cells to normal human umbilical vein endothelial cells (Balaj et al., 2011). Furthermore, the production of EVs seems to share resemblance with retroviral particle assembly, thus making retroviral-like particles a component of the EV contingent. Hence, we are now testing whether a high prevalence of the retrotransposon-like elements known as human endogenous retroviruses (HERVs) in tumour cell lines leads to selective packaging and transfer of exRNA through EVs. **Methods:** The assay is based on the BOGOTA construct (Contreras-Galindo et al., 2015), which includes parts of the HERV-K113 packaging signal, and is selected through a neomycin reporter gene. We have screened several cell lines for the presence of HERVs. Expression of neomycin is used to select for cell recipients and the initial confirmation of integration of the transferred retroviral element into the genome of the recipient. **Results:** Preliminary studies indicate that the transfer of retroviral elements is successful between different cell lines. The use of the BOGOTA construct results in a selective packaging of the reporter gene into EVs. The neomycin resistance encoding RNA is highly enriched in the resulting EVs compared with their cells of origin. **Conclusion:** The indication of retrotransposon transfer between different cell lines supports future studies to determine the role of retrotransposon transfer in tumorigenesis, and potentially as a means of generating therapeutic vehicles utilizing viral packaging signals.

OW1.2

Milk-derived exosomes – a platform nanocarrier to enhance anti-proliferative, anti-inflammatory and anti-cancer activities of small drug molecules against multiple human cancers

Ramesh Gupta¹, Farrukh Aqil¹, Manicka Vadhanam¹, Jeyaprakash Jeyabalan¹, Ashishkumar Agrawal¹, Ashley Mudd¹, Alhassan Kyakulaga¹, Divya Karukonda¹, Wendy Spencer² and Radha Munagala¹

¹University Of Louisville, Louisville, Kentucky, USA; ²3P Biotechnologies, Louisville, Kentucky, USA

Introduction: Exosomes are considered viable nano-transporters for carrying a payload of both small and macromolecules. We recently reported that bovine milk exosomes loaded with small molecules

are readily taken up by cells and show higher therapeutic response than free agents against lung cancer. Using curcumin (CUR), bilberry anthocyanidins (Anthos), withaferin A (WFA) and celastrol (CEL), we now report that this concept applies to multiple cancers. **Methods:** Human breast, lung, ovarian, pancreatic, prostate, cervical and colon cancer cells were treated in vitro with CUR, WFA, CEL and/or Anthos, as free agents or as exosomal (Exo) formulations. Inhibition of cancer cell proliferation and inflammation were measured by multi-table tournament assay and electrophoretic mobility shift assay, respectively. The antitumour activities of Exo formulations given orally were also determined against ovarian and lung tumour xenografts in nude mice. **Results:** All test agents as Exo formulation showed 2- to 20-fold higher antiproliferative activity against the various cancer cell lines versus free agents. A greater anti-inflammatory effect of Exo-CUR was observed versus CUR in lung cancer cells challenged with TNF- α . Oral delivery of Exo-WFA, Exo-Anthos and Exo-CEL showed enhanced anti-cancer effects in nude mice carrying lung and ovarian tumour xenografts versus the free agents. Tagging Exo-WFA with a tumour-targeting ligand, folic acid further enhanced the therapeutic activity against ovarian tumour. Exosomes alone showed intrinsic activity of 20–50% growth inhibition of all human cancer cells tested in vitro; normal cells were not affected. Finally, no systemic toxicity was observed following treatment of wild-type mice with the exosomes. **Conclusion:** Our data suggest that milk exosomes can serve as a nanocarrier platform to enhance activity of therapeutics by increasing cellular uptake and bioavailability against multiple cancers.

Financial support: From Duggan Endowment, Helmsley Trust Fund and the USPHS grant R41-CA-189517.

OW1.3

Exosomes transfer chemotherapeutic drug resistance between colorectal cancer cells

Stephanie Boukouris¹, Suresh Mathivanan², John Mariadason³ and Michael Liem¹

¹La Trobe University, Melbourne, Australia; ²Olivia Newton-John Cancer Research Institute, Heidelberg, Australia; ³Institute of Molecular Sciences, La Trobe University, Melbourne, Australia

Introduction: Chemotherapy is currently the mainstay for advanced colorectal cancer (CRC) with 5-fluorouracil (5-FU) being the most commonly used chemotherapeutic drug. While some patients respond to chemotherapy, some are inherently resistant for 5-FU. Among the responding patients, some acquire resistance and therefore fail to further respond to treatment. In this study, we investigated whether exosomes, a class of extracellular vesicles, can transfer chemotherapeutic drug resistance between cells. **Methods:** Exosomes were isolated by differential centrifugation followed by ultracentrifugation from colorectal cancer cell conditioned media and characterized by western blotting and electron microscopy. To identify regulators of chemotherapeutic resistance, label-free quantitative proteomics analysis was performed on both parental and 5-FU-resistant colorectal cancer cells including the respective derived exosomes. Fluorescence-activated cell sorting (FACS) cell cycle arrest, clonogenic assay and western blotting were carried out on SW620 and HCT116 upon incubation with exosomes secreted by resistant cells. **Results:** 5-FU-resistant CRC cells were established by continuous

culture of parent cells with increasing concentrations of 5-FU. Molecular biology and biochemical experiments confirmed the role of epithelial-mesenchymal transition (EMT) in this acquired drug resistance. Exosomes isolated from 5-FU-resistant cells were able to transfer the resistant phenotype to parental and other wild-type CRC cells as confirmed by cologenic and FACS apoptosis assays. Quantitative proteomic analysis of exosomes released by resistant and parental cells revealed the differential abundance of many proteins implicated in EMT, including YBX1. Western blotting analysis confirmed the induction of EMT in the recipient cells upon incubation with exosomes released by 5-FU-resistant cells. **Summary:** The results established that exosomes can transfer resistance to 5-FU in colorectal cancer cells therefore aiding in treatment failure.

OW1.4

Extracellular vesicles regulate gefitinib sensitivity in head and neck cancer cells

Dorival Rodrigues-Junior¹, D. P. Lau¹, F. T. Chong¹, H. S. Leong¹, D. S. Tan¹, S. S. Tan², S. K. Lim², Andre Luis Vettore³ and N. G. Iyer¹
¹National Cancer Centre of Singapore, Singapore; ²Institute of Medical Biology, Singapore; ³Federal University of Sao Paulo, Brazil

Tyrosine kinase inhibitors (TKIs) to epidermal growth factor receptor (EGFR) (gefitinib) have shown limited efficacy in the treatment of head and neck squamous cell carcinoma (HNSCC) patients despite pre-clinical data showing addiction to these pathways. Notwithstanding, a small subpopulation of patients has shown excellent response to TKIs, which does not correlate with EGFR expression or amplification. The objective of this study was to determine whether factors that confer TKI sensitivity could be transferred via extracellular vesicles (EVs). HNSCC cell lines Tm17 and Tm49, which were inherently sensitive to gefitinib (wild type, WT), and their isogenic gefitinib-resistant (GR) counterparts, generated through treatment with sublethal doses of gefitinib, were used in this study. EV isolation was based on binding properties: cholera toxin B chain (CTB) and annexin V (AV), which, respectively, binds GM1 ganglioside and phosphatidylserine. Both Tm17 GR and Tm49 GR were re-sensitized to gefitinib when cultured in EV-enriched conditioned media from Tm17 WT. Antibody arrays were performed to identify proteins in the EVs of Tm17 WT and Tm17 GR. For CTB-EVs, 16 and 69 proteins were overexpressed in WT and GR cells, respectively. For AV-EVs, 66 and 21 proteins were overexpressed in WT and GR cells, respectively, with a further 18 proteins exclusively found in WT. Ingenuity Pathways Knowledge Base analyses showed that differentially overexpressed proteins in the EVs of sensitive cells were related to apoptosis, cell death and inflammatory response ($p < 0.01$). These data indicate that WT HNSCC cells secrete EVs that have paracrine ability to re-sensitize their resistant counterparts to gefitinib, providing a potential treatment option of using EVs and improving clinical outcome for these patients. Interestingly, we identified 18 proteins exclusively present in EVs isolated from Tm17 WT that may contribute to the development of tools for prediction and assessment of gefitinib response.

OW1.5

Lipid and protein composition distinguishes stem cell EVs from cancer cell EVs and affects efficiency of EV-mediated small RNA cellular transfer

Reka Haraszti¹, Andrew Coles², Matthew Hassler², Julia Alterman², John Leszyk², Marie Didiot², Scott Shaffer², Hannah Rockwell³, Michael Kiebish³, Neil Aronin² and Anastasia Khvorova²
¹RNA Therapeutics, University of Massachusetts Medical, Worcester, MA, USA;
²University of Massachusetts Medical School, Worcester, Massachusetts, USA;
³Berg LLC, Farmington, Massachusetts, USA

Introduction: Extracellular vesicles (EVs) are implicated in the transfer of endogenous small RNAs between cells and are being explored as a formulation strategy for therapeutic RNA delivery. We hypothesize that EV trafficking is dependent on lipid and protein composition, which is defined by the source cell. **Methods:** Exosomes and microvesicles (MVs) were purified from different source cell types via differential ultracentrifugation. Lipidomic and proteomic profiles were assessed by MS/MS(ALL) and liquid chromatography coupled to mass spectrometry (LC-MS/MS), respectively. Purified vesicles were loaded with hydrophobically modified siRNA (hsiRNA) and applied to primary neurons in culture or directly infused into striata of mice. hsiRNA distribution and mRNA silencing were quantified by PNA (peptide nucleic acid)-hybridization and QuantiGene(r) assays, respectively. **Results:** All cell types tested produced EVs with characteristic lipid (~1900 species identified) and protein (~3500 proteins identified) profiles. Most importantly, stem cell EVs significantly differed from cancerous cell EVs with fibronectin, histone 3A, long-tailed glycolipids and sphingomyelins showing the largest difference. Furthermore, MVs were more similar to their source cells than exosomes were and displayed enrichment of certain mitochondrial and ER proteins as well as ceramides, cholesterol esters and diacyl glycerols. Meanwhile, exosomes were depleted in these and rather enriched in immune response and cell adhesion proteins as well as cardiolipins and glycolipids. Cellular uptake and hsiRNA potency were enhanced when formulated into EVs in a source cell-dependent manner. When source cells were serum-starved before EV purification, up to 15-fold enhancement in potency was observed. **Conclusions:** Functional as well as proteomic and lipidomic studies confirmed that source cells affect EV trafficking and composition. Serum-starved mesenchymal stem cell exosomes were the most potent delivery vesicles for therapeutic hsiRNA.

OW1.6

Extracellular vesicle-mediated reversal of castrate-resistant prostate cancer

Devasis Chatterjee¹, Michael Del Tatto¹, Joseph Renzulli², Justin Wang¹ and Peter Quesenberry¹
¹Rhode Island Hospital, Providence, Rhode Island, USA; ²The Miriam Hospital, Providence, Rhode Island, USA

Background: Castrate-resistant prostate cancer (CRPC) is the second leading cause of cancer-related death in men in the developed world. While androgen deprivation therapy is effective at the onset of treatment, nearly all men develop castrate resistance. Several new therapies, including enzalutamide (Enz)-based chemotherapy, have improved outcomes for CRPC. We have shown extracellular vesicles (EVs) isolated from non-malignant cells can inhibit the malignant phenotype. We investigated the role of EVs isolated from human mesenchymal stem cells (hMSC EVs) reversing the malignant phenotype and Enz resistance in prostate cancer (PCa). **Methods:** EVs isolated from human mesenchymal stem cells were co-cultured with Enz sensitive or resistant cells after which western blot analysis, anchorage independent and tumour xenograft growth were assessed and sensitivity to Enz was monitored via multi-table tournament assay. **Results:** We used Enz sensitive (C4-2B) and resistant (C4-2BR) PCa cells for our studies. hMSC EV treatment of C4-2BR cells restored sensitivity to Enz via the transfer of Raf kinase inhibitor protein (RKIP). RKIP inhibited STAT3 activation in C4-2BR cells. hMSC EV treatment induced apoptosis, inhibited anchorage independent growth, cell invasion and tumour xenograft formation. **Conclusions:** hMSC EVs can reverse the malignant "education" of recipient cells and provide the promising basis to investigate the therapeutic utility of hMSC EVs for the treatment of CRPC.

Room: van Weelde

Symposium Session 2 - EV proteomics and lipidomics

Chairs: Aled Clayton and Alissa Weaver

11:00-12:30 p.m.

OW2.1

Advanced molecular tools for proteomic analyses and characterization of microvesiclesMasood Kamali-Moghaddam¹, Liza LåF¹, Di Wu², Junhong Yan¹, Felipe Oliveira¹, Qiujiu Shen¹ and Wik Lotta¹¹Department of Immunology, Genetics and Pathology/Molecular Tools, Uppsala University, Uppsala, Sweden; ²Department of Biochemistry and Biophysics, Science for Life Laboratory, Solna, Sweden

Proteomic studies of extracellular microvesicles provide valuable knowledge about their origin and functions. However, the small size, low concentration and the heterogeneity are some of the features limiting characterization of these microvesicles. We have developed a large number of molecular tools such as different forms of proximity assays using two or more antibodies with conjugated DNA strands for single- or multiplex measurement of proteins in solution and in situ. Utilizing the multiplex proximity extension assay, we are able to analyse microvesicles for their protein content in minute amount of samples, and by applying a novel method, proximity barcoding assay (PBA), we are also able to characterize proteomic heterogeneity from individual microvesicles by targeting the surface proteins. In PBA, distinct DNA oligonucleotides with different protein-specific tags (ST) are conjugated to each of 24 different antibodies, directed against their cognate potential protein targets on the surface of microvesicle. Clusters of antibody-conjugated oligonucleotides are brought in proximity when the antibodies bind to the proteins on the surface of the same microvesicle, allowing hybridization and extension on a DNA concatemer of identical copies of a single-strand DNA molecule carrying a unique complex-ST. Thus, clusters of DNA oligonucleotides on the same microvesicle will incorporate the same complex-ST. The protein composition on individual microvesicle will then be revealed via next-generation sequencing by analysing the protein-ST and complex-ST from each sequencing read. Here, we illustrate the application of PBA to analyse thousands of individual microvesicles in each sample to differentiate between microvesicles from different origins. We also illustrate the application of the multiplex PEA for analyses of the protein content of different microvesicles using different protein panels. These methods can potentially be used to identify exosomes as disease biomarkers.

OW2.2

Proteomics-driven design of endothelial stress-based protein array for disease prognostics - applied to plasma and cerebrospinal fluidKenneth Kastaniegaard¹, Malene Møller Jørgensen², Rikke Bæk², Tobias Sejrbæk³, C. Wivie⁴, J. Baumbach⁴, Vladimir Zachar¹, Torben Moos¹, Søren Nielsen¹, John D. Nieland¹, F. Cotton⁵, C. Guttmann⁶, Zsolt Illes³ and Allan Stensballe¹¹Department of Health Science and Technology Aalborg University, Aalborg, Denmark; ²Aalborg University Hospital, Aalborg, Denmark; ³Department of Neurology, Odense University Hospital, Odense, Denmark; ⁴Institute of Clinical Research, University of Southern Denmark, Denmark; ⁵Service de Radiologie, Université de Lyon, France; ⁶Center for Neurological Imaging, Brigham and Women's Hospital, USA

Introduction: Endothelial dysfunction is central to multiple autoimmune diseases such as multiple sclerosis (MS). The stressed

endothelium may have a pro-inflammatory phenotype and can be characterized by biomarkers derived from different molecular pathways. The "barcode" of protein profiles, lipid composition and post-translational modifications (PTMs) from circulating classes of microvesicles (MVs) are important features for EVs. Our aim was to optimize the EV array, a well-functioning high-throughput method for EV and exosome phenotyping, for rapid improved diagnosis and prognosis of autoimmune diseases characterized by local endothelial dysfunction. **Methods:** Crude MVs and exosomes of different biological origins including endothelial cell lines (brain, lung), plasma and cerebrospinal fluid from MS patients and synovial fluid from rheumatoid arthritis patients were isolated using either ultracentrifugation or by immunoaffinity purification by custom-made immunobeads. The endothelial cell lines were subjected to oxidative stress, hypoxia or treatment with metabolism-modifying drugs. Purified MVs were validated and characterized by particle size determination and electron microscopy. The EV proteomes were characterized using quantitative proteome analysis and functional annotation. **Results:** Analysis of crude MVs and enriched exosomes by label-free quantification enabled detection of the EV-subproteome including surface-exposed proteins specific to treatment/disease. Putative prognostic markers for endothelial stress were used as part of the panel of the EV array, a sandwich ELISA type protein array highly optimized for EV quantification in biofluids. The applicability of the array and comparability of the quantitative data were compared to similar data obtained by mesoscale (41 cytokines) or quantitative mass spectrometry.

OW2.3

Proteomic analysis of extracellular vesicles isolated from airway epithelial cells using ultrafiltration followed by size exclusion chromatographyBirke J. Benedikter^{1,2}, Freek G. Bouwman³, Emiel F. M. Wouters², Paul H. M. Savelkoul^{1,4}, Gernot G. U. Rohde², Edwin C. Mariman³ and Frank R. M. Stassen¹¹Department of Medical Microbiology, Maastricht University Medical Center, Maastricht, The Netherlands; ²Department of Respiratory Medicine, Maastricht University Medical Center; ³Department of Human Biology, Maastricht University Medical Center; ⁴Department of Medical Microbiology & Infection Control, VU University Medical Center, Amsterdam, The Netherlands

Background: Situated at the vast lung surface, the airway epithelium is crucial for regulating pulmonary homeostasis. We and others have found that airway epithelial cells (AEC) secrete extracellular vesicles (AEC-EVs) under basal and pathological conditions. Getting insights into the composition of these AEC-EVs will help unravel their functions in lung homeostasis and pathology. Here, we characterized the proteomic composition of basal AEC-EVs, which were concentrated using ultrafiltration (UF) and then purified by size exclusion chromatography (SEC). **Methods:** Medium from BEAS-2B cells (120 mL, conditioned for 24 h by 2.3×10^7 cells) was depleted of cell debris and passed through a 0.22- μ m filter. Next, it was concentrated to 1 mL on a 10 kDa centrifuge filter and run over a sepharose CL-4B column. Fractions were assessed for protein content (Bradford assay, Silver staining) and EV content (CD63 + CD81 + bead-coupled flow cytometry). Proteomic composition of the pooled EV fractions was determined using mass spectrometry (nLC-MS/MS Q Exactive). Overrepresentation analysis was performed using ConsensusPathDB. **Results:** About 10 kDa UF concentrated the EVs with a recovery near 100%. The majority of EVs eluted in SEC fractions 6–11, while protein only became detectable at fraction 12. Mass spectrometry of the EV

fractions identified more than 500 different proteins, including 28 out of the 34 EV markers compiled by de Menezes-Neto et al. (JEV, 2015). Prominent pathways overrepresented in the AEC-EVs were metabolism of proteins, translation, protein folding and proteasomal degradation. **Conclusion:** UF followed by SEC efficiently separates EVs from free protein, which is ideal for proteomic analysis. The overrepresentation of pathways involved in protein metabolism suggests that AEC-EVs may contribute to regulating cellular proteostasis. Future research will focus on proteomic differences between AEC-EVs released under normal and pathological conditions, and their functional implications.

OW2.4

In-depth proteomics analysis of human breast milk-derived extracellular vesicles reveals a novel functional proteome distinct from other milk components

Martijn J. C. van Herwijnen¹, Marijke I. Zonneveld^{1,2}, Soenita Goerdal³, Esther N. M. Nolte-t Hoen¹, Johan Garssen^{2,4}, Maarten Altaelaar³, Frank A. Redegeld² and Marca H. M. Wauben¹

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Introduction: Breast milk contains macromolecular components with distinctive functions, whereby milk fat globules and casein micelles provide nutrition, while whey contains molecules that stimulate the infant's immunity. Interestingly, the importance and function of extracellular vesicles (EVs) in milk has not been addressed in detail. This in part is due to the difficulty to purify EVs from other milk components, and consequently, the molecular composition of human milk-derived EVs has not been fully elucidated. **Methods:** In this study, an extensive liquid chromatography coupled to mass spectrometry (LC-MS/MS) proteomic analysis was performed on EVs isolated via density-based separation from human breast milk of 7 individuals. The proteome of milk-derived EVs was analysed using FunRich and compared to the Vesiclepedia database, as well as to the whole milk proteome, which was constructed from 38 previously published milk proteomics studies. **Results:** A total of 1963 proteins were identified in milk-derived EVs, including EV-associated proteins like CD9, annexin A5 and flotillin-1. Overlap analysis revealed that 198 proteins were not yet listed in Vesiclepedia. Interestingly, these newly identified proteins included common milk proteins, like oleoyl-ACP hydrolase and parathyroid hormone-related protein. Next, the milk-derived EV proteome was compared to the whole milk proteome. Remarkably, 633 proteins identified in milk-derived EVs have not yet been identified in milk to date. Interestingly, these novel proteins included proteins involved in regulation of cell growth and control of inflammatory signalling pathways. **Conclusion:** These data show that milk-derived EVs harbour previously unidentified proteins that have the capacity to support the infant's developing gastrointestinal tract and its immune system. In addition, this study provides an expansion to the whole milk proteome, illustrating that milk-derived EVs are a distinct macromolecular component with a unique functional proteome.

OW2.5

Characterization of extracellular vesicles derived from hepatitis B virus producing liver cells

Kwang Pyo Kim, Kyung-Cho Cho, Raju Bandu, Joon Won Lee and Jae Hun Jung

Applied Chemistry, Kyung Hee University, Yongin, Korea

Infection of hepatitis B virus (HBV) is one of the major causes of hepatocellular carcinoma (HCC) in the world. The extracellular vesicles are expected to be involved in carcinogenesis, although their exact oncogenic functions remain controversial. In this study, we characterized extracellular vesicles from hepatitis B virus (HBV)-infected liver cell line (HepG2.2.15) and non-infected liver cell line (HepG2). The extracellular vesicles were isolated using the method of centrifugation with the sucrose cushion and density gradient solution. The western blot results revealed that the isolated extracellular vesicles were purely isolated without HBV, which have similar size as extracellular vesicles. It is noteworthy that the transmission electron microscopy (TEM) images and dynamic light scattering data showed that the extracellular vesicles derived from HepG2.2.15 cell have more size than the HepG2 cell-derived extracellular vesicles. Further, we identified and quantified specific extracellular proteins from both HepG2.2.15 and HepG2 cell lines by using Easy-nLC coupled Q-exactive mass spectrometric analysis and Maxquant search tool. In this study, a total of 1724 proteins were identified, and 364 proteins were selected as dominant proteins in which 256 EV proteins were significantly down-regulated and 108 EV proteins were up-regulated in HepG2.2.15 when compared with HepG2. The biological functional properties of the extracellular vesicles that are relevant to HBV-infected cell, including their roles as actin cytoskeleton organization, vesicle-mediated transport, generation of precursor metabolite and energy and translation.

OW2.6

Apoptotic cell-derived extracellular vesicles – towards defining a structure-function relationship

Ivana Milic^{1,2}, Roberta Liccardo^{1,2}, Allan Cameron^{1,2}, Helen Griffiths^{1,2}, and Andrew Devitt^{1,2}

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Introduction: Apoptotic cell-derived extracellular vesicles (ACdEVs) represent a vital link by which dying cells communicate their presence to enable their removal, thereby modulating immune responses. This process is of fundamental importance as failed clearance of dying cells influences inflammation, cardiovascular diseases and cancer. While a function of ACdEVs has been demonstrated, the molecular mediators are ill defined. Our work seeks to undertake the first comprehensive analysis of ACdEV molecular structure and function. A variety of cargo may be loaded into EVs, with miRNA and proteins being the most widely investigated. We aim to address fatty acid-derived lipid mediators, for example, lipoxins, resolvins, protectins and maresins, which have been identified as crucial mediators for the resolution of inflammation. **Methods:** Peripheral blood mononuclear cells (PBMCs) were isolated using lympoprep from defibrinated blood. Anti-Fas-induced apoptosis was determined by flow cytometry with annexin V and propidium iodide staining. ACdEVs were isolated by ultracentrifugation. Size characterization and concentration of EVs was determined using tunable resistive pulse sensing technology. The molecular composition of ACdEVs was analysed by liquid chromatography online coupled to tandem mass spectrometry (LC-MS/MS) operating in multiple reactions monitoring mode. **Results:** ACdEVs released from apoptotic PBMCs fall within the exosome size range and were demonstrated to be rich in pro-resolving lipid mediators. We quantified docosahexaenoic acid-derived resolvins (RvD1, RvD2) and maresin 1, eicosapentaenoic acid-derived RvE1, arachidonic acid-derived lipoxins LXA4 and LXB4, including prostaglandins PGE2 and PGD2, and leukotriene LTB4. The relative ratios of lipid mediators between ACdEVs from early and late apoptotic PBMCs differed and may underpin differential EV function. **Summary:** Our novel data suggest that ACdEVs and lipid mediators may play an important role in monocyte recruitment to sites of cell death and may provide new strategies for the control of inflammation.

Room: Jurriaanse

Symposium Session 3 - Cell biology and EVs I

Chairs: *Michael Graner and Matias Ostrowski*

11:00-12:30 p.m.

OW3.1

The role of S-palmitoylation in the biogenesis of skeletal muscle-derived extracellular vesicles and the structural organization of their membrane

Daniele P. Romancino¹, Valentina Buffa¹, Samuele Raccosta², Antonietta Notaro¹, Ines Ferrara¹, Alessandra d'Azzo³, Vincenzo Martorana², Mauro Manno² and Antonella Bongiovanni¹
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Introduction: Skeletal muscle (SkM) cells can release Alix-positive exosomes directly from their sarcolemma, suggesting a new paradigm for understanding how muscles communicate with adipose tissue, the brain or tumours. Our goals, here, were to understand how muscle cells generate these vesicles, to evaluate their heterogeneity and membrane structure, and to determine what regulates them. **Methods:** We have used an integrated biological/biophysical approach to determine whether protein lipidation (i.e. S-palmitoylation) affects the localization of extracellular vesicle (EV) modulator(s), EV biogenesis, cargo selection or other processes by (i) evaluating post-translational S-palmitoylation of EV proteins as well as their cell membrane and EV protein levels and (ii) characterizing EV heterogeneity and membrane structure in dispersion using dynamic light scattering (DLS), atomic force microscopy, small-angle X-ray scattering and small-angle neutron scattering. **Results:** By coupling these data, we were able to determine that Alix is S-palmitoylated and that palmitoylation inhibition altered its subcellular localization and protein interaction. Furthermore, the biophysical analyses of SkM-EVs showed that the structural organization of the lipid bilayer of palmitoylated-inhibited exosomes is qualitatively different compared to non-treated exosomes. **Conclusion:** We propose that S-palmitoylation might regulate the proper function of Alix in SkM EV biogenesis, support the interactions among the exosome-specific regulators/biomarkers and maintain proper EV membrane structural organization. Beside its biological relevance, our study sets the route for a comprehensive structural characterization of EV, which is expected to be crucial in EV-based therapies to be employed in the tissue regeneration field, for example, to help in recovery from muscle atrophy and/or injury.

Italian Ministry for Education, University, and Research in the framework of the Flagship Project NanoMAX.

OW3.2

Unexpected cytosolic protein localization on the surface of extracellular vesicles

Sarah Stewart, Florian Gessler, Stefano Pluchino and Kevin Moreau
 University of Cambridge, Cambridge, United Kingdom

Extracellular vesicles (EVs) contain many specific proteins, both cytosolic and membrane bound. The current model for extracellular vesicle biogenesis dictates that the subcellular location of proteins should be reflected in the EV architecture, where cytosolic proteins are in the lumen and cell surface proteins are on the outside of EVs. This is consistent with the conventional protein trafficking pathway for cell surface localization, whereby proteins destined for the plasma membrane (or secretion) must contain a signal sequence to target them to the secretory pathway. It is clear that receptors

anchored in the membrane are retained in the correct orientation and RNAs are in the lumen of EVs. However, there is limited data on the packaging and location of other proteins commonly identified in EVs. Annexin A2 is a cytosolic calcium-dependent membrane-binding protein consistently identified in EVs. Using biochemical approaches, we show that annexin A2 localizes to both the lumen and the surface of EVs. Based on this result, we are now investigating the function of annexin A2 on the surface of EVs. Additionally, we are using mass spectrometry analysis to identify other proteins expressed on the surface of EVs. The presence of cytosolic proteins on the surface of EVs raises questions about their trafficking and may provide further insight into EV packaging and function. It is possible that proteins are specifically packaged on the outer membrane of EVs. In which case, EVs may provide a pathway for unconventional protein trafficking to the cell surface. Understanding this unconventional protein trafficking pathway is extremely important and will uncover a novel fundamental cellular process.

OW3.3

Investigation of the molecular mechanism of cargo editing in human PMN-derived EVs

Balázs Bartos, Attila Mocsai, David Szombath, Márton Ákos Lorincz and Erzsébet Ligeti
 Department of Physiology, Semmelweis University, Budapest, Hungary

Introduction: In our previous studies, we demonstrated that the neutrophilic granulocytes are able to produce different types of extracellular vesicles (EVs) after different stimulation. EVs that were generated after stimulation by opsonized particles have an inhibiting effect on bacterial growth. The aim of this study was to investigate the role of certain immune-receptors (FcR, C3R) and src tyrosine kinases in the production of antibacterial EVs. **Methods:** We isolated polymorphonuclear cells from peripheral blood of healthy donors. After different biological stimulations, EVs were separated by two-step centrifugation and filtration. EVs were counted by EV-optimized flow cytometry. EV protein amount was measured by Bradford assay, and granule proteins were detected by western blotting. Antibacterial effect of different EVs was analysed by bacterial survival test. **Results:** We found that isolated stimulation of beta2 integrines or Fc receptors or pattern recognition receptors increased the EV production. Combined stimulation has additive effect on EV generation. In contrast to superoxide production and phagocytosis, inhibition of src kinases did not interfere with EV production. However, cargo protein and biological function of EVs depend on src kinases. **Conclusion:** EV production of neutrophils can be increased and modified both through integrine and Fc receptor stimulation, independent of the process of phagocytosis or superoxide production. Src kinases do not play a role in EV generation but through EV quality control and cargo editing they do influence the biological function of produced EVs.

OW3.4

YKT6 regulation of exosome release is mediated by microRNAs and impacts prognosis in non-small-cell lung cancer

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Introduction: Non-small-cell lung cancer (NSCLC) has a dismal prognosis, highlighting the need for new prognostic markers. The role of exosomes and their regulatory genes in intercellular communication is a promising research area. YKT6 is involved in the control of exosome release and has been linked to poor prognosis in breast cancer but its role in NSCLC has not been studied. We aim to examine the role of YKT6 in exosome release and to identify its regulating microRNAs (miRNAs) in vitro, and to evaluate the prognostic impact of YKT6 in NSCLC. **Methods:** After 48 h of YKT6 inhibition, exosomes were isolated by ultracentrifugation, characterized by nanoparticle tracking analysis and Cryo-TEM and quantified by western blot using the exosomal marker TSG101. miRNAs targeting YKT6 were validated by Renilla-Luciferase assay and western blot. In tumour and normal tissue specimens from 98 surgically resected NSCLC patients, YKT6 expression levels were assessed and correlated with disease-free survival (DFS) and overall survival (OS). Informed consent was obtained in accordance with the Declaration of Helsinki, and Hospital Clinic ethics committee approved the study. **Results:** A549 cells with YKT6 inhibition had an 80.9% reduction in exosome release ($p = 0.01$). Renilla-Luciferase assay identified miR-134 and miR-135b as miRNAs targeting YKT6. In A549 cells transfected with pre-miR-134 or pre-miR-135b, YKT6 protein levels decreased by 51.45% ($p = 0.01$) and 45.53% ($p = 0.02$), respectively, while exosome release decreased by 43.92% ($p = 0.03$) and 53.43% ($p = 0.008$), respectively. In patient samples, YKT6 expression was lower and miR-134 and miR-135b expression was higher in tumour than in normal tissue. Patients with lower YKT6 expression had longer DFS ($p = 0.02$) and OS ($p = 0.01$). **Conclusions:** Our findings are a first step towards a greater understanding of the role of exosome release and their regulatory genes in NSCLC and their potential role as new prognostic markers.

OW3.5

Scavenger receptor A blockade leads to tumour accumulation of systemically administered extracellular vesicles bearing interleukin-15
Dionysios C. Watson¹, Avinash Srivatsan², Defne Bayik^{3,4}, Gang Niu², Barbara K. Felber⁵, Ihsan Gursel⁴, Xiaoyuan Chen² and George N. Pavlakis¹
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Introduction: We identified the scavenger receptor A family (SR-A) as responsible for the rapid clearance by liver macrophages of systemically delivered therapeutic extracellular vesicles (EVs). We hypothesized that blocking SR-A would decrease liver uptake, resulting in improved tissue distribution of therapeutic EV bearing interleukin-15 (IL-15), which stimulate NK and T-cells, as an in vivo anti-cancer therapy. **Methods:** HEK293 cells expressing heterodimeric IL-15 (IL-15:IL-15Ra [hetIL-15]) were grown in a hollow-fiber bioreactor. EV-bearing hetIL-15 were purified from conditioned medium by differential ultracentrifugation. EVs were stained with SytoGreen

RNAselect or DiR, and unincorporated dye removed by gel filtration. Uptake of SytoGreen-stained EVs by cell lines and human primary cells was monitored in vitro by flow cytometry in the presence of specific scavenger receptor A and B blockers. Biodistribution of DiR-stained EVs delivered into healthy and 4T1 (mouse breast cancer cell line) tumour-bearing mice treated with dextran sulphate (SR-A blocker) was monitored in vivo by fluorescence imaging. **Results:** Dextran sulphate blocked EV uptake in the SR-A1 + mouse macrophage RAW264.7 cell line and in human primary monocytes, but had no effect in SR-A1(−) cell lines, such as 4T1. Combining selective SR-A and SR-B blockers decreased EV uptake additively. A single injection of dextran sulfate resulted in 50% reduction of EV liver uptake together with an increase of free plasma EVs 2 h after treatment. Finally, pretreatment with dextran sulfate of tumour-bearing mice led to an increase, compared to untreated control mice, of EV uptake by the tumour over 24 h. **Summary/conclusion:** SR-A is responsible for EV clearance by the liver. Blocking this receptor enhances EV pharmacokinetics and distribution to tissues, including tumours. These effects could be improved by combination of several SR inhibitors and the use of therapeutic EV bearing tissue-specific targeting ligands.

OW3.6

Binding of EVs is regulated by CD44-hyaluronan interactions and induce EMT-like changes in target cells

Uma Arasu¹, Ashik Jawahar Deen², Sanna Oikari², Riikka Kärrä¹, Arto Koistinen², Kai Härkönen², Kirsi Rilla², Pia Siljander and Elisa Lazaro-Ibanez⁴

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Hyaluronan (HA) is the most abundant and essential polysaccharide of the extracellular matrix for maintenance of normal tissues, but it also creates a favourable microenvironment for growth of tumour cells. HA is synthesized by specific plasma membrane-bound enzymes, hyaluronan synthases (HASs). The HASs activate growth of extremely long filopodia and shedding of HA-coated extracellular vesicles (EVs). EVs are suggested to interact with their target cells by utilizing receptors on the vesicular surface, such as CD44, which is the most common receptor for HA. The aim of this work was to study these interactions and their effects on target cells. The HA-coated EVs secreted by GFP-HAS3 overexpressing cells were shown to carry GFP-HAS3 (both as protein and mRNA) using techniques like immunoblotting and qPCR. Moreover, confocal microscopy and correlative light and electron microscopy showed that GFP-HAS3 containing EVs (i.e. HAS3-EVs) induced morphological changes and increased the size of pericellular HA coat of the target cells. HA secretion of the target cells to the culture medium was also increased as analysed by ELISA-like assay. Furthermore, adding an excess of HA oligosaccharides in the incubation medium to displace HA from the receptors, Streptomyces hyaluronidase digestion to remove the HA coat or treatment with CD44 blocking antibody regulated the vesicle binding to the target cell, thereby showing that HA-CD44 interactions are important for EVs binding to the target cells. HAS-induced EVs act as carriers for HA on their surface and are potential vehicles in preparing the premetastatic niches. Furthermore, CD44-HA interactions may act as universal mechanism facilitating cellular binding of EVs. This may result in signals to modify the target cells functions like paving way for benign cells to become metastatic. Moreover, HA-induced EVs could be utilized as diagnostic tools and targets of therapy.

Room: Willem Burger

Oral with Poster Session 1 - EV isolation, characterization and stem cells

Chairs: *Kenneth W Witwer and Andrew Devitt*

1:30-2:15 p.m.

OPW1.1 = PW7.02**A protocol for isolation and proteomic characterization of distinct extracellular vesicle subtypes by sequential centrifugal ultrafiltration**

Rong Xu, David Greening and Richard Simpson

Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Melbourne, Australia

Introduction: Extensive biochemical and biophysical validation with heterogeneous EV mixtures, along with disparate isolation schemes and annotations, has blurred the boundaries of defining EVs, raising the query whether these vesicles represent discreet entities with specialized functions or are they merely a size continuum of the same entity. It is well recognized that cells secrete essentially two EV subtypes that can be partially separated by differential centrifugation: the larger size class (referred to as “shed microvesicles” or sMVs) are heterogeneous (100–1,500 nm), while the smaller size class (“exosomes”) are relatively homogeneous in size (50–150 nm). A key issue hindering progress in understanding underlying mechanisms of EV biogenesis and cargo selectivity has been the technical challenge of isolating homogeneous EV subpopulations (exosomes and sMVs) suitable for molecular analysis. **Methods:** In this study, we reveal a novel method for the isolation, purification and characterization of exosomes and sMVs. This method, based on sequential centrifugal ultrafiltration (SCUF), affords unbiased isolation of EVs from conditioned medium from a human colon cancer cell model. **Results:** For both EV subtypes, this protocol details extensive purification, and characterization based on dynamic-light scattering, cryo-electron microscopy, quantitation, immunoblotting and comparative label-free proteome profiling, in addition to fibroblast invasion. We reveal TSG101, Alix/PDCD6IP and CD63, considered stereotypic protein markers of exosomes, to be exclusively enriched only in exosomes, while numerous proteins found exclusively in sMVs (KIF23, RACGAP1 and CSE1L/CAS) warrant further study as to their potential use as discriminatory markers for sMVs. **Summary/conclusion:** This analytical SCUF method developed is potentially scalable using tangential flow filtration and provides a solid foundation for future in-depth functional studies of EV subtypes from diverse cell types.

OPW1.2 = PF6.05**Label-free detection of extracellular vesicles in human breast milk compared to infant formula**L. De Rond^{1,2}, Edwin Van Der Pol^{1,2}, Auguste Sturk², Rien Nieuwland², Frank Coumans^{1,2} and T.G. Van Leeuwen¹¹Department of Biomedical Engineering and Physics, Academic Medical Centre, Amsterdam, The Netherlands; ²Department of Clinical Chemistry, Academic Medical Centre, Amsterdam, The Netherlands

Introduction: Extracellular vesicles (EVs) in human breast milk carry immune relevant structures (J Immunol. 2007; 1;179(3)), and breast milk has a beneficial long-term effect on infants’ health when compared to infant formula. Therefore, we study the relation between EV and infants’ health. For this study, the concentration of EVs has to be determined. However, detection of EVs in breast milk and infant formula is complicated by the abundance of lipoprotein particles. We recently demonstrated that scatter signals of a flow cytometer uniquely define the diameter (d) and refractive index (RI) of particles below ~500 nm. RI detection can discriminate EVs (RI 1.37–1.42) from lipoproteins (RI 1.43–1.50). Therefore, we determine the EV to lipoprotein ratio (EV:LP) of breast milk, cow milk

and (cow milk-derived) infant formula. **Methods:** Forward and side scatter signals of particles between 200 and 500 nm are measured on an A50-Micro (Apogee, UK). To validate the method, we compared the measured d and RI to the specified d and RI for two silica and three polystyrene bead populations. Breast milk (4 donors, 11–20 weeks postpartum) was obtained with informed consent. The EV:LP was derived from the RI distribution. **Results:** Validation with beads showed that d and RI could be determined with a measurement error between 1.5% and 9.5% for d and between 0.17% and 2.7% for RI. The EV:LP of the breast milk samples was between 1.3 and 3.9, which was much higher than the EV:LP of infant formula (0.22). The EV:LP of infant formula was comparable to cow milk (0.34). **Conclusion:** Calibrated measurement of the forward and side scatter signals on a flow cytometer enables label-free determination of the number of EVs and lipoproteins in milk. The average EV:LP of breast milk is 9-fold higher than that of infant formula. The observed difference indicates that EVs may contribute to human breast milk’s beneficial effect on the infants’ health.

OPW1.3 = PF6.01**Characterization of EVs by flow cytometry**

John Tigges, Camacho Virginia and Toxavidis Vasilis

Flow Cytometry Science Center, Center for Extracellular Vesicle Research, Beth Israel Deaconess Medical Center, Boston, USA

Characterization of extracellular vesicles (EVs) is greatly impeded by several factors: their size (below 100 nm), their overlapping size distribution, particle polydispersity and an overall low refractive index. Flow cytometry is an advantageous tool for the analysis and characterization of EVs because of its robust statistical power and its multiparametric capabilities. The goal of nanoscale analysis and sorting, termed small particle flow cytometry (SPFC), is to accurately represent the size distribution and scatter profiles of these vesicles. The most challenging elements have been the lack of sufficient forward scatter resolution and precision optics to delineate populations from background and instrument noise. Because conventional flow cytometers are not equipped with necessary optical capacities to reach this lower threshold, instruments that are used for SPFC typically have specific adaptations. In the light of these enhancements, there are a number of modifications to the conventional form of analysis that should be considered. Here we describe a comprehensive methodology for the set up and standardization of EV analysis using SPFC. Controls of different size ranges, fluorescent intensities and materials are required to set up distribution curves. These are then used for instrument optimization and as a reference guide. Using these controls, FACS instruments can be primed for the detection, analysis and sorting of specific EV populations. This allows for cross platform comparison and the ability to monitor QC, QA. The method here will describe the use of nanoparticles to optimize a flow cytometer for small particle detection. It will also outline the procedures necessary to recover EVs for downstream applications.

OPW1.4 = PW7.09**Comparative analysis of urine storage, exosomes/EV purification and normalization methods for the selection of most appropriate solutions for biomarker discovery, clinical diagnostics and biobanking**Natasa Zarovni¹, Antonietta Corrado¹, Giorgia Radano¹, Elisa Lari¹, Davide Zocco¹ and Riccardo Vago²

¹Exosomics Siena, Spa, Siena, Italy; ²Urological Research Institute, Istituto di ricovero e cura a carattere scientifico Ospedale San Raffaele, Milan, Italy

Background: Non-invasive urine harvesting as well as its enrichment with extracellular vesicles (EVs) from urinary-genital tract makes it ideal for detection of diagnostic markers for a set of clinical conditions. Unlike blood whose composition and volume is tightly regulated, urine concentration varies with nutrition, hydration and environmental factors. Biobanks usually comprise low volumes and spot, rather than time normalized urine samples. Conservation and purification impacts the detection of some analytes, including EV subsets, and the normalization approach further introduces bias in inter- and intra-individual/population comparisons. We have evaluated (i) the stability of urine upon storage at room temperature (RT), (ii) the appropriate method and minimal volume for recovery of relevant EV proteins and RNAs and (iii) normalization method including creatinine (CT) and protein measure and EV count. **Methodology:** Ultracentrifugation, precipitation, immuno/affinity capture and SEC column were used for EV isolation from healthy donor urine that was either frozen or stored for 6 months at RT. EV proteins and miRNAs were quantified by western blot, ELISA, FACS, qPCR, while CT and protein levels, and EV number (nanoparticle tracking analysis) were measured in row urine and in EV pellets. **Results:** Tested long-term storage of urine at RT was equivalent or superior to standard freezing protocol. Creatinine levels do not correlate with EV content, while EV number and protein load have similar trends. The correlation of latter with canonical EV proteins (CD9/CD63, Alix, Tsg101) depended on the purification method. Relative abundances of selected EV proteins/miRNAs are likely to give true information on underlying condition/pathway. Immuno and affinity precipitation methods provided most efficient and specific recovery of urine EV molecules from as few as 1 mL of urine. **Conclusions:** We describe realistic urine processing and normalization solutions for unbiased readout of EV biomarker studies and realistic routine clinical sampling and diagnostics.

OPW1.5 = PT3.15

Quick screening for microparticle content in platelet-rich plasma of cardiovascular patients

Elisabeth Maurer-Spurej¹ and Audrey Labrie²

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Introduction: Cardiovascular disease is associated with elevated plasma microparticle content (MPC). For routine clinical screening of plasma samples, an inherently standardized test that requires minimal sample preparation is desirable. Although dynamic light scattering (DLS) cannot characterize particle origin or function, it enumerates particles by size without need for calibration. ThromboLUX is a DLS device specifically designed for quick screening of platelet-rich plasma (PRP) and platelet concentrates. It detects particles in the size range of 20 nm to 20 µm (100–1100 nm defined as microparticles). This study tested the performance characteristics of the ThromboLUX microparticle assay and its utility to screen patients before and after stenting. **Methods:** ThromboLUX microparticle assay precision was determined over 20 days. For linearity, MPC between 4 and 57% was tested with ThromboLUX and flow cytometry (N = 21). Following an approved clinical protocol, PRP was obtained from sodium citrate anticoagulated whole blood from fasting patients requiring percutaneous coronary intervention (PCI) (N = 17) and healthy subjects (N = 8). Baseline samples before PCI were from the arterial sheath and 1 day after PCI from peripheral venipuncture. PRP samples were fixed and frozen until use. **Results:** Over 20 days ThromboLUX measured low, medium or high MPC in platelet concentrates at $4.4 \pm 1.8\%$, $19.5 \pm 2.6\%$ and $53.8 \pm 5.8\%$, respectively. MPC was linear (CV = 11.8%) and correlated highly with flow cytometry ($r = 0.99$). MPC in pre- and post-PCI samples was significantly higher ($32 \pm 11\%$ and $40 \pm 9\%$) than in healthy samples ($16 \pm 8\%$, $p = 0.001$) corresponding to MP concentrations of 3.8 ± 2.2 , 4.8 ± 2.2 and $1.5 \pm 1.0 \times 10^{12}$ MP/L, respectively.

Summary/conclusion: The ThromboLUX microparticle assay is repeatable and linear without the need for platelet removal or dilution. In combination with other parameters, elevated MPC ($> 23\%$ or 2.5×10^{12} MP/L) might be a diagnostic biomarker for cardiovascular disease.

OPW1.6 = PT4.10

Effect of ageing on pro-inflammatory micro-RNAs contained in MSC-derived extracellular vesicles

Juan Antonio Fafian Labora¹, Fons A. J. Van De Loo², Onno J. Arntz², Pablo Fernández Pernas¹, Miranda Bennink³ and María C. Arufe¹

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The promising role of mesenchymal stem cells (MSCs) in cell-based therapies and tissue engineering appears to be limited due to a decline of their regenerative potential with increasing donor age. Recently, it has been reported that micro-RNAs play a role in ageing and immunosenescence. miRNAs are also present in the extracellular vesicles from MSCs and their composition may change during ageing. It is believed that extracellular vesicle (EV)-based therapies will reduce the risks and drawbacks associated with cell-based therapies. For that we decided to analyse whether ageing affects the miRNA profile in MSCs-derived exosomes. EVs from conditioned medium of bone marrow MSCs obtained of Wistar rats at 4 ages (newborn, young, pre-pubertal and old) using ultracentrifugation. The size of EVs was characterized using nanoparticle tracking analysis (NTA), electron microscopy and for exosome marker CD63 by flow cytometry, and for the presence of micro-RNAs (miR-155, miR-146a, miR-132 and miR-21) in MSC-derived EVs using a commercial kit. We found that production of EVs by MSCs in old age group was increased with ageing $25.6 \pm 1.2\%$ but their expression of pro-inflammatory miRs related to Toll-like receptor 4 decreased $93.3 \pm 3\%$ as compared to the other age groups. Our extracellular vesicles had a size of 160.3 ± 18.26 nm and their expression of CD63 was $0.4 \pm 0.1\%$. In MSC-derived EVs, the expression of miR-155, miR-146a and miR-132, all known inhibitors of the TLR-4 signalling pathway, decreased with age of the donor. MSC-derived EVs from the pre-pubertal group had the highest level of miR-21, a known miRNA that enhances the anti-inflammatory IL-10 cytokine production. We conclude that ageing of rat donors affects the production of extracellular vesicles by mesenchymal stem cells and also changed their content of miRNAs. This an important finding as the age of the donor may determine the therapeutic potential of MSC-derived EVs to inhibit the innate immune response.

OPW1.7 = PF5.07

Extracellular vesicles released by induced pluripotent stem cells modulate cellular function of recipient cardiac endothelial cells

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Introduction: Stem cell-derived extracellular vesicles (EVs) have recently gained attention as highly bioactive, acellular carriers in a wide variety of biomedical applications. The molecular composition and regenerative potential of EVs derived from murine-induced pluripotent stem cells (miPSCs) have not been investigated. Thus, we characterized RNA and protein content of miPSC-EVs and investigated their effects on target cell behaviour. **Methods:** EVs were purified by differential centrifugation of conditioned media collected from the serum- and feeder-free culture of miPSCs. The morphology

and size of EVs were characterized with atomic force microscopy and nanoparticle tracking analysis. Western blot, high-sensitivity flow cytometry, real-time RT-PCR and liquid chromatography coupled to mass spectrometry (LC-MS/MS) analysis were used to characterize EV content. Cardiac endothelial cells were exposed to miPSC-EVs in the culture. **Results:** We established that miPSC-EVs are rich in mRNAs, miRNAs and proteins carried by donor cells. In particular, miPSC-EVs contained transcripts regulating pluripotency, self-renewal and differentiation, including Oct4, Nanog and Rex1. Among 282 miRNAs detected in iPS cells, 199 were simultaneously present in EVs. Interestingly, 33 miRNAs were expressed exclusively in EVs, including embryonic stem cell-specific miR-302a-5p. Endothelial cells treated with miPSC-EVs exhibited greater migratory, proliferative and angiogenic activity. **Summary/conclusion:** Our data show for the first time that miPSC-derived EVs are natural nanocarriers capable of transferring bioactive contents to mature cells affecting target cell function as well as regenerative potential. We conclude that miPSC-EV may represent a safe therapeutic alternative to whole cell-based therapy for cardiovascular repair. In order to further assess regenerative capacity of miPSC-EVs, we are currently studying their effect for the repair of ischemic tissue injury in murine in vivo model.

OPW1.8 = PT3.10

Pro-coagulant activity of platelet-derived extracellular vesicles

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Introduction: The biological activity of human platelet-derived micro-vesicles (MVs) was investigated with respect to their association with tissue factor (TF) and their potential to initiate the clotting cascade. We compared MVs from physiological platelets to MVs from lipopolysaccharide (LPS)-stimulated monocytic THP-1 cells with respect to amount and activity of TF. **Methods:** Platelet concentrates from healthy volunteer donors were collected using the Trima Accel[®] system (Version 5.0, Gambro BCT). After removal of platelets (1500 g, 15 min, RT), MVs were enriched by centrifugation (20000 g, 30 min, 4°C) and characterized by flow cytometry (FC) and nanoparticle tracking analysis (NTA). In FC, MVs were identified as annexin V-positive events in the MV gate after calibration with fluorescent beads (0.1, 0.3, 0.5, 0.9 µm). TF expression was evaluated by western blot (clones: HTF-1 and TF9-10H10). The Technothrombin TGA kit (Technoclone GmbH) and the Enzygnost TAT micro (DADE Behring) were used to assess thrombin generation and formation of thrombin-antithrombin complex. **Results:** The mean size of MVs was 173 ± 15 nm. FC showed 70 ± 9.7% CD41⁺ AV⁺ events in the MV gate. Both platelets and platelet-derived MVs expressed TF to different extent and supported the generation of thrombin in a dose-dependent manner. Thrombin generation was strongly inhibited by pre-incubation with annexin V and partially inhibited by anti-TF (HTF-1 clone, 10 µg/mL). When compared to THP-1-derived MVs, MVs of platelet origin differed with respect to the lag phase rather than to the amount of thrombin generated. **Conclusion:** Our findings support the presence of TF in platelet-derived MVs as well as its correlation to their pro-coagulant activity. Differences between physiological platelet-derived and LPS-stimulated THP-1-derived MV preparations suggest that there are not only quantitative but also qualitative dissimilarities between TF on physiological MVs and on MVs released during LPS-stimulation.

Room: van Weelde

Oral with Poster Session 2 - EVs, immunity and more

Chairs: *Erzsébet Ligeti and Susanne Gabrielsson*

1:30-2:15 p.m.

OPW2.1 = PW2.13**Induction of tolerogenic dendritic cells by nasopharyngeal carcinoma-derived exosomes**

Sarah Renaud

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Background: A characteristic of the nasopharyngeal carcinoma (NPC) micro-environment is the presence of immunosuppressive exosomes released by tumour cells. Our team has recently shown that NPC-derived exosomes, which carry galectine-9, favour the recruitment and suppressive activity of human regulatory T cells (Treg), thus contributing to NPC immune escape (Mrizak et al., JNCI, 2015). In this study, our objective is now to evaluate whether these NPC-derived exosomes could promote the emergence of tolerogenic immature dendritic cells (tolDC) able to induce regulatory T cells from nave CD4+ T cells ultimately contributing to the tolerance of tumour cells. **Methods and results:** We performed a complete phenotypical and functional study comparing the effect of NPC and healthy donor-derived exosomes on DC maturation. This study includes (i) cell morphological analysis by photonic microscopy, (ii) transcriptional study by RT-qPCR, (iii) flow cytometric analysis of the expression of specific makers (phenotypic DC and co-stimulatory markers), (iv) a preliminary DC functional study by western blotting (IDO) and finally (v) a secretome analysis by ELISA (IL-10; TGF- β , TNF- α). Taken together, our results strongly suggest that the presence of NPC-derived exosomes favours the emergence of semi-mature DCs seemingly tolerogenic. **Conclusion:** Despite the importance of immature DCs as mediators of cancer immune escape, no other studies have shown the impact of tumour exosomes on the maturation of human DCs. Thus, these promising results should open new prospects for antitumour immunotherapies based on the inhibition of factors involved in the emergence and activation of Treg.

OPW2.2 = PF3.01**Immunogenic and tolerogenic dendritic cells release extracellular vesicles that differ in small noncoding RNA content**Tom Driedonks, Susanne Van Der Grein, Henrike Jekel, Tom Groot Kormelink, Marca Wauben and Esther Nolte-'t Hoen
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Introduction: Depending on environmental cues, dendritic cells (DC) differentiate into potent stimulators of T-cell-mediated adaptive immune responses or into tolerogenic DC that downregulate immune responses. We hypothesize that the molecular composition of extracellular vesicles (EVs) released by tolerogenic and immunogenic DC affects modulation of T-cell activation by these DC subtypes. We previously reported that EVs from DC-T-cell co-cultures are enriched in (fragments of) structural RNAs vault RNA, Y-RNA and 7SL-RNA, which may have the potential to regulate transcription and/or translation processes. **Methods:** Murine bone marrow-derived DC were rendered immunogenic by lipopolysaccharide (LPS) stimulation, whereas tolerogenic DC were generated by 1 α ,25-dihydroxyvitamin D3 (VitD3)-treatment. EVs were isolated from culture supernatant by density gradient centrifugation. EV numbers were determined by high-resolution flow cytometry. The small RNA content of the DC

subtypes and their corresponding EVs was compared for structural RNAs and immune-relevant miRNAs by RT-qPCR using carefully selected reference genes. **Results:** Immunogenic DC released more EVs, which contained higher levels of endotoxin responsive miRNAs (miR155a, miR146a and miR29a) than tolerogenic DC. The relative differences in EV miRNA content reflected differences in cellular miRNA levels in the DC subtypes. Strikingly, tolerogenic and immunogenic EVs varied in 7SL-RNA, vault RNA and Y-RNA contents and these RNAs were either enriched or underrepresented in EVs compared to cellular levels. **Conclusions:** The EVs released by tolerogenic and immunogenic DC vary in quantity and in (immune) regulatory RNA content. Whereas levels of miRNAs in EVs reflected the cellular levels, 7SL-/vault-/Y-RNAs were differentially incorporated into EVs. These structural RNAs may therefore play a role in immune regulation via EVs. Future studies will address whether and how the EV-RNA released by these functionally different DC contributes to DC-T-cell communication.

OPW2.3 = PF3.02**Exosomes released by regulatory T cells modulates T cell polarization**Sim Tung, Akansha Agarwal, Marilena Letizia, Giorgia Fanelli, Robert Lechler, Giovanna Lombardi and Lesley Smyth
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Introduction: Regulatory T cells (Tregs) maintain immune tolerance to self-antigens and prevent excessive pro-inflammatory actions of T-effectors (Teffs) and antigen presenting cells (APCs) responses. These cells inhibit in a variety of ways including the release of extracellular vesicles (EVs) called exosomes. We have previously shown that the immune modulatory role attributed to mouse Treg-derived exosomes was the expression of the ectoenzyme CD73. To date, whether human Tregs also use this mechanism has not been investigated. Given our mouse data, we hypothesized that human Tregs release exosomes that can prevent Teffs function. **Methods:** Human CD4+ CD25+ T cells were isolated from blood and were stimulated in the presence of rapamycin using a protocol previously shown to expand and stabilize Tregs. These cells inhibited Teffs proliferation as expected. To isolate exosomes, Tregs were activated using plate-bound anti-CD3/CD28 antibodies for 24 h. Culture supernatant was collected, cells removed and supernatant passed through a 0.22- μ m filter and either ultracentrifuged at 100,000 g or was mixed with ExoQuick solution. EVs were characterized by transmission electron microscopy, nanoparticle tracking analysis, CD63 ELISA and flow cytometry. Functional assays were performed by co-culturing exosomes with carboxyfluorescein succinimidyl ester (CFSE)-stained Teffs stimulated using anti-CD3/CD28-coated beads, and CFSE dilution and cytokine production were assessed by flow cytometry. **Results:** Activated human Tregs release cup-shaped EVs with a diameter size of approximately 50–100 nm. They were CD63+ CD81+ and also expressed molecules associated with Tregs including CD25 and CD39. Excitingly, Treg exosomes were immune suppressive. Teffs co-cultured with these EVs were less responsive to activation and exhibited an anti-inflammatory cytokine profile. **Summary/conclusion:** Our data highlight exosomes release as a novel mechanism by which human Tregs modify immune responses.

OPW2.4 = PT3.11

Impact of leukocyte-derived procoagulant extracellular vesicles on endothelial cells: significance in blood-mediated pancreatic islets graft reaction

Ali El Habhab¹, Malak Abbas², Mohamad Kassem¹, Guillaume Kreutter¹, Fatiha Zobairi¹, Valérie Schini-Kerth², Laurence Kessler¹ and Florence Toti²
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Introduction: Microparticles (MPs) are procoagulant extracellular vesicles that expose phosphatidylserine and are shed from the plasma membrane. In blood flow, they are biomarkers of vascular disease and act as inflammatory mediators. The active form of tissue factor (TF), the cellular initiator of coagulation, exposed by MPs shed from inflamed endothelium and activated leukocytes. During instant blood-mediated inflammatory reaction (IBMIR), activated leukocytes are recruited at the vicinity of transplanted islets. We aimed at deciphering the impact of MPs from stimulated leukocytes on endothelial senescence and inflammatory response. **Methods:** Porcine coronary artery young P1 endothelial cells (ECs) were incubated with leukocytes-derived MPs (1–30 nM) isolated from rat splenocytes treated by lipopolysaccharide (5 µg/mL) or phorbol 12-myristate 13-acetate (25 ng/mL)-A23187 ionophore (1 µM). Senescence-associated β-galactosidase activity (SA-b-GALact) was assessed by C12FDG probe, senescence markers, oxidative stress, local angiotensin system proteins and TF by western blot, apoptosis by double propidium iodide/annexin V (IP/AV) staining and caspase-3 protein expression. **Results:** MPs induced a significant raise in SA-b-GALact in P1 ECs (18 ± 5 vs. 58 ± 6 MFI, p < 0.05) after 48 h associated with p53, p21, p16 (up to 3-fold) expression level. The 2-fold up-expression of NADPH oxidase subunits (GP91, P47 and P22phox) and 3-fold down-expression of eNOS indicated MP-mediated oxidative stress. MPs prompted procoagulant TF up-expression (up to 3-fold) and a secondary generation of MPs. AT1 and ACE expression (up to 1.5-fold) was increased. No significant variation in IP/AV labelling (8% vs. 13%) nor caspase-3 activation was detected. **Conclusion:** Leukocyte-derived MPs induce premature senescence and thrombogenicity in young primary ECs. Our data suggest that MPs prompt premature endothelial ageing during IBMIR and possibly contributing to poor islets survival.

OPW2.5 = PW5.13

Lupus nephritis was associated with a high increase in the urinary amount of miR-146a via exosomes compared to microvesicles or cellular pellet

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Introduction: Previous studies have reported that microRNA-146a is involved in renal inflammation and its cellular levels were related with lupus nephritis (LN). Here we investigated the distribution pattern of urinary miR-146a into exosomes (Exo) and microvesicles (MVs) in a glomerular disease as LN and compared to levels in the urinary sediment. **Methods:** We used urine of 33 patients with systemic lupus erythematosus (SLE) (10 active, 10 inactive and 13 absence of LN) and 20 healthy controls. We quantified miR-146a by RT-qPCR in urinary cellular pellet (uCP), microvesicles (MV, mean size 200 nm) and exosomes (Exo, mean size 60 nm). Vesicles were isolated by differential ultracentrifugation, then pellets were treated with dichlorodiphenyltrichloroethane (DDT) and RNase, and characterized by electron microscopy and western blot. **Results:** To compare urinary miR-146a levels inside the different types of vesicles analysed, we obtained higher amounts of miR-146a into exosomes (20-fold change, p = 0.005) compared to MVs in active LN patients. No differences were found in the other groups (inactive or in absence of LN). Furthermore, when comparing miR-146a levels in the uCP and vesicles fractions among pathological groups with controls, we only

observed a significant increase in the active LN group (p < 0.001), being miR-146a in exosomes more augmented (86-fold change, p < 0.0001) than in MV (5-fold change, p = 0.009) and uCP (15-fold change, p = 0.003). **Summary/conclusion:** Our results show a different pattern of distribution of miR-146a in urine of lupus nephritis patients. Therefore, in active disease state, the highest increase of urinary miR-146a was found into exosomes when compared to MV and uCP. Our findings suggest a potential selective cellular release of miR-146a mainly packaged into exosomes to regulate inflammation in an autoimmune disorder, such as LN.

OPW2.6 = PT3.01

Neutrophil-derived microvesicles could play a role in the early stages of atherosclerosis

Ingrid Gomez¹, Ben Ward¹, Paul Hellewell², Paul Evans¹ and Victoria Ridger¹

¹Immunity, Infection and Cardiovascular Disease Department, University of Sheffield, Sheffield, UK; ²Life Sciences, College of Brunel, Brunel, UK

Atherosclerosis is the most common cause of cardiovascular disease. Despite their rare detection in plaques, depletion of neutrophils from the circulation reduces plaque formation and monocyte recruitment to the vessel wall. One possible mechanism for this is through the release of microvesicles (MVs) that contain cargo such as microRNAs (miRNAs). **Hypothesis:** Neutrophils influence atherosclerotic plaque formation through the release of MVs. **Aim:** To determine the role of neutrophil microvesicles in early atherogenesis. **Methods and results:** To investigate plaque formation, mouse peripheral blood neutrophils were isolated and stimulated with fMLP in order to obtain MVs. ApoE mice were fed a western diet for 6 weeks and injected twice a week with phosphate-buffered saline (PBS) or neutrophil MVs. Plaque formation was investigated using oil red O and was more advanced in mice injected with MVs (% lesion area PBS = 0.45 ± 0.09, MVs = 1.64 ± 0.38; p = 0.0118, n = 7). En face staining was performed on aorta to quantify NF-κB. A significant increase was observed in the atherosclerotic region from mice injected with MVs (mean fluorescence intensity: PBS = 10395.7 ± 3871.7, MVs = 41181.7 ± 16682.4; p = 0.0335, n = 3). To investigate the role of miRNA, neutrophils isolated from healthy human donors were stimulated with fMLP to obtain MVs. Human coronary artery endothelial cells (HCAEC) were incubated with MVs and miRNA extraction and RT-qPCR performed. miR-155, which is known to be involved in NF-κB regulation, was enhanced in HCAEC after incubation with MVs (2.24 ± 0.14-fold increase, p = 0.0008). ICAM-1, VCAM-1 and MCP-1 were analysed by qPCR and a significant increase in expression was observed in HCAEC in response to MVs (fold increase: ICAM-1 = 2.26 ± 0.11, p = 0.0003; VCAM-1 = 2.30 ± 0.26, p = 0.0073; MCP-1 = 2.94 ± 0.26, p = 0.0017; n = 5). **Conclusion:** Neutrophil MVs enhance plaque formation by activating the pro-inflammatory transcription factor NF-κB in endothelial cells. The mechanism may involve transfer of miR-155, which is known to modulate NF-κB.

OPW2.7 = PW4.16

Novel therapeutic inhibitor cocktail suppresses extracellular vesicle-mediated inflammation induced by micro-RNA in a humanized mouse model of lupus

Nicholas Young, Giancarlo Valiente, Jeffrey Hampton, Holly Steigelman and Wael Jarjour
 The Ohio State University Wexner Medical Center, Columbus, Ohio, USA

Introduction: Systemic lupus erythematosus (SLE) is a prototypic autoimmune disease affecting multiple organs. We have previously shown that toll-like receptor (TLR)7 and TLR8 are significantly up-regulated in peripheral blood mononuclear cells (PBMCs) of SLE patients and activation of TLR8 stimulates extracellular vesicle (EV) secretion. TLR7 and TLR8 bind to single-stranded RNA of viral origin to stimulate innate inflammatory responses, but recent studies have discovered that micro-RNAs (miRs) packaged and secreted in

exosomes can also activate these receptors. *Methods:* To explore the therapeutic potential of antagonizing TLR7 and TLR8 inflammatory pathways induced by EV-derived miRs, PBMCs from active SLE patients were injected into immunodeficient NOD-scid IL-2r (null) mouse recipients to produce chimeras containing PBMCs from SLE patients. Prior to injection, PBMCs were incubated with synthetically produced liposomal EVs containing either a cocktail of locked nucleic acid miR antagonists or a nonsense control. After 21 days, blood was collected for both flow cytometry and cytokine analysis and tissues were processed for histopathological examination by H&E and immunohistochemistry. *Results:* Human T-cells (CD4+ and CD8+), B-cells, monocytes and NK cells were all successfully recovered from whole blood of chimeric mice at similar levels in both treatment groups. However, levels of human IL-2, IL-6, IL-10 and TNF- α were reduced with miR inhibition when compared to control treatment. Although histopathological analysis revealed little to no inflammation in the skin and ear, a robust response was detected in the small intestine, liver and kidney, which was markedly reduced with miR inhibition. Additionally, staining for human CD3 confirmed T-cell presence in these infiltrates. *Summary/conclusion:* These results establish a novel chimeric model to study the inhibition of EV-encapsulated miRs that bind to TLR7 and TLR8 and to explore their therapeutic efficacy in suppressing innate immunity in SLE.

OPW2.8 = PT7.08

Characterization of mycobacterial membrane vesicles

Vanessa Chang, James Dalton, Priscila Dauros Singorenko, Cherie Blenkiron, Anthony Phillips, Siouxsie Wiles and Simon Swift
University of Auckland, New Zealand

Outer membrane vesicles released by Gram-negative bacteria have well-documented roles in infection. Release of similar bodies from acid fast bacteria is drawing attention. Mycobacterial membrane vesicles (MMVs) range from 60 to 300 nm in size and contain lipoproteins and polar lipids. To better understand MMV biology, we analysed 3 mycobacterial species: *Mycobacterium smegmatis* (non-pathogenic, fast grower), *M. abscessus* (pathogenic, fast grower) and *M. marinum* (opportunistic pathogen, slow grower). *M. marinum*-zebrafish model is proposed to be one of the best models to mimic and study human tuberculosis. Mycobacteria were grown in Sauton's defined media, avoiding protein components like bovine serum albumin (BSA) that interfere in downstream vesicle proteomics. MMV composition, size, concentration and release with respect to cell growth and viability were studied. We surveyed the macromolecular contents of MMVs to quantify proteins and nucleic acids. Nanoparticle tracking analysis technology was used to determine MMV size and concentration. Vesicle sampling and live/dead staining at time points along the mycobacterial growth curves were done to observe release with respect to cell viability and growth phase. Electron microscopy was used to image MMVs and their release. We isolated MMVs with mean diameters between 80 and 250 nm and similar protein banding patterns among replicates across all three mycobacterial species. DNA and RNA concentrations of 5–42 μg and 1–23 μg , respectively, were obtained from MMVs isolated from 14-day mycobacterial culture in 1 L of Sauton's media. MMVs were produced throughout growth, with most produced at the transition from exponential to stationary phase. Stationary phase MMVs from *M. abscessus* were the largest and contained more DNA than RNA. MMVs from *M. smegmatis* and *M. marinum* contained equivalent levels of DNA and RNA. Live/dead staining demonstrated peak vesicle release with culture viability >99%, supporting the notion that release is an active process of living cells.

Room: Jurriaanse

Oral with Poster Session 3 - EVs in cancer and other diseases

Chairs: *María Yáñez-Mó and Marjolein van Driel*

1:30-2:15 p.m.

OPW3.1 = PT5.05**Circulating RNAs as biomarkers in Epstein–Barr virus-associated nasopharyngeal carcinoma**

Octavia Ramayanti¹, Sandra A.W.M Verkuijlen¹, Lisa Beckers¹, Jordy Meijer¹, Debora Martorelli², Chantal Scheepbouwer³, Michael Hackenberg⁴, Ruud H. Brakenhoff⁵, Marlinda Adham⁶, Danijela Koppers-Lalic³, D. Michiel Pegtel¹ and Jaap M. Middeldorp¹
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Nasopharyngeal carcinoma (NPC) is an aggressive tumour associated with Epstein-Barr virus (EBV), which has a poor prognosis when detected late. Easy methods for detecting NPC at earlier stages are highly needed. Host and viral miRNAs are perturbed in NPC, a fraction of which are included in extracellular vesicles and/or in ribonucleo-protein complexes that enter into the circulation. Measuring circulating miRNAs may provide a robust and direct insight of the oncogenic process. Here we analysed whether host and viral circulating miRNA fingerprints may reflect tumour burden and behaviour. Defined circulating microRNAs were analysed from total serum and plasma (NPC, non-NPC patients and healthy controls). Selected NPC sera and plasma were fractionated by size-exclusion chromatography into extracellular vesicle and lipoprotein fractions. Exosome-like vesicles released from EBV-infected NPC were characterized by western blot and transmission electron microscopy. Deep RNA sequencing (RNA-seq) was done for NPC cell lines, exosomes and selected sera. EBV DNA load was measured as well. Viral miRNA (mirBART7, 9, 13), host miRNA (miR16, 21, 155) and small RNAs (EBER1 and vaultRNA) were detected by stemloop reverse transcription-polymerase chain reaction (RT-PCR). We detected viral miRNAs in NPC sera and plasma, which levels seem to increase with TNM stage despite absence of detectable EBV DNA in some. EBER1 RNA was absent in virtually all NPC sera and plasma, despite high levels in NPC tissues. Cellular miR155, which expression is induced by EBV-LMP1, was significantly elevated in NPC when compared to controls. Fractionation from NPC sera and plasma revealed that miR155 and vault RNA are selectively enriched in extracellular vesicles, whereas viral miRNAs are more randomly distributed. The pattern of tetraspanin (CD63, CD81) protein expression is heterogeneous in NPC patients compared to healthy controls. Further, RNAseq and RT-PCR analysis are being performed to determine the diagnostic potential of circulating miRNAs for NPC.

OPW3.2 = PF4.01**Gastric cancer cell-derived extracellular vesicles modulate E-cadherin expression, invasion and migration of normal cells from the tumour microenvironment**

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*These authors equally contributed to this work

Tumours need to modify the surrounding tissue to grow and disseminate. Extracellular vesicles (EVs) secreted by tumour cells have emerged as main modulators of the tumour microenvironment; however, the phenotypes induced in normal recipient cells remain poorly known, as well as the underlying mechanisms. Our aim was to identify and characterize the behavioural modifications that gastric cancer cell-derived EVs (GC-EVs) promote in the adjacent normal epithelia, which facilitate local dissemination of cancer cells. EVs were isolated from 4 GC cell lines (GC-EVs), and their effects were analysed in recipient normal epithelial (E) and mesenchymal (M) states of the same cell line. GC-EVs were isolated by differential ultracentrifugation and characterized by TEM, NTA and imaging flow cytometry for the presence of exosomal markers (e.g. CD9). Proliferation, migration and invasion abilities of E and M recipient cells were assessed before and after treatment with GC-EVs, and the expression of epithelial/mesenchymal markers was followed by immunofluorescence. GC-EVs (50–150 nm) expressed CD9, CD81 and flotillin and were able to reduce E-cadherin expression and promote invasiveness of E-recipient cells. The opposite effect was observed in M-recipient cells, which regain E-cadherin expression and lose invasion and migration ability. However, different GC-EVs induce different levels of E-cadherin modulation and invasion ability. These findings suggest that (1) GC-EVs do modulate the behaviour of normal adjacent cells, (2) recipient cells respond differently to the same EVs cargo depending on the recipient cells' state and (3) each cancer cell line secretes EVs with different efficiencies in inducing phenotypic alterations in recipient cells. miRNA and proteomic profiling of GC-EVs is ongoing to identify the EVs-factors likely involved in the intracellular reprogramming of normal recipient cells of the tumour microenvironment.

OPW3.3 = PF2.09**D-type CpG oligonucleotide encapsulating and antigen-harboring exosomes improve therapy of tumours**

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Introduction: Exosomes are membranous nanovesicles functioning as natural conveyors of intracellular signalling. They are also known to play important roles in immune modulation. CpG ODNs are strong candidates for treatment of plethora of diseases including cancer. Unfortunately, labile nature of synthetic ODNs hampered their clinical performance. This study aims to investigate whether exosomes could be harnessed as a delivery vehicle improving CpG ODN adjuvant activity against cancer. **Methods:** D-type CpG ODN was encapsulated into exosomes isolated from EG7 cell supernatants known to stably express ovalbumin as the model cancer antigen. Immunostimulatory activity of exosomal D-ODN on mouse splenocytes was assessed by ELISA and flow cytometry for cytokine induction and cell surface marker upregulation, respectively. C57/B16 mice were immunized with EG7 exosomes harbouring D-ODN. Anti-OVA IgG levels were assessed from mice sera. Next, immunized mice were seeded with EG7 cells 2 months after booster immunization.

Tumour development was followed. *Results:* Stimulation assays revealed that D-ODN encapsulation into exosomes significantly elevated IL6, IL12, IFN γ and IFN α production in comparison to free D-ODN. FACS analysis implicated that CD86 and MHC-II expressions were 2–4-fold higher in cell treated with exosomal form rather than free form of D-ODN. Th1-biased anti-OVA immune response was established in mice treated with exosomal D-ODN as evidenced by significantly higher anti-OVA IgG2a titres. More than 85% of immunized mice with exosomal D-ODN cleared tumours following EG7-thymoma induction, whereas all naive mice failed to control tumour formation. *Conclusion:* Our data suggest that D-ODN encapsulation into exosomes harbouring tumour antigen is capable of tumour clearance via improving innate immune activity as well as cancer antigen-specific immunity in mice.

OPW3.5 = PW4.09

Repurposing glioblastoma exosomes as personalized multi-antigenic antitumour vaccine

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Introduction: Among multiple immune escape mechanisms operated by glioblastoma (GBM) are glycosylation-dependent interactions with Siglec and C-type lectin receptors on tumour-infiltrating immune cells. Receptor triggering results in both cargo internalization and signalling regulating the immunogenic/tolerogenic balance of the cellular response. The composition of tumour-derived exosomes (TEX) reflects plasma membrane and cytosol-derived proteins of the parent cell and represents interesting candidates for antitumour vaccination. However, literature reports potent immunosuppressive characteristics of TEX that should be carefully considered. Our group has shown glycosylation-dependent uptake and processing of glycan-modified liposomes. The aim of this study is to investigate whether repurposing of GBM TEX for vaccination can be achieved by de novo glycosylation of their surface. *Methods:* Prior to their chemo-enzymatic modification, TEX were carefully characterized by ELISA and electron microscopy using a panel of human and plant lectins of known specificity. Chemo-enzymatic modifications were aimed at reducing Siglec ligands and enhancing DC-SIGN ligands. TEX uptake and downstream routing was assessed by imaging flow cytometry analysis. *Results:* GBM-derived TEX were 50-200 nm in size and expressed high levels of CD63. The surface glycan profile of U251, U87 and GBM8-derived TEX was dominated by a-2,3 and a-2,6 sialic acid-capped N-glycans, high mannose glycans and truncated Tn-bearing O-glycans. These TEX appeared to have low affinity for DC-SIGN and MGL. In contrast, glycan-modified TEX were efficiently taken up by human dendritic cells and fused with the endosomal pathway. *Conclusion:* GBM-derived exosomes exhibit glycans that are typically associated with tolerogenic responses, indicating a possible role in GBM immune escape. Repurposing through de novo glycan modification could provide a strategy for personalized, multi-antigenic, anti-GBM vaccination.

OPW3.6 = PW4.06

Suppression of breast cancer metastasis by targeting cancer-derived extracellular vesicles with antibodies

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Introduction: Metastasis is the main cause of cancer mortality, but it is still difficult to prevent metastasis. It has been recently reported that cancer-derived extracellular vesicles (EVs) contribute to cancer

metastasis. Thus, therapeutic strategies targeting cancer-derived EVs hold great promise due to the possibility of EVs to influence cancer microenvironment towards metastasis. Here, we provide a novel strategy of therapeutic antibody treatment targeting cancer-derived EVs to inhibit metastasis of breast cancer in mouse model. *Methods:* We prepared a mouse xenograft model for metastatic breast cancer by transplanting human highly metastatic breast cancer cell line, MDA-MB-231-D3H2LN to 6-week-old scid/scid female mice, orthotopically. Forty-five micrograms of anti-human CD9 or anti-human CD63 antibody, which specifically recognize human CD9 or CD63 at the surface of EVs from MDA-MB-231-D3H2LN, was injected to the mouse xenograft model intravenously, for 3 times with a 3-day interval. The metastatic cancer cells were detected by in vivo imaging system of bioluminescence from luciferase-expressing cancer cells or immunohistochemistry. Phagocytotic assay of EVs incubated with anti-CD9 or anti-CD63 antibody was performed by mouse macrophage cell line, RAW264.7, to check opsonization ability of the antibodies. *Results:* Administration with human specific anti-CD9 or anti-CD63 antibodies significantly decreased metastasis to lung, lymph nodes and thoracic cavities, although it did not make obvious effect on the xenografted primary tumour size or neovascular formation in tumour tissue. The EVs incubated with the targeted antibodies were preferentially engulfed by macrophages in vitro, suggesting that cancer-derived EVs were eliminated by phagocytosis to prevent metastasis in mouse model. *Summary/conclusion:* Our results provide a novel therapeutic method to prevent tumour metastasis by therapeutic antibody administration targeting cancer-derived EVs.

OPW3.7 = PW1.11

The involvement of microvesicle miRNA in gestational vascular complications

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Introduction: Gestational vascular complications (GVC) are a major cause of maternal morbidity and foetal mortality. They are characterized by vascular and placental dysfunction. The major aim of this study was to explore the role of microvesicle (MV) miRNA of women with gestational vascular complications and evaluate their effects on endothelial cells (EC) and early-stage trophoblast (EST) cell function. *Methods:* Plasma and placental samples were collected from healthy pregnant (HP) women, women with GVC and pregnant women treated with low molecular weight heparin (LMWH) after obtaining informed consent. MV miRNAs were screened using NanoString technology and further validated by RT-PCR. Effects of MV miRNA derived from the study groups on EST and EC miRNA expression and cell function were evaluated. *Results:* NanoString screening of samples drawn from the same person demonstrated significant differences in the profile of miRNA packed in MVs and those freely circulating in plasma, including different expression levels in some of them. Differences in expression clusters were also observed between the study groups. RT-PCR findings showed that exposure of EC and EST cells to the MVs of the study groups affected miRNA expression. Changes were seen in different miRNAs known to be involved in the MAPK signalling pathway, which is altered in GVC, as shown in our previous study. *Conclusion:* MV content and effects on endothelial and trophoblast cells vary depending on the physiological/pathological state of a pregnant woman. Our findings may point to a special mechanism related to miRNA packaging in MVs and to the role of the packed miRNAs upon their penetration to target cells. Furthermore, the current results support our previous findings showing reduction in trophoblast invasion and increase in apoptosis under the exposure to GVC-MVs, while MVs of LMWH-treated women reversed these effects.

OPW3.8 = PW8.11

Exosome DNA: a new promising liquid biopsy-based diagnostic tool for personalized management of aggressive prostate cancer (PCa) patients

Chiara Foroni¹, Natasa Zarovni², Simona Bernardi³, Davide Zocco², Domenico Russo⁴, Francesca Valcamonico⁵ and Alfredo Berruti⁵
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Introduction: Metastatic castration-resistant prostate cancer (mCRPC) is associated with poor prognosis and response to hormonal or conventional treatments. While novel drugs increase the therapeutic options, they bring up elevated costs, over-treatment and side effects. There is a pressing need to develop non-invasive tests for stratification and surveillance of post- and in-therapy patients for increased cost-efficiency of mCRPC treatments. Our aim was to assess the comparative advantages of liquid biopsy methods based

on extracellular vesicle-associated DNA (EV-DNA) and cell-free DNA (cfDNA) as an input for digital PCR (dPCR), a high sensitivity/accuracy method for detection of the low abundant targets and promises to improve the power of detecting AR gene status in biofluid samples. **Methods:** Serum samples were collected following informed consent from 10 mCRPC patients, progressing on systemic therapy, at 3 time points of relapse. Total cfDNA and DNA extracted from immunocapture-enriched tumour-derived exosomes were analysed for AR T878A point mutation by QuantStudio 3D dPCR test, calibrated using positive controls (LNCaP and PC-3 cell line genomic DNAs). **Results:** AR T878A mutation was detected in 3/10 patients. In one of AR mutation positive patients, the mutation was detected at all time points and using both cfDNA and EV-DNA samples, while in the other two patients, AR mutation was detected at all time points when EV-DNA was used, and only in later time points by using cfDNA. An increment of AR T878A mutational events could be appreciated in EV-DNA fraction comparing with cfDNA from the same sample. **Conclusion:** Enrichment of AR somatic mutation in tumour-associated exosomes suggests that tumour EV-DNA is more appropriate than total cfDNA for specific and sensitive liquid biopsy approaches. This small pilot study provides insights into the potential of tumour exosomes as robust monitoring tool for the clinical implementation of novel personalized strategies for PCa patients.

Room: van Weelde Symposium Session 4 - EVs of the CNS

Chairs: *Shilpa Buch and Lawrence Rajendran*

2:15-3:00 p.m.

OW4.1

Glia-derived exosomes mediate neuroprotection and promote axonal transport

Wen-Ping Kuo-Elsner, Dominik Fröhlich, Carsten Frühbeis
and Eva-Maria Krämer-Albers
Molecular Cell Biology, Johannes Gutenberg University Mainz, Mainz, Germany

Introduction: Oligodendrocytes myelinate central nervous system axons and mediate maintenance of axonal integrity. We have shown that neuronal electrical activity controls glial exosome release resulting in subsequent neuronal uptake and functional retrieval of exosomal content. Functional assays revealed that glial exosomes desensitize neurons towards cellular stress. Mouse mutants lacking the oligodendrocyte-specific proteins PLP or CNP reveal a secondary axonal degeneration due to absent glial support. To study the potential role of exosomes in glial support, we analysed oligodendroglial exosome secretion in these mutants and their interaction with target neurons. Furthermore, we investigated the influence of exosomes derived from wild-type and mutant oligodendrocytes on axonal transport. **Methods:** Mutant exosomes were isolated by differential centrifugation and characterized by nanoparticle tracking analysis, western blotting as well as by proteomics. Transfer of exosomes to neurons was assessed by a Cre-reporter assay. The metabolic activity of neurons treated with wild-type or mutant exosomes was measured using the MTT assay. To study the influence of exosomes on axonal transport, we performed time-lapse microscopy. **Results:** Analysis of mutant-derived exosomes revealed that mutant oligodendrocytes release less exosomes of an abnormal content. Furthermore, mutant exosomes did not share the neuroprotective characteristics of wild-type exosomes. Analysis of axonal transport demonstrated that wild-type exosomes facilitate axonal transport by reducing the pausing time and the number of static vesicles. In contrast, mutant-derived exosomes did not promote axonal transport. **Conclusion:** Our results emphasize that oligodendroglial exosomes exhibit a beneficial influence on the neuronal metabolism and physiology. We propose that oligodendroglial exosomes facilitate axonal maintenance. Exosome-mediated neuron-glia communication appears to represent a novel mechanism of neuroprotection.

Supported by DFG.

OW4.2

Cerebrospinal fluid-derived extracellular vesicles as a novel source of potential biomarkers for multiple sclerosis

Joanne Welton¹, Sam Loveless², Tim Stone³, Neil Robertson² and Aled Clayton⁴

¹Biomedical Sciences, Cardiff Metropolitan University, Cardiff, UK; ²Institute of Psychological Medicine and Clinical Neurology, Cardiff University; ³Central Biotechnology Services, Cardiff University; ⁴Division of Cancer and Genetics, School of Medicine, Cardiff University, Cardiff, UK

Introduction: Multiple sclerosis (MS) is one of the most common neurological disorders in young adults, affecting over 100,000 people in the United Kingdom. There are currently no reliable biomarkers to aid in the diagnosis or monitoring of the disease. Here we developed methods of isolation and proteomic analysis to examine the potential of extracellular vesicles (EVs) as a novel source of biomarkers for the relapsing remitting form of MS (RRMS). **Methods:** A method was developed for EV isolation from cerebrospinal fluid (CSF), using precipitation and size-exclusion chromatography (SEC). EV-enriched fractions were selected based on their characteristics, including

elevated presence of tetraspanins CD9/81. EVs were isolated from RRMS patient (n = 4) and control (n = 3) CSF. The EVs and paired CSF were subsequently analysed using a novel aptamer-based protein assay, providing semi-quantitative information for 1128 proteins. **Results:** The isolation method removed 75% of contaminating protein in the EV-enriched fractions. These EV isolates were confirmed compatible with the proteomics platform, despite the potential for remaining precipitant. Around 350 and 580 proteins were identified in CSF-derived EVs and CSF, respectively, of which 50 proteins were significantly and exclusively enriched in RRMS-derived EVs. Some of the RRMS-EV-enriched proteins were further evaluated, such as KLKB1, and were found to be enriched in CSF EVs compared to CSF, by western blot. Enrichment analysis of the 50 proteins (performed via g:Profiler) revealed enriched biological themes visualized in Cytoscape. Strongly interconnected terms included complement and coagulation, vesicles and extracellular regions. **Summary:** This study demonstrates that EVs can be isolated from the CSF of MS patient and highlights the advantage of EV enrichment for identifying novel disease-related proteins within CSF. This proof of concept study has identified several candidate proteins for further evaluation as biomarkers of MS.

OW4.3

Secondary release of exosomes from astrocytes contributes to neural plasticity and improvement of functional recovery after stroke in rats treated with IA administration of exosomes harvested from miR-133b-enriched MSCs

Hongqi Xin
Henry Ford Health System, Detroit, USA

The exosomes from blank control vector transfected control mesenchymal stem cells (MSCs) and from pre-miR-133b expressing vector transfected miR-133b-enriched MSCs were harvested and administered via intra-arterial (IA) injection to rats (3 × 10¹¹ particles per rats, n = 6 per group) subjected to 2 h of middle cerebral artery occlusion (MCAo). All rats were sacrificed at 28 days post-MCAo. Compared with phosphate-buffered saline treatment, both exosome treatment groups exhibited significant improvement of functional recovery (p < 0.05 after day 14). Compared with control MSC exosomes, treatment with miR-133b-enriched MSC exosomes significantly increased functional improvement (p < 0.05 after day 21) and neurite remodelling and brain plasticity in the ischemic boundary area (p < 0.05). Quantification analysis showed that miR-133b-enriched MSC exosome treatment increased the presence of exosomes in brain extracellular space (3.68 ± 0.58 × 10⁹ vs. 2.71 ± 0.34 × 10⁹, particles/mg tissue, p < 0.05). In vitro oxygen and glucose-deprived (OGD) cultured astrocytes were incubated with exosomes harvested from naive MSCs and miR-133b level modified MSCs. Compared with that by naive MSC harvested exosome treatment, the exosome release from OGD astrocytes was significantly enhanced (1.88 ± 0.33-fold, p < 0.05) by miR-133b-enriched MSC exosome treatment and significantly decreased (0.37 ± 0.04-fold, p < 0.01) by miR-133b down-regulated MSC exosomes treatment. We also found that OGD astrocytic exosomes harvested from miR-133b-enriched MSC exosome treated significantly increased neurite outgrowth and elongation compared with that from control MSC exosome treated. In addition, the effect of astrocytic exosomes on neurite outgrowth and elongation was dose-dependent. Although we do not exclude a possible contribution from other parenchymal cells, our data suggest that exosomes from miR-133b-enriched MSCs improve neural plasticity and functional recovery after stroke by increasing the exosome release from astrocytes.

Room: Jurriaanse Symposium Session 5 - EV isolation

Chairs: Jaesung Park and Andreas Möller

2:15-3:00 p.m.

OW5.1

Novel methods of polysaccharide-based EV isolation: a new opportunity for EV-based diagnostics and therapeutics

Anirban Ghosh¹, David Barnett¹, Sebastien Fournier¹, Nguyet Nguyen¹, Andrew Joy¹, Ian C. Chute¹, Rodney J. Ouellette¹, M. Vicki Meli² and Biji Anish¹

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Introduction: Circulating extracellular vesicles (EVs) have tremendous potential for minimally invasive diagnostics and therapeutic applications. The interactions between the polysaccharides, polysaccharide-proteins and polysaccharide-lipids on the outer membrane surfaces of cells and EVs are essential for their biological activities. These biological interactions prompted us to hypothesize and test polysaccharide-based EV capture. Ultracentrifugation, ultrafiltration or antibody-based EV isolation methods provide significantly less yield compared to polymer-based EV precipitation. Currently available polymer-based (polyethylene glycol or PEG and its derivatives) EV isolation technologies are not compatible with therapeutic applications due to the presence of remaining polymers in the EV preparation. Polysaccharides such as chitosan and hyaluronic acid are FDA approved for multiple formulations, wound-healing and internal uses in human medicine. Therefore, an EV-capture technology that uses chitosan and/or hyaluronic acid strategically fits the demand for EV isolation technologies that are applicable to therapeutic uses. **Methods:** We used hyaluronic acid and chitosan to enrich EVs from cell culture-conditioned media and human body fluids using a standard laboratory/clinical setup. We standardized low-speed centrifugation and bead-conjugated batch purification methods of EV isolation using these polysaccharides. **Results:** We validated the EVs captured with hyaluronic acid and chitosan using electron microscopy, atomic force microscopy, proteome-based cellular component ontology analysis, nanoparticle tracking analysis and immunoblotting. Furthermore, our results also indicate that both hyaluronic acid- and chitosan-captured EVs are biologically active. **Summary/conclusion:** These polysaccharide-based EV capture technologies open new opportunities to develop subsequent manipulation methodologies to explore EV-based therapeutics and provide new tools for diagnostics.

OW5.2

Prostate cancer diagnosis using extracellular vesicles isolated by aqueous two-phase systems

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Introduction: Prostate-specific antigen (PSA) is commonly used to diagnose prostate cancer (PCa). However, PSA shows low specificity, such that benign hyperplastic conditions can also be associated with a PSA increase. To overcome this limitation of PSA, a new approach that detects cancer extracellular vesicles (EVs) has been introduced. However, diagnosis using EVs to date have been limited by inefficient process in isolating extracellular vesicles and lack of effective purification strategies. We developed a simple strategy employing aqueous two-phase system (ATPS) to isolate extracellular vesicles from urine sample. In this study, we isolated EVs from urine with

efficiency of 100%; total processing time was ~35 min. After isolating EVs from patients' urine, we successfully distinguished benign prostate hyperplasia (BPH) patients from PCa patients. **Method:** We optimized the ATPS to get highest quality and quantity of EVs from urine by controlling polymer concentration. Additionally, dextran phase volume was minimized to obtain highly concentrated EVs. After ATPS had been optimized, 20 BPH patients were recruited. Ten millilitres of each patient's urine was collected, and EVs were isolated using the ATPS. The expression level of PCA3 (PCa mRNA marker) and actin was measured using nested PCR and analysed by comparing clinical data. **Result:** The ATPS isolation method optimized by adjusting polymers concentration recovered ~100% of EVs from the urine, whereas ultracentrifuged-pellet methods recovered only < 30% of total EVs. After isolating EVs from the patients' urine by ATPS, high quality and quantity of EVs can be obtained. We measured an expression level of PCA3 of the EVs and normalized the level using actin. Significant differences between benign and PCa in PCA/ACTIN was identified. **Conclusion:** Using the proposed method using ATPS, prostate cancer can be distinguishable from benign prostrate hyperplasia successfully.

OW5.3

Flow cytometric detection and sorting of EVs: analysis and characterization of background noise sources that may impact EV fluorescence or scatter detection

Carley Ross¹ and Jennifer Jones²

¹Beckman Coulter Life Sciences, Fort Collins, Colorado, USA; ²National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA

Introduction: Extracellular vesicle (EV) detection via flow cytometry in fluorescence or scatter reaches the limits of current flow cytometry. Measurements of Q and B using an LED pulser indicate scatter and detection limits on the flow cytometer and are useful in understanding cytometer capabilities. However, understanding how electronic and optical noise sources may individually contribute to the system instrument noise pinpoints specific areas of instrument improvement for better EV detection. With high-resolution electronics at and below the photomultiplier (PMT) dynamic range for the system, the noise background may be fully visualized, characterized and reduced. This poster describes both optical and electronic noise sources that may impact EV detection, characterization and sorting. **Methods:** An MoFlo Astrios EQ (BEC) with a quantiFlash LED pulser (APE) installed was used to study noise sources such as electronic (ADC, Preamp and PMT) and optical (laser, stream, nozzle tip distance, autofluorescence, nozzle tip sizes 25–100 um and drop drive frequency and amplitude). **Results:** Optical and electrical noise determined the noise floor. The fluidic stream added 5–10% optical noise in fluorescent channels. Larger nozzle tip sizes reduced drop drive noise, whereas smaller nozzle tips reduced sort volume and optical noise but require higher pressure. About 5–10% of the optical noise is due to laser cross-talk. **Conclusions:** Characterization and analysis of the sorter noise background provided insight into instrument EV characterization capabilities. Improvements to drop drive amplitude and frequency, laser selection and location and nozzle tip will reduce the noise floor. Optimal EV detection, isolation and characterization depend on measuring not only the Q value, but also the B value and true noise floor of the flow cytometer.

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Room: Willem Burger

Symposium Session 6 - EVs in cancer transcriptomics

Chairs: Guido Jenster and Irene Bijnsdorp

2:15-3:00 p.m.

OW6.1**NGS analysis of EVs RNA in ageing and cancer**

Hidetoshi Tahara, Megumi Okada, Naoki Sako, Shigeyuki Teranishi, Yukie Nishiyama, Masaki Kinehara and Akira Shimamoto
Hiroshima University, Hiroshima, Japan

Extracellular vesicles (EVs) are 50–100 nm in size and carry various cargo, including microRNA and other small RNAs, released into the extracellular environment and can be used for cell-to-cell communication in vivo. Recently, we found that the secretion of EVs in senescent fibroblast cells is significantly higher than those in young cells, and senescence-derived EVs inhibit cell proliferation, colony formation and invasion of cancer cell lines. In contrast, EVs from highly metastatic cancer cell lines significantly induce apoptosis in senescent fibroblasts. To understand the function of these EVs from young and senescence cells, we analysed EVs RNA by using a next-generation sequencer (NGS), Ion PGM. Interestingly, microRNA profiling of EVs is significantly altered between senescent cells and young cells. Several significant tumour suppressive microRNAs, which contained in senescence-derived EVs, significantly inhibit tumour invasion and cell growth. We also analyse EVs RNA in cancer cell lines and circulating small RNA in serum using NGS and found specific microRNAs are altered in cancer. Most miRNAs are generated as a family of related isomers that differ by a small number of bases at the 5' and the 3' end of the miRNA, termed 5' or 3' isomiRs. We also focus on the isomiRs difference in ageing and cancer. Surprisingly, we found specific isomiRs between cancer patients and healthy volunteer. We believe that these specific isomiRs are powerful tools for identifying EVs from specific origin of cells.

OW6.2**BRAF inhibition regulates miR-211 expression in melanoma extracellular vesicle subsets with functional consequences**

Taral Lunavat¹, Yong Song Gho², Jonas Nilsson³, Andrew Francis Hill⁴, Jan Lötvalld³, Roger Olofsson Bagge³, Cecilia Lässer³, Robyn Sharples⁵, Berglind Einarsdottir³ and Lesley Cheng⁴

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²Postech; ³University of Gothenburg, Sweden; ⁴La Trobe University, Melbourne, Australia; ⁵University of Melbourne, Australia

Introduction: In melanoma, more than 50% of the tumours harbour BRAFV600 mutations. Vemurafenib is a BRAF-V600 inhibitor used for the treatment of late-stage melanoma. Here we determine the effects of vemurafenib on melanoma cells and the RNA content in their subsets of extracellular vesicles (EVs) including apoptotic bodies, microvesicles and exosomes. **Methods:** EVs were isolated using differential centrifugation, and ion torrent sequencing was used to sequence small RNAs. Xenograft models were established by transplanting patient tumour cells or cell lines into mice. Proteins and miRNA from cells and EVs subsets were validated by using western blot and quantitative PCR, respectively. **Results:** Vemurafenib

treatment of melanoma cells harbouring the BRAF mutation significantly increases the RNA and protein cargo in the EV subsets. The RNA profile also changes substantially in apoptotic bodies and microvesicles, with significant alterations in the ribosomal RNA ratio. With small RNA sequencing, we found that EVs from treated cells harbour unique miRNAs. The significantly different miRNAs detected were validated in vitro, confirming upregulation of miR-211 in cells as well as in EVs subsets after vemurafenib treatment. Additionally, miR-211 was significantly upregulated in tumours tissues harvested from patient-derived xenografts as well as cell line-derived xenograft and their respective vesicular subsets. Furthermore, treatment of BRAF WT cells with treated exosomes showed significant post-transcriptional downregulation of KCNMA and IGF2R genes that are known to play a role in tumour growth and metastasis. **Conclusions:** Overall, BRAFi induces miR-211 upregulation in melanoma cells and xenografts, and in their subsets of EVs. Upregulation of miR-211 may be a biomarker of response in patients carrying BRAF mutant melanoma. This study also provides the framework for further investigations of miR-211 functions in melanoma cells and EVs as well as in cells receiving this cargo.

OW6.3**Selective release of circular RNAs in platelet-derived extracellular vesicles**

Christian Preußner, Lee-Hsueh Hung, Silke Schreiner and Albrecht Bindereif
Institute of Biochemistry, Justus Liebig University Giessen, Germany

Introduction: Circular RNAs (circRNAs) are a new class of noncoding RNAs present in all eukaryotes investigated so far. They are generated by a special mode of alternative splicing from pre-mRNAs, whereby single or multiple adjacent and spliced exons are released in circular form. CircRNAs are cell-type specifically expressed, are unusually stable and can be found in various body fluids such as blood and saliva. **Methods:** We isolated extracellular vesicles from purified and in vitro activated human platelets, using density-gradient centrifugation, followed by RNA-seq analysis and bioinformatic identification of circRNAs; validation of platelet- and exosome-associated circRNAs was based on semi-quantitative RT-PCR and northern blotting. **Results:** Our analysis of circRNAs and the corresponding linear splice isoforms revealed that – compared to other hematopoietic cell types – circRNAs are particularly enriched in platelets. By activating platelets and thereby inducing the generation of extracellular vesicles, we also showed that platelet-specific circRNAs are packaged and released within exosomes. Interestingly, we observed a selective sorting of circular and linear RNA species into exosomes. **Summary:** CircRNAs associated with exosomes are released from activated platelets, representing yet another class of extracellular RNAs that circulate in the body and may be involved in signalling pathways. These findings will greatly help to extend the potential of circRNAs as prognostic and diagnostic biomarkers since platelets are essential for central physiological processes such as hemostasis, wound healing, inflammation and cancer metastasis and play an important role in the pathogenesis of cardiovascular disease.

Room: van Weelde

Biotech Sponsored Session: CARIS Life Sciences

Chairs: *Juan Falcon Perez and Gyongyi Szabo*

3:30-3:45 p.m.

BW2

Adaptive Dynamic Artificial Poly-ligand Targeting (ADAPT): A Novel Platform for Profiling Exosomes from Prostate Cancer Cells

Mark R. Miglarese, Tassilo Hornung, Stephen C. Logie, Aniket S. Bondre, Jelena Zarkovic, Teresa T. Tinder, Varun Maher, Melissa N. Richards, Heather A. O'Neill, and David B. Spetzler
Caris Life Sciences, Phoenix, AZ, USA

Introduction: Exosomes are small (40–100 nm) vesicles secreted by various cell types detected in a multitude of body fluids. Exosomes have protein profiles characteristic of their cells of origin and play a role in cell-to-cell communication making them attractive targets to identify early disease stage biomarkers. Due to the heterogeneity of cancer, it is unlikely to find a single cancer marker that will identify the majority of individuals with a morphologically-defined cancer type. We developed the highly multiplexed ADAPT platform to capture systems-based biological signatures that may reflect the molecular heterogeneity of various cancer types. Exosomes from two prostate cancer cell lines, VCaP and LNCaP, were used to train ssDNA libraries to discriminate them. *Methods:* A library of 10^{12}

oligonucleotides (ODNs) was subjected to five rounds of positive and negative selection against exosomes from VCaP and LNCaP prostate cancer cell lines. From the remaining $\sim 3 \times 10^5$ sequences, ODNs that were bound preferably to exosomes from VCaP cells were identified by NGS. A subset of individual ODNs was resynthesized and binding of co-precipitated ODNs to VCaP exosomes was verified by qPCR. ODN-bound proteins were identified by affinity purification and LC-MS/MS. *Results:* Cognate binding partners of ODNs bound to VCaP exosomes were identified, including ESCRT endosomal sorting proteins (CHMP1b/2a/4b, VPS28, Syntenin-1). Components of ESCRT participate in exosomes biogenesis and are overexpressed in human cancers. In addition, we found the chemokine I-TAC, which is overexpressed in blood and tissue of men with advanced prostate adenocarcinomas. Finally, we identified hnRNP-1, a cancer associated splicing factor, and the cold shock proteins RNPL and A18 hnRNP. Knock-down of these cold shock proteins has been shown to enhance chemotherapeutic cell killing of prostate cells. *Conclusions:* ADAPT is an unbiased profiling platform that identifies cancer associated proteins expressed on exosomes. This platform can be deployed against multiple cancer types and offers broad potential applications in biomarker discovery.

Room: van Weelde

Symposium Session 7 - EVs in metabolic syndrome, Fatty Liver Disease and Cancer

Chairs: Juan Falcon Perez and Gyongyi Szabo

3:45-5:00 p.m.

OW7.1

Hepatocytes-derived extracellular vesicles from metabolic syndrome models influence adipocytes metabolismJustyna Mleczo¹, Juan Manuel Falcon Perez¹ and Silvia Mora²¹CIC bioGUNE, Bizkaia, Spain; ²University of Liverpool, England

Introduction: Metabolic syndrome is a clinical condition affecting up to 25% of all adults worldwide. It influences many cellular systems including adipose tissue, hepatocytes and macrophages. Main characteristics include impaired insulin signalling leading to abnormal glucose and lipid metabolism and systemic inflammation. Up to date, most cell types were described to secrete extracellular vesicles (EVs) and their function was mostly associated with intercellular communication. In the present work, EVs secreted by cellular models of metabolic syndrome were characterized and their effect on 3T3-L1 adipocytes metabolism was studied. **Methods:** EVs were isolated from hepatic mouse cell lines and rat hepatocytes in normal, drug-induced liver injury (DILI) and obesity-resembling conditions. The effect of these EVs on metabolism of 3T3-L1 adipocytes was evaluated by adipokine array and Seahorse mitochondrial and glycolysis stress test. **Results:** EVs secreted by hepatocytes are altered under DILI and obesity-resembling conditions displaying significant differences in several EVs membrane markers including CD81 and CD63. Furthermore, the different EVs have different effects on the cytokine secretion ejected by adipocytes. Seahorse assays also evidenced significant changes on the adipocyte metabolism caused by hepatocytes-derived EVs under normal and damaging conditions modifying the adipocyte capacity to handle mitochondrial stress. **Summary/conclusion:** Our results suggest that hepatocytes in metabolic syndrome conditions secrete EVs that influence adipocyte metabolism. This intracellular communication mediated by EVs could play a role in the development and progression of metabolic syndrome.

OW7.2

Proteomic profiling of extracellular vesicles derived from adipocytes reveals the presence of key metabolic proteins involved in obesity-associated metabolic dysfunctionsAudrey Fleury^{*1}, Maeva Durcin^{*1}, Zuzana Krupova², Sandrine Truchet³, Céline Henry⁴, Martin Trötz Müller⁵, Harald Köfeler⁵, Guillaume Mabilieu⁶, Ramarosan Andriantsitohaina¹, Patrice Martin² and Soazig Le Lay¹

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Extracellular vesicles (EVs) are biological vectors that can modulate the metabolism of target cells by their ability to convey signalling proteins. Plasmatic EVs rate is significantly increased in cardiometabolic diseases associated with obesity, suggesting their possible participation in the development of metabolic dysfunctions. Few data exist on adipocytes-derived EVs. The purpose of this study is to qualitatively and quantitatively characterize EVs secreted by fat cells. Adipocyte-derived EVs were isolated by differential centrifugations

of conditioned media collected from 24h-cultured 3T3-L1 adipocytes in the absence of serum. Based on morphological and biochemical properties as well as quantification of secreted EVs using nanoparticle tracking analysis, we distinguished two subpopulations of adipocyte-derived EVs, namely, microparticles (MP) and exosomes, with different mean size (285.3181 nm and 39.915 nm, respectively). Proteomic study using high-resolution mass spectrometry analysis (LC-MS/MS) revealed differential expression of proteins associated with these EVs. Indeed, MP and exosomes exhibit specific protein signatures allowing not only their sorting but also the prediction of their subsequent function(s) with respect to metabolic responses in recipient cells. By contrast, MP and exosomes present similar phospholipid patterns with respect to quantitative mass-spectrometry high-throughput lipidomic analysis. Our results demonstrate the ability of adipocytes to secrete substantial quantities of EVs. They also highlight the ability of adipocytes to produce the two subpopulations of EVs that can be differentially sorted by their protein contents known to regulate the glucidolipidic metabolism. These adipocyte-derived EVs could therefore participate in metabolic dysfunctions commonly associated with obesity.

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OW7.3

Extracellular vesicles, extracellular vesicles-contained miRNAs and total miRNAs as plasma-based biomarkers for metabolic syndromeSadhbh O'Neill¹, Søren Gregersen², Mette Larsen², Kjeld Hermansen² and Lorraine O'Driscoll¹

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Introduction: Metabolic syndrome (MetS) is the compounding of several risk factors that increase the risk of cardiovascular disease, type 2 diabetes and cancer. Reliable, minimally invasive biomarkers for diagnosing MetS are needed. In an effort to identify biomarkers, we investigated extracellular vesicles (EV), EV-contained miRNAs and total miRNAs isolated from plasma of obese and MetS individuals. **Methods:** EVs were isolated by filtration and ultracentrifugation and characterized by bicinchoninic acid assay, immunoblotting, nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM). RNA was extracted by TriReagent, and miRNAs were assessed using miRNA panels and qPCR assays. **Results:** Evidence of successful EV isolation included immunoblots showing CD81, PDC61, with Grp-94 undetected. Also, NTA and TEM showed vesicles to be ~100 nm. The amounts of EVs (mg/mL) was significantly ($p=0.004$) increased in MetS (250 ± 34) compared to obesity (136 ± 19). Total plasma miRNA profiling identified miR-758-3p to be significantly (-2.75 ± 0.44 -fold, $p=0.09$) reduced in MetS ($n=19$) compared to obesity ($n=19$). This was confirmed (-23.2 ± 1 fold, $p=0.03$) by qPCR in an independent cohort ($n=7$ MetS, $n=7$ obese). Considering EV-miRNAs, miR-758-3p was undetectable in EVs from the same cohorts, suggesting that it is not carried by EVs. Conversely, miRNA profiling of EV-RNA indicated miR-15a-5p (22.7 ± 0.5 -fold, $p=0.01$) and miR-100-5p (3.42 ± 0.4 -fold, $p=0.02$) to be significantly increased in MetS ($n=12$) compared to obesity ($n=12$). **Summary/conclusion:** This study shows, for the first time, that EVs, EV-miRNAs and total miRNAs all have potential as

minimally invasive biomarkers to decipher between obesity and those who go on to develop full-blown MetS. Simply assessing “total miRNAs” or “EV-carried miRNAs” may not be most beneficial, but a combination of both maybe of most value. Further studies in larger cohorts, including appropriate healthy controls, and co-analysing each of these are now warranted.

OW7.4

Leukocyte microvesicle as fibrosis biomarkers in non-alcoholic fatty liver disease

Joshua Welsh, Christopher Byrne, Nicola Englst, Geraldine Clough and Eleonora Scorletti
University of Southampton, England, UK

Introduction: Non-alcoholic fatty liver disease (NAFLD) is an increasingly common, progressive inflammatory fibrotic liver condition, with a variety of stages (steatosis, steatohepatitis (NASH), fibrosis and cirrhosis). Better non-invasive liver fibrosis tests are required that allow monitoring of fibrosis and testing of therapeutic interventions. Microvesicles (MVs) are submicron vesicles released upon cell activation or damage and may be useful for assessing the stage of liver disease in NAFLD. The aim of this study was to determine whether leucocyte MV production changes with severity of liver fibrosis. **Methods:** Twenty-five adults with NAFLD diagnosed by liver biopsy were recruited as part of a sub-study of the WELCOME Trial 1. Liver fibrosis severity was graded 0–4 as part of Kleiner scoring of NAFLD severity. Citrated plasma samples were centrifuged twice at $2000 \times g$ and stored at -80°C prior to analysis using flow cytometry. Monocyte microvesicles (MMVs) between 200 and 1000 nm were counted and categorized as the sum of CD14+ and CD14+CD16+ events. An ANOVA with a post hoc least significance difference (LSD) test for multiple comparisons was undertaken to test differences between groups and associations. **Results:** Cohort characteristics (meanSD): age = 49.4 ± 12.7 years, men = 13, type 2 diabetes n = 12, Kleiner score = 6.02.5. Individuals with a fibrosis score of 0 (n = 5) were shown to have significantly more MMVs than those with a score of 3 (n = 5, p = 0.009) and 4 (n = 4, p = 0.005), with a mean decrease in the MMV number from no fibrosis of 61.4 and 67.8%, respectively. **Conclusion:** MMV numbers may differentiate individuals with severe fibrosis from those with little or no fibrosis in NAFLD, suggesting further exploration of MMVs as NAFLD biomarkers is merited (1).

Acknowledgements: This study was supported by Diabetes UK (BDA 09/0003937)

Reference

1. Scorletti, E. et al. *Contemp Clin Trials* 37, 301–11 (2014).

OW7.5

High-density lipoprotein-like nanoparticles target SR-B1 and inhibit melanoma cell-derived exosome uptake to block the pre-metastatic niche

Michael Plebanek, Olga Volpert and C. Shad Thaxton
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Introduction: Exosomes are natural nanovesicles produced by all mammalian cells. They function as intercellular delivery vehicles, to transport an array of molecular cargo. Exosomes are critical for the pathogenesis of many diseases. In cancer, tumour exosomes generate pro-metastatic microenvironments termed the “pre-metastatic niche.” Exosome uptake depends on cholesterol-rich membrane microdomains called lipid rafts and is blocked by non-specific depletion of cholesterol in the plasma membranes. Our laboratory has developed a synthetic nanoparticle mimetics of high-density lipoprotein (HDL NP) that, like HDL, binds scavenger receptor type B-1 (SR-B1), but, unlike HDL, they destabilize lipid rafts through enhanced cholesterol efflux. We hypothesized that HDL NPs inhibit exosome uptake and block the pre-metastatic niche. **Methods:** Exosomes from A375 or B16F10 melanoma were labelled with fluorescent lipophilic dyes. The ability of HDL NPs to block the functions of melanoma exosomes was assessed in vitro and in vivo. The methods included fluorescent microscopy and flow cytometry, assessment of endothelial cell migration and quantifying mobilization of the endothelial progenitors and myeloid-derived suppressor cells in vivo. **Results:** We showed that HDL NPs inhibit uptake of melanoma exosomes by tumour cells, endothelial cells and macrophages via SR-B1-dependent modulation of cholesterol flux and disruption of lipid raft homeostasis. We also show that HDL NPs prevent endothelial cell migration caused by melanoma exosomes in vitro and block exosome-dependent mobilization of endothelial progenitor and myeloid-derived cells from the bone marrow in vivo. **Summary/conclusions:** Our study implicates SR-B1, as a critical mediator of exosome uptake, and points to HDL NPs as a means to inhibit exosome uptake. Importantly, because HDL NPs prevent exosome-dependent pro-metastatic, they may be developed into much needed therapy for metastasis.

Room: Willem Burger

Symposium Session 8 - EVs and tumour environment I

Chairs: Irina Nazarenko and Riccardo Alessandro

3:30-5:00 p.m.

OW8.1**Immunomodulatory properties of large oncosomes from prostate cancer cells**Dhong-Hyun Lee¹, Valentina R. Minciacci¹, Umberto Foresta¹, Rebecca Porritt², Maretti Ana², Mandana Zandian¹, Michael R. Freeman², Gislaine Martins² and Dolores Di Vizio¹¹Department of Surgery, Cedars Sinai Medical Center, Los Angeles, USA;²Department of Medicine, Cedars Sinai Medical Center, Los Angeles, USA

Introduction: Metastasis is the leading cause of prostate cancer (PCa)-related death in the United States. Immunotherapy confers survival advantages for men with asymptomatic to minimally symptomatic metastatic PCa, but not with symptomatic disease. Development of new strategies will require a better understanding of the complex cross-talk between PCa cells and the immune system. We recently discovered cancer-specific extracellular vesicles (EVs) called large oncosomes (LO), which may play an important role in the cross-talk. LO contain tumour-derived macromolecules. MicroRNA 1227 (miR1227) emerged as enriched in EVs from tumorigenic but not from benign cells. **Methods:** Ultracentrifugation for EV isolation; confocal imaging to follow EV uptake; lentiviral overexpression of miR1227; western blot; RT-qPCR. **Results:** miR1227 was predicted by multiple algorithms to target the ring finger protein 125 (RNF125), an E3 ubiquitin protein ligase and a known inhibitor of the retinoic acid inducible gene 1 (RIG-I). Overexpression of miR1227 in Jurkat cells induced significant decrease in RNF125 mRNA and a modest increase in RIG-I protein levels. We found that macrophages and dendritic cells (DC) take up LO avidly. RNF125 mRNA levels were significantly reduced in DC treated with LO from parental or miR1227 over-expressing PC3 cells. In the same conditions, DC exhibited altered mRNA levels of select cytokines downstream of the RIG-I pathway (CCL5, IL6, IFNB1 and TNF). These results suggest that LO might inhibit RNF125 that, in turn, can activate the RIG-I pathway, thus reprogramming the immune system in favour of tumour progression. **Conclusion:** Our study has identified a new molecular mechanism underlying the modulation of the immune response to PC. This can potentially contribute to the improvement of the current therapies as well as the development of new immunotherapeutic strategies specifically targeting pathways underlying impairment of the immune response to PC and other types of cancer.

OW8.2**Characterization and functional evaluation of distinct exosome subpopulations**E Willms¹, HJ Johansson², I Mäger^{1,3}, Y Lee¹, KE Blomberg⁴, M Sadik⁵, A Alaarg¹, CI Smith⁴, J Lehtiö², S El Andaloussi^{1,4}, MJ Wood¹ and P Vader^{1,5}¹Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, United Kingdom; ²Cancer Proteomics Mass Spectrometry, Science for Life Laboratory, Department of Oncology-Pathology, Karolinska Institutet, Stockholm, Sweden; ³Institute of Technology, University of Tartu, Tartu, Estonia; ⁴Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden; ⁵Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, Utrecht, The Netherlands

Cells release nano-sized membrane vesicles that are involved in intercellular communication by transferring biological information between cells. It is generally accepted that cells release at least three types of extracellular vesicles (EVs): apoptotic bodies, microvesicles and exosomes. While a wide range of putative biological functions have been attributed to exosomes, they often are assumed to

represent a homogenous population of EVs. Here, we demonstrate the existence of subpopulations of exosomes with defined molecular compositions and biological properties. Exosomes isolated from B16F10 melanoma cells consisted of two distinct subpopulations, as demonstrated by both density gradient centrifugation and size exclusion chromatography. Further characterization of the exosome subpopulations, obtained after density gradient centrifugation, revealed their distinct differences in biophysical properties, and their proteomic and RNA signature. Exposure of HSV endothelial cells to the exosome subpopulations induced differential changes in gene expression. In conclusion, we demonstrate that cells release distinct exosome subpopulations that differ in their composition, and more importantly biological effects on recipient cells. Further dissection of exosome heterogeneity will advance our understanding of exosomal biology and function, and accelerate the development of exosome-based diagnostics and therapeutics.

OW8.3**Extracellular vesicles regulate soluble secreted factors and epigenetic profile of the tumour microenvironment**Michael Liem¹, Suresh Mathivanan¹ and Kalra Hina²¹La Trobe University, Melbourne, Australia; ²La Trobe Institute of Molecular Sciences, Melbourne, Australia

Introduction: For a tumour to progress, a bidirectional crosstalk of the cancer cells and its surrounding tissue is critical, and various soluble factors released in the tumour microenvironment (TME) are known to induce tumour growth. Extracellular vesicles (EVs) contain various oncogenic proteins and RNAs, which can potentially regulate the TME. We hypothesize that EVs derived from colorectal cancer (CRC) cells promote cancer progression by reprogramming the cells in the TME by altering the soluble secreted proteins and DNA methylation. **Methods:** Quantitative proteomics analysis was carried out to identify the changes in soluble secreted factors during exosomes treatment. In vitro scratch assay was performed. Methylation profiling of cells in the tumour microenvironment was performed using Infinium HumanMethylation450 BeadChip and targeted bisulfite sequencing. **Result:** In this study, SILAC-based quantitative proteomic analysis highlighted the EV-based transfer of various oncoproteins and histones from the cancer cells into the recipient cells. A follow-up subcellular fractionation confirmed the intercellular transfer of proteins into the recipient cell nucleus. EV-mediated transfer of proteins regulated the soluble secreted proteome of the recipient cells in the TME (fibroblast, pericytes, endothelial, monocytes, NK and T cells). More importantly, the altered secreted proteins induced more aggressiveness of cancer cells thereby aiding in tumour progression. To further study the potential of EVs in educating the TME, variety of cells in TME were treated with EV and analysed for DNA methylation profile. Upon the uptake of cancer-derived EVs, the recipient cells exhibited differential DNA methylation status of many genes again aiding in tumour progression. **Summary:** The results confirm that EVs can regulate the secretome and the DNA methylation profiles of the recipient cells in favour of tumour progression.

OW8.4**Tumour-derived extracellular vesicles captured by mesenchymal stromal cells induce an ERK1/2-dependent pro-tumorigenic inflammatory reaction**

Yves Declerck

Children's Hospital Los Angeles-University of Southern California, USA

The bone marrow (BM) is a microenvironment that promotes survival, dormancy and therapeutic resistance in tumour cells. Central to this function are mesenchymal stromal cells (MSC), precursor cells of osteoblasts, chondrocytes and adipocytes that contribute to the BM niche. Here, using neuroblastoma (NB) as a model of communication between tumour cells and MSC, we demonstrate that tumour cells release two populations of extracellular vesicles (EVs). One rich in CMGA/chromogranin A, and a second, rich in proteins present in exosomes such as HSP90 and HSP70, tetraspanin CD63/melanoma1 antigen, PDCD6IP/ALIX and SDCBP/syntenin and galectin-3 binding protein (Gal-3BP), a sialoglycoprotein we had previously reported to induce the expression of interleukin (IL)-6 in MSC. In contrast, these EV contain few cytokines and chemokines. We then show that the capture of these EV by MSC stimulates the production of several pro-tumorigenic cytokines and chemokines like IL-6, CXCL8/IL-8, vascular endothelial cell growth factor (VEGF), stromal-derived factor-1 (SDF1/CXCL12) and monocyte-chemotactic protein-1 (MCP-1/CCL2) by MSC. Exposure of MSC to NB-derived EV stimulates ERK1/2 and Akt activation, and inhibition of ERK1/2 but not Akt prevents the stimulation of IL-6 and IL-8 production by MSC. Thus, we describe here a new mechanism by which EVs produced by NB cells educate MSC towards a pro-tumorigenic role contributing to a favourable microenvironment for NB cells in the BM.

OW8.5

Disruption of the LINC complex in cancer cells drives the genesis of extracellular vesicles with nuclear content

Mariana Reis-Sobreiro¹, Samantha Morley², Kenneth Steadman¹, Jie-Fu Chen³, Sungyong You¹, Edwin M. Posadas¹, Dolores Di Vizio⁴ and Michael R. Freeman⁵

¹Cedars Sinai Medical Center, Los Angeles, CA, USA; ²Boston Children's Hospital, Boston, MA, USA; ³Samuel Oschin Comprehensive Cancer Institute, Cedars Sinai Medical Center, Los Angeles, CA, USA; ⁴Department of Surgery, Cedars-Sinai Medical Center, University of California, Los Angeles, CA, USA; ⁵Department of Surgery and Department of Biomedical Sciences, Cedars-Sinai Medical Center, University of California, Los Angeles, CA, USA;

Introduction: Amoeboid tumour cells are phenotypically plastic, move rapidly through extracellular matrixes, shed extracellular vesicles (EVs) and have propensity to metastasize. For amoeboid cells to squeeze into narrow spaces, nuclear deformations are critical, as the nucleus is the largest and most rigid organelle. Protein assemblies that link the cytoskeleton with the nucleoskeleton govern nuclear shape and stiffness. Collectively, these structures are known as the linker of nucleoskeleton and cytoskeleton (LINC) complex. We tested whether alterations in the LINC complex are associated with the amoeboid phenotype and shedding of EVs. **Methods:** Immunoprecipitation-mass spectrometry (IP-MS), flow cytometry, differential ultracentrifugation, iodixanol gradient, confocal 3D reconstruction, life cell imaging, western blot and nanovelcro. **Results:** Loss of the cytoskeletal regulator DIAPH3 drives the transition of cancer cells to an amoeboid phenotype. We found that DIAPH3 directly interacts

with the LINC complex. DIAPH3 silencing in breast and prostate cancer cells destabilizes the complex, promoting nuclear envelope blebbing and shedding of non-apoptotic EVs with nuclear content. LINC complex disruption through the silencing of emerin and lamin A/C promotes increased migration, formation of nuclear envelope blebs, increased shedding of non-apoptotic EVs and ROCK pathway activation. Consistent with the idea that disruption of the LINC complex is important in metastasis, lamin A/C is downregulated in metastatic prostate cancer patients, and low levels of emerin are associated with poor survival outcomes in breast cancer patients. Furthermore, circulating tumour cells (CTCs) isolated from prostate cancer patients express low emerin levels. **Conclusions:** Our study provides new markers of lethal disease in prostate and breast cancer and suggests that LINC complex disruption is a critical step for metastatic spread. It also identifies a mechanism that promotes inclusion of nuclear content into EVs.

OW8.6

Extracellular vesicles as a signalling system involved in kidney morphogenesis and disease

Anatoliy Samoylenko, Mirja Krause, Khem Giri, Tuomas Nurmi, Aleksandra Rak-Raszewska, Prateek Singh, Genevieve Bart, Timo Pikkarainen and Seppo Vainio

Faculty of Biochemistry and Molecular Medicine, University of Oulu, Oulu, Finland

Cells secrete different types of membrane-enclosed extracellular vesicles (EVs), which are widely present in the body fluids and incorporate mRNA, miRNA, proteins and signalling molecules. One of the most studied groups of these vesicles are exosomes (EXs), characterized by small size (30–100 nm) and endosomal origin. Increasing amounts of evidence suggest that EXs play an important role in cell-to-cell communication. EXs secreted by kidney cells control nephron function and are involved in kidney diseases and cancers. In our study, EVs were purified from Renca renal carcinoma and mK4 metanephric mesenchyme-derived cell culture media by sequential and sucrose gradient ultracentrifugation and analysed by electron microscopy (EM), confocal microscopy, nanoparticle tracking analysis (NTA) and western blotting using EV-specific markers. Metanephric mesenchyme (MM) cells were isolated from E11.5 mice embryos by micro-dissection and cultured as a monolayer. Morphology, proliferation and viability of monolayer MM cells and mK4 cells treated with EVs were assayed by immunofluorescent staining and live cell imaging using IncuCyte ZOOM (Essen BioScience) and Operetta (PerkinElmer). We found that conditioned media as well as isolated EVs influence growth of MM and mK4 cells in culture. EM and NTA showed hypoxia treatment (1% oxygen for 24 h) greatly increased the EV count in collected culture media. Hypoxia also changed protein content of Renca-derived exosomes, as identified by liquid chromatography–mass spectrometry. Thus, the *in vitro* cell and organ culture models have proven to be useful tools in finding out the roles of EV during inductive events and organogenesis.

Room: Jurriaanse

Biotech Sponsored Session IZON

Chairs: *David Lyden and Stefania Raimondo*

3:30-4:00 p.m.

BW.1**Integrated methodology for purification, detailed biophysical characterization and phenotyping of extracellular vesicles from biological fluids**

Julien Muzard¹, Anoop K. Pal², Robert Vogel³ and Murray Broom³
¹Nanobiochemistry, Izon Science Ltd, Oxford, UK; ²Nanobiochemistry, Izon Science Ltd, Cambridge, MA, USA; ³Nanobiochemistry, Izon Science Ltd, Christchurch, New Zealand

Extracellular vesicles (EVs) have been shown to play important role in broad range of physiological and pathological processes. The size, number, membrane composition and contents of EVs are highly heterogeneous, dynamic and depend on the cellular source, state and environmental conditions. Thus, understanding the biophysical diversity in EV population is paramount for linking the impact of EV properties to its biological role and function. Here, we outline an integrated methodology that utilizes simple size exclusion chromatography (SEC) based qEV columns for rapid EV purification from variety of biological fluids (urine, serum, plasma, CSF, cell culture etc.) followed by accurate, high-resolution particle-by-particle EV biophy-

sical characterization (size, size range, concentration and surface charge) through qNano that utilizes Tunable Resistive Pulse Sensing (TRPS) technology. The SEC columns provide a convenient, reproducible and highly effective means of eliminating >99% of non-vesicular protein from biological fluid samples. Moreover, the integrated methodology has been further developed and utilized for specific immuno-labelling and phenotyping of EV surface markers (CD9, CD63 and CD142) from bio-fluids. Here, specific EVs are isolated post SEC purification through antibody conjugated to magnetic beads. The resulting EV-magnetic complex (immunoprecipitate) is then directly analysed and the extent of aggregation monitored through TRPS. Our results showed a proportional increase in size, volume and surface charge of the EV-magnetic bead complexes over a defined dose-range. Changes in physical properties indicated the positive selection of specific sub-population, and these results were confirmed with secondary endpoints. Tissue factor activity assay for CD142 confirmed our findings. Thus, proposed methodology provides a simple, rapid, reliable and cost-efficient approach that has tremendous utility for biophysical characterization and EV-based diagnostics.

Room: Jurriaanse

Symposium Session 9 - EV biomarkers for cancer I

Chairs: David Lyden and Stefania Raimondo

4:00-5:00 p.m.

OW9.1

TM9SF4 expression in tumour tissue and in circulating exosomes: a novel diagnostic biomarker for gastrointestinal tumoursPaolo Guazzi¹, Sergejs Isaevs², Natasa Zarovni³, Giorgia Radano³, Antonietta Corrado³, Pietro Ferruzzi³, Antonio Chiesi³, Marcis Leja² and Francesco Lozupone⁴¹HansaBioMed, Tallinn, Estonia; ²Faculty of Medicine University of Latvia, Riga, Latvia; ³Exosomics Siena, Italy; ⁴Istituto superiore di Sanità

Background: The identification of novel biomarkers for the early detection and monitoring of gastric (GC) and colorectal cancer (CRC) is of paramount importance. TM9SF4 is a transmembrane protein highly expressed in Golgi and endosomal membranes of cancer cells which interacts with V-ATPase. The aim of this study was the evaluation of TM9SF4 expression in GC and CRC patients and in GC and CRC deriving exosomes, as potential new biomarker for early detection and monitoring of GC and CRC. **Methods:** Tissue samples of 108 cancer patients were analysed by Immunohistochemistry. Exosomal expression of TM9SF4 was assessed by western blot and ExoTEST analysis of blood plasma deriving exosomes from a cohort of 162 cancer patients (80 with GC and 82 with CRC) and 174 control subjects. **Results:** TM9SF4 was significantly increased in both non-advanced and advanced GC and CRC, compared to the control group and premalignant lesions, with a sensitivity that is significantly higher than commonly used tumour markers such as CEA and CA19-9. Furthermore, TM9SF4 expression is also related with disease stage in both cancer types. The analysis of TM9SF4 in tumour patients deriving exosomes and in exosomes collected from healthy donors clearly suggested that TM9SF4 is an exosome protein, strongly expressed in tumour cell and in cancer patients' plasma deriving exosomes, with a 2.5- and 2.7-fold increase, respectively, for GC and CRC as compared to control group. Noteworthy, a significant correlation between TM9SF4 expression in tissue samples and in exosomes isolated from peripheral blood plasma of the same patients has also been observed. **Conclusions:** TM9SF4 can be proposed as a new, highly specific cancer biomarker exploitable for the disease evaluation of gastrointestinal cancers patients. TM9SF4 bearing exosomes reflecting the pathological changes in GC and CRC patients are applicable for the development of new and promising tool for a non-invasive early diagnosis of GC and CRC.

OW9.2

Circulating tumour cells, tumour extracellular vesicles and serum cytokeratins in castration-resistant prostate cancerAfroditi Nanou¹, Guus van Dalum¹, Leonie Zeune¹, Frank Coumans², Wendy Onstenk³, Stefan Sleijfer³, Mateus Crespo⁴, Johann de Bono⁴ and Leon WMM Terstappen¹¹Department of Medical Cell BioPhysics, MIRA Institute, University of Twente, Enschede, The Netherlands; ²Department of Biomedical Engineering and Physics, Academic Medical Center, University of Amsterdam, The Netherlands; ³Department of Medical Oncology, Erasmus MC Cancer Institute, Rotterdam, The Netherlands; ⁴Institute of Cancer Research, Royal Marsden Hospital, London, UK

Introduction: Presence of circulating tumour cells (CTC) in castration-resistant prostate cancer (CRPC) is associated with poor outcome and may serve as a liquid biopsy and an intermediate surrogate endpoint of treatment efficacy in CRPC. Herein, we investigated the relation of CTC, tumour-derived extracellular vesicles (tdEV) and serum cytokeratins (CK) with clinical outcome. **Methods:** CTC

and cytokeratin+, EpCAM+, CD45-tdEV smaller than 4 µm were determined with the CellSearch system in 7.5 mL of blood of 87 CRPC patients and 16 healthy donors. The levels of circulating cytokeratin 18 (M65) and cleaved cytokeratin 18 (M30) were evaluated with ELISA in the corresponding plasma samples. **Results:** The median number of CTC and tdEV in 7.5 mL blood of 87 CRPC patients was 5 (IQR: 53.5) and 275 (822.4), whereas the respective numbers of CTC and tdEV in 7.5 mL blood of 16 healthy donors were 0 (0) and 11 (11). The median concentrations of M65 and M30 were 582 (349) and 268 (119) U/L in the plasma of CRPC patients. The respective values in the plasma of healthy donors were 158 (21) and 70 (22) U/L. CTC, tdEV, M30 and M65 values of patients were all significantly higher compared to the values in healthy donors (Mann-Whitney U test, $p < 0.001$). The presence of ≥ 700 U/L of M65, ≥ 5 CTC and ≥ 500 tdEV in 7.5 mL blood of CRPC patients predicted significantly shorter overall survival in the patient cohort; M30 could not predict survival. **Conclusion:** CTC, tdEV and circulating cytokeratin 18 (M65) are detected in significantly higher amounts in blood of CRPC patients. Cut-off values for M65 and tdEV were determined. Multivariate Cox regression showed that the hazard ratios of M65 and tdEV in blood of patients are equivalent to prostate-specific antigen and CTC and are potential markers to predict the outcome of CRPC patients. The cut-off values presented for each biomarker will be validated in another set of CRPC patients to determine their potential clinical utility.

OW9.3

Detection and functional implications of tumour-derived microvesicles in cancer patientsKerstin Menck¹, Annalen Bleckmann¹, Astrid Wachter¹, Bianca Hennies¹, Tobias Pukrop¹, Dirk Wenzel², Florian Klemm¹ and Claudia Binder¹¹University Medical Center Göttingen, Germany; ²Max Planck Institute for Biophysical Chemistry, Göttingen, Germany;

Introduction: Plasma membrane-derived microvesicles (MVs) are known as important mediators of intercellular communication. Especially in cancer, many in vitro studies already showed that tumour cells use MVs to create a favourable tumour niche that promotes tumour progression. However, in vivo studies on tumour-derived MVs (T-MVs) are still missing due to the lack of suitable markers. Previously, we had described the protein EMMPRIN/CD147 as a novel marker for T-MVs and demonstrated an increase of EMMPRIN+ MVs in the blood of a small number of breast cancer patients. Now, we aimed to evaluate which markers can be used to detect T-MVs in peripheral blood of a large cohort of patients with different tumour entities and whether T-MVs are associated with tumour progression in vivo. **Methods:** MVs were isolated by differential ultracentrifugation from peripheral blood of 274 cancer and 103 control patients. All samples were collected upon informed consent with approval from the local ethics committee. **Results:** We demonstrate a significant increase in the number of MV positive for the tumour markers EMMPRIN, MUC1 and EpCAM in the blood of cancer versus control patients. Flow cytometry revealed a population of EMMPRIN/MUC1 double positive MVs that was significantly elevated in cancer patients and almost undetectable in control patients. In vitro, MVs from cancer patients increased cancer cell invasion, which was antagonized by blocking peptides directed against the highly glycosylated form of EMMPRIN. In contrast, MV from control patients had no such effect on tumour invasion. Interestingly, stimulation of human macrophages with cancer patient-derived MVs induced expression of WNT5A and VEGF, two proteins known to promote tumour invasion and angiogenesis. **Conclusion:** T-MVs can

be detected in the blood of cancer patients by conventional flow cytometry with a combination of different markers. Apart from being potentially useful biomarkers, they also seem to play a role in cancer progression in vivo.

OW9.4

Identification of Del-1 on circulating exosomes as a liquid biopsy to detect early breast cancer

Eun Ju Im and Moon-Chang Baek

Department of Molecular Medicine, School of Medicine, Kyungpook National University, Daegu, Republic of Korea

Introduction: Emerging evidence suggests that cancer cell-derived exosomes containing proteins linked to the disease pathogenesis may provide early biomarkers for various cancers. Here we report on Del-1 protein on circulating exosome (cirExo) as an early diagnostic biomarker for breast cancer (BC). **Methods:** To understand the mechanism of BC metastasis and identify early biomarkers of this

cancer, we studied exosomes secreted from BC cells. We focused on the Del-1 protein based on the fact that this protein is highly expressed in MDA-MB-231 exosomes. Enzyme-linked immunosorbent assays (ELISAs) were used to measure Del-1 in plasma samples from healthy controls, patients with BC, BC patients after surgical resection, patients with benign breast tumours and patients with non-cancerous diseases, in two cohorts. **Results:** These exosomes are sufficient for enhancement of BC cells invasion, which is most likely mediated via the integrin-FAK signalling cascade in cancer cells and for acceleration of lung metastasis at the initial stage of cancer mouse models. However, these effects are significantly suppressed when Del-1 is inactivated, providing evidence for a critical role of Del-1 in development of cancer. Consistently, in human patients with BC, the levels of Del-1 on cirExo are significantly elevated at the early stage of breast cancer, including ductal carcinoma in situ (DCIS), but return to normal levels after removal of the tumour, as determined by a developed ELISA with >96% high sensitivity and specificity using 1 μ L of plasma. **Summary/conclusion:** Taken together, these results identify an exosomes-mediated mechanism of invasion and an early diagnostic biomarker for BC using Del-1 protein on circulating exosomes.

Poster Presentations

Room: Willem Burger Foyer/Van Der Mandele and Arcadis Zaals

Poster Session 1 - Cell biology of EVs: biogenesis and transfer

Chairs: *Pascale Zimmermann and Charles Lai*

5:00-8:00 p.m.

PW1.01

New insights into sortilin regulation in lung cancer

Cornelia Wilson, Triantafyllos Liloglou and Amelia Acha-Sagredo
University of Liverpool, England, UK

Lung cancer is the leading cause of cancer death worldwide killing over 1.2 million people each year with non-small-cell lung cancer (NSCLC) being the most common form (75–85% lung cancer patients). This is mainly due to the lack of early screening and efficient tools for early diagnosis of the disease. Exosomes are small membrane-bound microvesicles (or extracellular vesicles) released from most eukaryotic cells, containing a plethora of nucleic acids and proteins that are thought to facilitate microenvironment communication and control. In cancer, the number and content of exosomes changes affecting the microenvironment and inducing immune suppression, angiogenesis and pre-metastatic niche formation. Microenvironment communication through neurotrophin signalling could lead to the progression of cancer, while an imbalance in expression of neurotrophin receptors such as sortilin is strongly linked to different types of cancer. Sortilin is a member of the Vps10 family, having a multifaceted role both as a co-receptor and as a trafficking molecule in the trans-Golgi network (TGN). Sortilin can form a novel complex with TrkB and EGFR (TES complex), found in exosomes that are released from NSCLC cells conveying a microenvironmental control upon endothelial cells. We hypothesize that an imbalance of sortilin's role in protein trafficking and exosome biogenesis may contribute to cancer progression. We demonstrate that sortilin exists as two variants, sharing high homology with each other, the variant 1 is well described in the literature while the identity and function of variant 2 is still unknown. We show that the expression of the two sortilin variants is deregulated in early stages of lung cancer. Hence, the deregulation of sortilin expression in NSCLC could provide the first insights of its function in exosomes as an early diagnostic and prognostic marker.

PW1.02

Biogenesis of exosomes derived from macrophages exposed to *Mycobacterium avium* subsp. *paratuberculosis* is altered in relation to infection dosage and time of exposure

Matt Johansen, Kumudika De Silva, Karren Plain, Richard Whittington and Auriol Purdie
The University of Sydney, Australia

Introduction: Johne's disease is a chronic granulomatous enteritis caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), primarily affecting a variety of agriculturally significant ruminant species such as sheep, cattle, deer and goats. The pathogenesis of paratuberculosis resembles other chronic mycobacterial diseases. While the importance of exosomes and the role they play in the development of tuberculosis are documented, their role in paratuberculosis is unknown. The aim of this study was to investigate the biogenesis of exosomes released from MAP-exposed murine macrophages associated with changes in the infection dosage and length of exposure in vitro. **Methods:** Exosomes were studied using nanoparticle

tracking analysis, flow cytometry and transmission electron microscopy. Mass spectrometry analysis was used to identify proteomic constituents of exosomes. **Results:** The infection dose influenced the concentration of exosomes produced at 24 h post-exposure. An increasing bacterial load resulted in a linear increase in exosomes produced. Examination of a series of increasing durations of bacterial exposure revealed that the production of exosomes was associated with specific timepoints post-exposure. Furthermore, the crucial timepoints identified for the production of exosomes changed significantly in relation to the bacterial load and the exosome isolation method used. While protein quantification is typically used to estimate the abundance of exosomes, it was found there was no correlation between protein concentration and exosome particle concentration. Mass spectrometry analysis identified a number of MAP-specific proteins within exosomes that changed in relation to infectious dosage and length of time post-exposure. **Summary/conclusion:** This study demonstrates that the infection dosage and time post-exposure have significant impacts on the production and composition of exosomes from MAP-exposed macrophages.

PW1.03

Extracellular vesicles in pharmaceutical bioprocess – comparison of two cell lines

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Introduction: Recognition of the role of extracellular vesicles (EVs) in a variety of diseases mostly stimulates their investigation for clinical purposes. In this work, we present EVs from a different point of view, as a tool for the bioprocess monitoring. EVs are rapidly released by cells during cell activation and cell stress; therefore, the amount of released MVs could be used for the identification of cell state and cell condition during the bioprocess. However, the release of EVs depends on cell type also. We have compared the vesiculation of two different cell lines during pharmaceutical bioprocess. **Methods:** We have used two different cell lines, both derived from a Chinese hamster ovary (CHO) cell line. EVs were quantified by flow cytometry. EVs were measured in samples from the bioreactor after cells were centrifuged off. **Results:** The absolute amount of EVs in the bioreactor increased highly during the bioprocess for both cell lines; besides, the amount of EVs in comparison to cells increased obviously during the bioprocess. During the early stages of the bioprocess, the amount of EVs with regard to cells increased only slightly, but the amount of EVs in comparison to cells increased significantly at the end of the bioprocess because of the increase in cell dying. The amount of dead cells continuously increased during the bioprocesses of both cell lines. The amount of EVs continuously increased with one of the cell lines; however, the release of EVs of the cell line with higher proportion of dead cells throughout the bioprocess was highly dynamic, and enormous amount of EVs were released on the bioprocess days 9 and 12. **Conclusions:** The amount of EVs increased during the bioprocesses of both cell lines and indicates deteriorating conditions in bioreactor. However, while the release of EVs continuously increased during the bioprocess of one of the cell lines, the

other cell line with decreased viability showed dynamic and hostile reaction of the cell vesiculation.

PW1.04

Inhibitors of microvesicle release with the potential to enhance effectivity of cancer chemotherapy

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Introduction: Microvesicle (MV) release from tumour cells plays an important role in cancer drug resistance. It is essential that chemotherapeutic drugs are retained within target cells for increased effectiveness in inducing apoptosis. Microvesiculation influences drug retention so minimizing drug efflux is important. Based on MV biogenesis pathways, a range of potential inhibitors were tested. **Methods:** The NanoSight LM10 was used to analyse MV release from PC3 human prostate cancer cells. Cells were maintained in serum-free RPMI 1640. Washed cells were seeded in triplicate at 3.8×10^5 cells/well initially treated with 300 μ M BzATP for 1 h, and further 1 h with relevant concentrations of MV-inhibitors. MV count, annexin V staining and cell viability were assessed. **Results:** The numbers of MVs released were compared to control (BzATP alone). Up to 60% clear inhibition of MV release was shown with all reagents used. The maximum MV inhibition was with 500 μ M ethylene glycol tetraacetic acid (EGTA) and 10 μ M bisindolymaleimide I, resulting in 48 and 34%, respectively. Cell viability was >80% in all cases except for panthethine, which only resulted in 25%. Annexin V staining confirmed the vesicles identified were indeed MVs. **Summary/conclusion:** EGTA and bisindolymaleimide I are potent inhibitors of MV release. Both novel and classic reagents described may be used individually or in combination. This could be similar to calpeptin and chloramidine, which enabled prostate cancer cells to sensitize to docetaxel treatment previously. This study extends the range of inhibitors that can be utilized to enhance novel combinatory treatment options.

PW1.05

Extracellular vesicles derived from SW480 cancer colon cells are internalized by human primary monocytes but not by lymphocytes

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Introduction: Extracellular vesicles (EVs) carry a range of nucleic acids, proteins and metabolites and play an important role in cell-to-cell communication. Cells have been shown to internalize EVs by a variety of endocytic pathways that may depend on specific proteins found on the surface of both the vesicle and the target cell. Importantly, EV uptake seems to influence the phenotypic status of cells. **Aim:** To investigate the endocytic pathways of extracellular vesicles in human primary monocytes and lymphocytes. **Methods:** Elutriation-purified, cryopreserved human monocytes and lymphocytes were thawed and incubated (150,000 cells per well in 96-well plates) with and without the actin polymerization inhibitor cytochalasin D (1–10 μ g/mL) or with the PI3K-inhibitors LY294002 (5–500 μ M) or Wortmannin (1–100 μ M) for 30 min at 37°C. Cells were further incubated with approx. 1×10^9 PKH67-labelled SW480 EVs per well (based on particle concentrations obtained by a NanoSight NS500 instrument) for 4 h. Subsequently, the cells were trypsinized

(0.25% trypsin/ethylenediaminetetraacetic acid) for 5 min, washed 3 times with phosphate-buffered saline and further analysed by flow cytometry (BD Accuri C6) to determine the degree of EV internalization (reported as mean fluorescence intensity, MFI), as well as by fluorescence microscopy (Nikon Eclipse Ti). **Results:** PKH67-labelled SW480 EVs were internalized by human primary monocytes, but not by lymphocytes, as observed by both flow cytometry and fluorescence microscopy. The internalization of EVs in monocytes was inhibited to 15–70% of control by the 3 compounds cytochalasin D, LY294002 and Wortmannin, in a dose-dependent manner, as shown by MFI measurements. **Conclusions:** Our findings show that SW480 EVs are internalized by human primary monocytes, but not by lymphocytes, thus suggesting that cells have different requirements for internalizing EVs. In addition, our results suggest that EVs are internalized by an actin- and PI3K-dependent endocytic pathway in primary monocytes.

PW1.06

Preferential uptake of circulating extracellular vesicles by lung-margined Ly6C-high monocytes during systemic inflammation

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Introduction: Under normal conditions, circulating extracellular vesicles (EVs) are taken up in the liver and to a lesser extent in the lungs and spleen (1,2); however, the profile of EV clearance during inflammatory conditions has not been described. During subclinical endotoxaemia, lung uptake of circulating microbial particles is increased due to enhanced "margination" of monocytes and neutrophils within the pulmonary microcirculation (3). Here we hypothesize that lung capture of EVs would increase during systemic inflammation, with implications for their vascular activity and function. **Methods:** EVs were prepared by ATP stimulation of J774A.1 macrophages and differential centrifugation. EVs labelled with the fluorescent membrane-intercalating dye, DiD, were injected intravenously (i.v.) into untreated or low-dose lipopolysaccharide (LPS) (20 ng, i.v. 2 h) pre-treated C57BL/6 mice. After 1 h, single-cell suspensions were prepared from excised lungs, liver and spleen for flow cytometric analysis of cell-associated DiD-fluorescence as a measure of EV uptake. **Results:** In untreated mice, EV uptake was primarily in the liver by Kupffer cells and endothelial cells, while EV uptake in the lungs was low and mainly by monocytes. Systemic LPS pre-treatment enhanced Ly6C-high monocyte subset margination to the lungs and liver and resulted in substantial increases (3-fold) in their EV uptake. EV uptake remained low in lung Ly6C-low monocytes, neutrophils and endothelial cells, while uptake in the liver by Kupffer and endothelial cells was substantially reduced. DiD-fluorescence in the spleen was negligible in both groups. In additional experiments using ex vivo isolated buffer-perfused lungs from LPS-treated mice, EV uptake was also limited to Ly6C-high monocytes. **Conclusion:** These findings indicate that EV-cell interactions within the vasculature are dynamic, with inflammation resulting in a significant redistribution of uptake from the hepatic to the pulmonary microcirculation.

PW1.07

Nogo/RTN4 as an extracellular vesicle-associated ligand

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Nogo-A (RTN4A) is a ~200 kDa member of the reticulon (RTN) protein family identified in the late 1980s as a myelin-associated inhibitor

of axonal regeneration after central nervous system (CNS) injury. It consists of a C-terminal trans-membrane reticulon homology domain (RHD) and an almost 1000 amino acid-long soluble N-terminus unique to Nogo-A with the exception of the first ~170 amino acids, which are also present in a splice variant, Nogo-B. The current view of Nogo signalling is that plasma membrane-bound full-length Nogo initiates signalling cascades through binding to receptors on cells in direct contact with the Nogo expressing cell. However, the presence of Nogo sequences in body fluids such as the cerebrospinal fluid (CSF) has been anecdotally reported and recently found in a proteomic study (Chiasserini et al., 2014). As the presence of functionally active Nogo in the CSF and other body fluids would have major implications, for example, for the administration of anti-Nogo-A antibodies as therapies, we sought to investigate whether either full-length or cleaved fragments of Nogo are released into the supernatant of cultured neuron-like cells. We found that full-length Nogo-A, full-length Nogo-B, as well as cleaved fragments, which include the shared N-terminus of Nogo-A/B, are found in the culture supernatant of N2a neuroblastoma cells. Both the full-length proteins and the cleaved fragments were associated with extracellular vesicles rather than free in solution. Broad spectrum inhibition of metalloproteases with the compound GM6001 almost completely eliminated the N-terminal fragments, indicating that Nogo-A/B is cleaved by metalloproteases. Furthermore, preliminary data indicate that full-length Nogo-A is also found in extracellular vesicles from the CSF of adult rats. Further studies will assess the functional relevance of vesicle-bound Nogo-A and evaluate the significance of the proteolytic processing of Nogo-A/B in the context of Nogo signalling.

PW1.08

Thiol-based labelling of prostate-derived exosomes for analysis of cellular uptake and intracellular traffic

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Introduction: A major goal in drug delivery is transportation of macromolecular therapeutics to the insides of cells. These include nucleotides, proteins and peptides, which are normally cell impermeable requiring vectors for intracellular delivery. More effective delivery vectors than those currently available are needed to meet the challenge of overcoming diseases such as cancer. Exosomes could be viewed as natural vectors capable of delivering signal mediators and macromolecular cargo and could be manipulated as vectors for therapeutic applications. How exosomes deliver cargo, however, is not fully understood. Exosome cell uptake is typically studied following labelling with fluorescent lipids such as Paul Karl Horan (PKH) dyes that could modify their structure and function. Our aims were to develop new exosome labelling methods and high-content endocytosis assays to assess their internalization and intracellular traffic. **Methods:** Here we describe a novel thiol-based conjugation method for the attachment of Alexa-488 to well characterized prostate cancer (Du145) exosomes. The involvement of different endocytic pathways in cell uptake was investigated using siRNA targeting specific endocytic proteins and pharmacological inhibitors of endocytosis, together with live cell confocal microscopy. Uptake was analysed in fibroblasts and HeLa cells. **Results:** Alexa488-exosomes retained their function (to differentiate fibroblasts to myofibroblasts) and were effectively internalized into fibroblasts and HeLa cells as motile punctate cytoplasmic structures. siRNA and inhibitor studies in HeLa cells highlighted actin-dependent macropinocytosis as a major pathway mediating cell uptake suggesting that exosomes may be activating their own internalization. **Conclusions:** The use of this novel labelling technique and endocytosis assays will help characterize how exosomes gain intracellular access to mediate their effects and allow for further assessment of their potential as drug delivery vectors.

PW1.09

New molecular approaches to quantitatively assess exosome uptake by specific target cells

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Introduction: Exosomes (Exo) deliver specifically their cargo from the donor to recipient cells. Thus, approaches for the application of Exo as tool for a cell-specific delivery of signalling molecules need to be tested in *in vitro* and *in vivo* models. Here we describe experimental design for the assessment and the quantification of Exo uptake by target cells. **Methods:** *C. elegans* species specific Cel-miR-39 was overexpressed in human cardiac progenitor cells (CPC) or human dermal fibroblasts (DF) as control. Exo containing Cel-miR-39 (Exo-Cel39) were isolated, and the amount of the miRNAs has been quantified by real-time polymerase chain reaction (RT-PCR). Cardiomyocytes (CM) were incubated with Exo-Cel39 for 5 h. After incubation, total CM RNA was isolated and Cel-miR-39 quantified. As second approach, we used the "fluorescent complementation technology." With this technique, CPC-producing Exo were transfected with a small green fluorescent protein (GFP) fragment fused with Exo-specific marker (Exo-smGFP). The large GFP fragment is expressed by CM as target cells. Following the uptake of Exo by CM, the small and large GFP fragments spontaneously associate, resulting in GFP folding and formation of the fluorophore. **Results:** we found that CPC and DF similarly sort Cel-miR-39 into Exo even if cell transfection efficiency was different. Five hours after incubation, Cel-miR-39 expression levels were significantly higher in CM incubated with Exo-Cel39 from CPC then DF ($3.8 \times 10^5 \pm 4.3 \times 10^3$ vs. $4.1 \times 10^3 \pm 1.9 \times 10^3$ folds increase, $p < 0.05$, in CPC vs. DF). Constructs carrying the GFP fragment fused with Exo-specific marker have been obtained and they are currently undergoing validation *in vitro* in rat CM. **Conclusions:** Using a specific and quantitative method, we were able to demonstrate that Exo have specific uptake by target cells. Particularly Exo derived from CPC have higher tropism to CM when compared to DF-derived Exo. Thus, CPC-derived Exo can be thought as a potential tool for a specific delivery of functional molecules in the heart.

PW1.10

Cell-non-autonomous neurogenesis by convective exosomal transport of microRNA

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Various biological processes are regulated by cell-non-autonomous function, which is crucial for the change of physiological cellular behaviour. However, it is unclear whether exosome-mediated cell-non-autonomous fashion contributes to regulation of neurogenesis. Here, we found a new mode of cell-non-autonomous miRNA/exosome-mediated differentiation of neural progenitor cells and visualized exosomes carrying the neurogenic miRNA from leading to neighbouring cells. MiR-193a facilitated neuronal differentiation in Ngn1-induced F11 cells. In addition to 2D co-culture and transwell culture setting, time-lapse live-cell confocal imaging using microfluidic assay visualized the convective transport of exosomes from differentiated to undifferentiated F11 cells. Individual exosomes containing miR-193a from differentiated donor F11 cells reached the recipient cells and were taken up by undifferentiated F11 cells to lead them to neuronal differentiation. These findings indicate that

exosomes of neural progenitors and neurogenic miRNA within these exosomes propagate cell-non-autonomous differentiation to neighbouring progenitors, to delineate the roles of extracellular vesicles mediating neurogenesis of population of homologous progenitor cells.

PW1.11

The involvement of microvesicle miRNA in gestational vascular complications

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Please see OPW3.7

PW1.12

Endometrial extracellular vesicles are important for maternal-embryo communications

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Successful pregnancy can only be achieved when communications between the endometrium and the embryo occur synergistically during the receptive phase of the menstrual cycle. Endometrial extracellular vesicles (eEVs) released into the uterine microenvironment are proposed to play an essential role in this process. In this study, we investigated eEVs molecular content cargo and examined their functions. eEVs were isolated, purified and characterized from ECC1-derived serum-free conditioned media using differential ultracentrifugations, cryo-electron microscopy and western blot analyses and subjected to molecular profiling. We have shown that eEVs contained distinct subset of miRNAs compared to their parental cells and their protein cargo profiles are hormonally regulated. Mass spectrometry of highly purified eEVs found 258 and 131 proteins responsive to oestrogen and progesterone, respectively. Live cell imaging showed an accumulation of fluorescently labelled eEVs in HTR8 trophoblast cells and enhanced their adhesive response (24%, $p < 0.001$) by 1.5 h and maintained throughout the 4 h of real-time cell analysis recording using XCELLigence. SytoRNA-Green was used to selectively label the RNA content of eEVs and were localized to the mitochondria compartment of trophoblast cells, suggesting their role in energy production during implantation. This study demonstrated that molecular content cargo packaged within endometrial EVs are selective and are important modulator of maternal-embryo communications. This data set provides new evidence that eEVs could be used as biomarker of endometrial receptivity and improve pregnancy outcomes.

Poster Session 2 - EVs in tumour metastasis

Chairs: *Jameel Inal and Mercedes Tkach*

PW2.01

Identifying RNA and protein cargo that is responsible for EV-mediated transfer of malignant properties in vivo

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Introduction: Tumour cells release a wide variety of extracellular vesicles (EVs), including multivesicular body (MVB)-derived exosomes, limiting plasma membrane-derived microvesicles, and apoptotic bodies. EVs contain proteins, lipids and diverse RNA species that reflect the cell of origin and which can be functionally transferred to recipient cells. A growing number of studies show that uptake of EVs can change the gene expression and behaviour of recipient cells, thereby influencing tumour progression and metastasis. **Methods:** Here, to study functional EV exchange between less malignant and more malignant mouse melanoma cells in vivo, we use the Cre-LoxP reporter method (1, 2) to differentiate between cells that have and have not taken up EVs from a predefined cell population. **Results and conclusion:** Using intravital imaging, we show that less malignant tumour cells that have taken up EVs from more malignant tumour cells have a higher migration speed compared to less malignant tumour cells that have not taken up these malignant EVs. This shows that EVs can phenocopy malignant behaviour from more malignant to less malignant tumour cells. Current experiments focus on identifying the vesicular RNAs and proteins that are responsible for the transfer of this malignant phenotype.

References

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PW2.02

Developing high-throughput tools to evaluate exosome-induced motility in breast cancer cells

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Stimulating cellular motility is a key step in promoting metastasis, which causes cancer cells to migrate from the initial tumour to a distal site. One method of triggering cellular motility is through interactions with exosomes released from cancer-associated fibroblasts (CAFs) found in the tumour microenvironment. These exosomes interact with breast cancer cells, modifying the CAF-exosomes through the addition of Wnt11. This allows the CAF-exosomes to activate non-canonical Wnt signalling within the cancer cells, leading to increased cellular motility and metastasis in vivo. Further research has determined that the motility is achieved through planar cell polarity pathways. Although the phenotype is extensively characterized, the exact mechanisms involved are unknown. I am developing a high-throughput methodology, which will allow me to screen for essential proteomic components of this exosome-induced motility in the cancer cells. Utilizing MATLAB, I have developed a script that can track cells over an imaging series and determine their individual velocity. Expressing H2B-Clover in the cancer cell line, MDA-MB-231, the programme can detect individual nuclei and produce continuous

tracks between each captured image of nuclei. Optimization of the plating and imaging conditions produced a 2-fold dynamic range. Analysis shows the programme can successfully differentiate between conditions containing CAF-exosomes and controls when compared to manual tracking. Additionally, the script could detect differences in cellular motility when the MDA-MB-231 cells were treated with siRNA against key motility proteins, SMURF2, WNT11 and PK1, when compared to the knockdown of GAPDH. Currently, I am treating the motility-stimulated MDA-MB-231 cells against a kinase inhibitor library to further evaluate the script as well as develop my final screening methodology.

PW2.03

Autologous tumour-derived extracellular vesicles affect tumour cells

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Background: Tumour cells release extracellular vesicles (tumour-derived microvesicles or TMV) and this ability was shown to protect them from immunological surveillance; however, no reports are available on the effects that TMV may exert on the tumour cells themselves. **Methods:** TMV from the supernatants of three gastric cancer cell lines (GC1401, 1415 and 1436) were isolated by differential centrifugation. For size analyses, nanoparticle tracking analysis (NTA) was used. Impact of autologous TMV on tumour cells was assessed by PCR arrays and western blotting. **Results:** The mean size of GC1401, GC1415 and GC1436 TMV were 120.2, 186.3 and 101.4 nm, respectively. The mode size for the GC cell lines were as follows: 19.6, 40.2 and 15.2 nm. The size distribution range for GC1401 TMV was 13–237.2 nm, GC1415 TMV: 40–364.6 nm and GC1436 TMV: 10.2–169.2 nm. The following genes were overexpressed in the respective GC tumour cells after stimulation with autologous TMV: GC1401 – IL-18, SRC, HRAS, BRMS1, RORB and MMP13; GC1415 – TCF20, TIMP3, METAP2, MDM2 and TSHR; GC1436 – TIMP2, MET, CXCR2, CXCR4 and TGFB1. The following genes were underexpressed in the respective GC tumour cells after stimulation with autologous TMV: GC1401 – MET; GC1415 – VEGFA, TP53, CHD4, MMP2, CCL7 and TRAIL; GC1436 – NME1, CD44, PNN and NME4. **Conclusion:** The NTA analyses revealed that mean and the modal size values of TMV from all of the three GC cell lines were similar. The modal value, however, suggests that the predominant TMV population is composed of exosomes (as defined by size criterion). The PCR array analyses showed that autologous TMV stimulate tumour cells to produce mRNA transcripts of genes whose protein products may aid further tumour growth, angiogenesis and metastasis. These results suggest that besides the impact that the released TMV exert on the tumour microenvironment (i.e. extracellular matrix and infiltrating leukocytes), they also affect the tumour cells themselves in a manner that promotes a more aggressive tumour progression.

PW2.04

Selective packaging of certain microRNAs into tumour microvesicles impacts tumour cell as well as adjacent stroma via TGFBR1

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Microvesicles are small, secreted vesicles that have previously been reported to play a role in specific extracellular exclusion of several molecular factors, including microRNAs (miRNAs). Emerging data suggest that packaging of miRNAs into microvesicles is a selective process targeting specific miRNAs, rather than an indiscriminate process. It follows then that miRNAs selectively excluded or retained by cancer cells may play key roles in malignant processes. We have identified selective exosomal packaging of multiple miRNAs in a panel of oral dysplasia and oral squamous cell carcinoma cell lines. Inhibition of exosome export protein Rab27A increased intracellular expression of these miRNA candidates and prevented their packaging into microvesicles. Increased intracellular expression of miR-142-3p specifically was found to target TGFBR1, causing a decrease in its expression and a reduction of malignant features. Conversely, increased exosomal release of miR-142-3p from cancer cells was found to activate TGFBR1 and promote growth in endothelial cells, supporting a possible cell signalling role for this miRNA in promoting malignancy. In vivo analysis demonstrated that tumours overexpressing miR-142-3p – and subsequently releasing more miR-142-3p via microvesicles – exhibited decreased tumour growth. However, increases in angiogenesis caused tumours to be more vascular as well as less hypoxic and necrotic. Untangling the complex role of selective exome-mediated miRNA release in these model systems will enhance our understanding of oral cancer biology, potentially leading to identification of new druggable cell processes.

PW2.05

A model to study the role of extracellular vesicles in the interaction between ovarian tumour cells and the peritoneum

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Introduction: In advanced stages of epithelial ovarian cancer, small clusters of tumour cells spread through the abdominal cavity and adhere to the peritoneal surface. The underlying molecular mechanisms of this interaction are poorly understood. Since tumour-derived vesicles are abundantly present in the ascites, these vesicles may play a role in the adhesion of tumour cells to the peritoneum. A model to study the role of extracellular vesicles (EVs) in this interaction, however, does not exist. Therefore, we developed an in vitro model to image EVs and study their role in tumour cell interaction with the peritoneum. **Methods:** In the developed model, two fluid-containing compartments are separated by a specimen of abdominal peritoneum of ovarian cancer patients. The interaction between tumour cells and the peritoneum was studied using human ovarian cancer cells (SKOV-3). Cells were labelled with a fluorescent dye (PKH67) and added to the model. Adhesion of tumour cells was imaged by fluorescence microscopy, and trans-peritoneal passage of tumour cells was monitored by flow cytometry. **Results:** In our model, we were able to mount the peritoneum specimen onto gauze. Morphology and immunohistochemistry confirmed that the peritoneum specimen was still vital after 5 days. With fluorescent microscopy, tumour cell adhesion to the peritoneum tissue was shown. Trans-peritoneal passage of tumour cells could not be detected by flow cytometry. **Conclusions:** The developed system enables studies on the interaction between ovarian tumour cells and peritoneum. The next step will be high resolution microscopy of the interaction between tumour cells, EVs and the peritoneum. Additionally, concentration measurements of EVs in supernatant may confirm involvement of EVs in this interaction. In the future, this model can also be applied to other studies.

PW2.06

Exosomes enriched in stemness/metastatic-related mRNAs promote oncogenic potential in breast cancer: specific prognostic exosome signature

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Introduction: Tumour-derived exosomes are a significant mechanism of intercellular communication, affecting tumour-related pathways, such as angiogenesis, drug resistance and immune response. This study analysed the release of exosomes from breast tumour cells with different capacities of stemness/metastasis based on CXCR4 expression and evaluated their involvement in the capacity to generate oncogenic features in recipient cells. **Methods:** Oncogenic effects of breast cancer cells-derived exosomes were evaluated in in vitro and in vivo models. Exosome cargo in mRNAs involved in stem cell differentiation and metastasis were analysed by specific quantitative PCR arrays. Finally, stemness- and metastatic-related mRNA most differentially detected in exosomes were analysed in plasma of breast cancer patients assessing its capacity as a prognosis marker. **Results:** In vitro, exosomes released from CXCR4-tumour cells modify stemness markers and proliferation, migration and invasion capacities of neighbouring cells. Accordingly, inoculation of CXCR4-cells-derived exosomes in immunocompromised mice mediated primary tumour growth and metastatic potential. Exosomes isolated from CXCR4 cells and stem-like cells were highly enriched in stemness- and metastatic-related mRNAs and showed high homology between them. Furthermore, comparative analysis of mRNAs contained in exosomes isolated from patients revealed a gene signature of mRNAs highly enriched in exosomes of patients with worse prognosis. **Summary/conclusion:** Our data support the view that exosomes released by CXCR4-cancer cells that exhibit stem-like properties are capable of transferring these oncogenic features to recipient cells. In addition, our results show the involvement of “stemness and metastatic signature” mRNA in exosomes from plasma in breast cancer patient survival.

PW2.07

Malignant extracellular vesicles derived from ovarian cancer cells promote peritoneal metastasis by the breakdown of peritoneum

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Introduction: Ovarian cancer is the most lethal gynecological malignancy, and the lethality is mainly attributable to the frequency of peritoneal metastasis. However, the molecular mechanisms of dissemination and implantation in the peritoneal cavity remain unclear. **Methods:** The mice model of peritoneal metastasis was orthotopically established by injecting the A2780 ovarian cancer cell line. To assess the metastatic effect of extracellular vesicles (EVs) derived from ES2 high-metastatic ovarian cancer cell line, the EVs was injected intraperitoneally. To investigate the effect of EVs from cancer cells to mesothelial cells, which are main components of peritoneal membrane, 2 types of mesothelial cells (Met5A cells and HMPC cells) were used. **Results:** It was clearly demonstrated that EVs derived from ES2 cells promote peritoneal metastasis in vivo. On the other hand, there was no significant change in primary tumours. Next, it was found that the mesothelial cells, which were treated with the EVs from ES2 cells, were broken in vitro and in vivo because the EVs induced apoptosis. From microarray gene expression analysis in mesothelial cells, which were treated with various types EVs, a lot of genes significantly changed. The gene, which is known as the metastasis-associated gene, was validated by RT-qPCR at high reproducibility. Interestingly, the gene mRNA also packaged in the EVs from ES2 cells. The gene is confirmed as a prognostic factor for ovarian cancer patients by referring to open access database. In addition, the EVs that contain

the gene mRNA also exist in the EVs derived from ovarian cancer patients' ascites, and furthermore, the EVs also induced apoptosis in vitro. *Summary/conclusion:* These results indicated the existence of the malignant EVs, which promote peritoneal metastasis by the breakdown of peritoneum and which contain the specific gene mRNA. Furthermore, this fact will lead to elucidation for novel mechanisms of peritoneal metastasis involved in EVs from cancer cells.

PW2.08

Live imaging of exosomal chemokine receptor release
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Introduction: Chemokine receptors play a key role in the trafficking of immune cells, but are also involved in the progression of a large number of human cancers. By activating oncogenic signalling pathways, these receptors can contribute to tumour growth, angiogenesis and metastasis. Recently, we observed the presence of chemokine receptors on membrane vesicles of chemokine receptor-expressing cells. However, the nature of these vesicles was not clear. *Methods:* We developed pH-sensitive fluorescent chemokine receptor mutants that enable live-cell imaging of the fusion of receptor-containing endosomes with the plasma membrane. Moreover, we used stimulated emission depletion (STED) super resolution microscopy to visualize the membrane vesicles containing the chemokine receptor. *Results:* In this study, we showed the colocalization of intracellular chemokine receptors with the late endosomal/multivesicular body marker CD63. Furthermore, we observed the fusion of acidic endosomal compartments containing pH-sensitive receptor mutants with the plasma membrane. Moreover, super resolution microscopy revealed that the receptor mutants were released on membrane vesicles with a size corresponding to what has previously been reported for exosomes. *Summary/conclusion:* In this study, we provide evidence that chemokine receptors are released via exosomes that are generated in acidic endosomal compartments, presumably multivesicular bodies. The pH-sensitive fluorescent receptor mutants can be used in future studies to elucidate the mechanism behind the exosomal release of chemokine receptors.

PW2.09

Towards exosome-based cancer therapy: rejuvenation of exosomes derived from normal bone marrow stromal cells

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Introduction: Multiple myeloma (MM) is a refractory hematologic malignancy. Because MM is a disease of the elderly, stem cell transplantation is rarely used to treat it. Novel therapies for MM need to be developed. The bone marrow stromal cell (BMSC) is one of the most promising sources of cell therapy. However, the biological properties of BMSCs, such as ageing, are largely unknown. Recently, exosomes derived from normal BMSCs have been found to be efficacious for treatment of graft versus host disease. Therefore, we investigated the biological properties of BMSC-derived exosomes to establish exosome-based therapy by engineering the incorporation of therapeutic miRNA. *Methods:* Human BMSCs obtained from bone marrow of young healthy donors (yBMSCs; age of donors: 19–21 years) and elderly healthy donors (eBMSCs; age of donors: 68–72 years) were purchased from Lonza. The exosomes were isolated from conditioned medium of BMSCs using Exoquick-TC (SBI). Exosomal miRNA profiling was performed using a TaqMan miRNA array (ABI). The candidate miRNA mimics were directly introduced into exosomes by Exo-fect (SBI). Nude mice were subcutaneously injected with 400 µL Matrigel containing a hypoxia-resistant myeloma cell line (HR-MM cells) established in our laboratory with BMSC-derived exosomes. *Results:* Nanoparticle-tracking assays revealed that

there were no significant differences in the size and amount of exosomes among yBMSCs and eBMSCs. The yBMSC exosomes significantly reduced the endothelial cells in Matrigel. We compared exosomal miRNA profiles of yBMSCs and eBMSCs. Four miRNAs were obtained as specific miRNAs of yBMSC exosomes. The engineered exosomes transfected with two miRNA mimics significantly inhibited HR-MM-induced angiogenesis in Matrigel plug. *Conclusion:* Our results suggest that BMSC exosomes are able to transfer miRNAs, which have the ability to inhibit angiogenesis and progression of MM. The present study explores the therapeutic potential of exosome-derived BMSCs in MM.

PW2.10

MicroRNA-containing exosomes derived from human gallbladder cancer cell inhibit HUVEC tube formation

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Introduction: Exosomes are small vesicles (40–100 nm in diameter) secreted by most of cells. They contain a variety of molecules including microRNAs (miRNAs) and are suggested to participate in intercellular communication. Recently, tumour-derived exosomal miRNAs were shown to be involved in tumour microenvironment, tumour angiogenesis, metastasis and tumour immunity. We isolated human gallbladder cancer cell-derived exosomes and characterized their function. *Methods:* Exosomes are isolated from culture media of human gallbladder cancer cell lines NOZ and G-415 by means of ultracentrifugation. Extracted RNAs from the exosome fraction were applied to miRNA array analysis. We further examined whether the tumour-derived exosomes containing miRNAs affect angiogenesis by employing human umbilical vein endothelial cell (HUVEC) culture systems. *Results and summary:* miRNA array analysis revealed enrichment of miR125b, miR-320, miR-494 and miR-638 in exosomes. These miRNAs are reported as an inhibitor of angiogenesis. Supplemented the tumour-derived exosomes into the medium, HUVEC declined its tube formation on Matrigel, but did not change its proliferation. The results suggest that gallbladder cancer cell-derived exosomes may inhibit tumour angiogenesis, through transmission of these miRNAs into endothelia.

PW2.11

Jagged1 extracellular vesicles influence endothelial cell behaviour by downregulating VEGFR2

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Notch signalling is an evolutionary conserved pathway that is essential in development. Jagged1 (Jag1), a Notch ligand, is over-expressed in several cancers. It promotes tumour cell survival, proliferation and metastasis. It also influences angiogenesis by controlling endothelial cell differentiation. Dll4, another Notch ligand, was previously shown to be packaged into extracellular vesicles, bypassing its canonical contact-dependent signalling, to influence Notch signalling. Together, these observations suggest the possibility that tumour-derived Jag1 could be packaged into extracellular vesicles, whereby it could influence cells in the tumour microenvironment. Here, we aim to study the effect of Jag1 extracellular vesicles on angiogenesis. In order to study the effect of Jag1 extracellular vesicles (Jag1-EVs) on endothelial cells, we purified Jag1-EVs from Jag1-overexpressing HEK293T cells. We found that Jag1-EVs are internalized by endothelial cells. Jag1-EVs induce a downregulation of VEGFR2 in endothelial cells, thereby inhibiting endothelial cell proliferation, migration and sprouting. Although these phenotypes are consistent with Notch activation, Jag1-EVs did not stimulate Notch signalling directly. This suggests that Jag1 might function differently

when packaged into EVs. Our findings show a novel paracrine function of Jag1-EVs on angiogenesis and prompts further studies into extracellular vesicle-mediated signalling.

PW2.12

Role of osteosarcoma-derived exosomes in tumour angiogenesis in vitro

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Introduction: Osteosarcoma (OS) is the most common primary malignant bone tumour in childhood and adolescence and is characterized by a high metastatic potential. Angiogenesis is a pivotal process during OS development, as it is involved in cancer cells growth, invasion and metastasis (1). Angiogenesis involves a number of different players among which exosomes have been recently proposed as efficient cargoes of pro-angiogenic mediators (2,3). In cancer other than sarcoma, acidity of tumour microenvironment, developed by the peculiar metabolism of tumour cells, has been associated with an increased exosome release and uptake (4). In this study, we investigated the role of OS-derived exosomes on tumour angiogenesis in vitro and the influence of cancer-induced extracellular acidic pH in this process. **Methods:** Exosomes were isolated by differential centrifugation of culture media from 143B OS cells grown at different pH (6.5 or 7.4). Successful isolation was confirmed by transmission electron microscopy. To test the effect of exosomes on angiogenesis, human umbilical vein endothelial cells (HUVEC) were stimulated with exosomes, and cell proliferation, migration and tubule-like structure formation were analysed. **Results:** Exosomes isolated by OS cells displayed the expected size range (30–100 nm). The release of exosomes by OS cells was significantly increased at acidic compared to neutral pH ($p=0.009$). HUVEC proliferation and migration was not significantly affected by the treatment with OS-derived exosomes. Exosomes derived from OS cells at pH 6.5 induced a higher tubulogenesis when compared to OS-derived exosomes at pH 7.4. **Conclusions:** Our findings suggest that OS-derived exosomes may be involved in tumour angiogenesis and that acidic tumour microenvironment may increase this process.

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PW2.13

Induction of tolerogenic dendritic cells by nasopharyngeal carcinoma-derived exosomes

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Please see OPW2.1

PW2.14

Breast cancer cell migration is induced by exosomes via surface interaction and through the activation of FAK signalling

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Introduction: Tumour metastasis accounts for the vast majority of cancer-related deaths. Metastatic dissemination of cancer is mediated by concordant activation of matrix degradation and cell movement. There are a number of different mechanisms by which exosomes have been shown to modulate pericellular proteolysis (Sevenich L. et al., 2014). Exosome-induced pathways regulating cancer cell movement are less studied. Internalization of exosomes was shown to be associated with cancer cell spreading (Koumangoye R et al., 2011). However surface interaction of exosomes and target cells may play an important role as well and may produce a rapid effect. We observed fast activation of breast cancer cell movement by exosomes and hypothesized that this effect is mediated by surface interaction. The study was aimed to evaluate molecular mechanism of this phenomenon. **Methods:** We used MDA-MB-231 breast cancer cell line. Exosomes were concentrated by ultracentrifugation and analysed by western blotting, NTA and RAMAN spectroscopy and treated with trypsin to alter a molecular composition of their surface. **Results:** Incubation of cells with concentrated exosomes induced their random movement (2D) and FBS-directed migration (3D) in vitro. Breast cancer cells with over-expressed focal adhesion kinase (FAK) were more sensitive to stimulation by exosomes, whereas cells with FAK stably knocked down were almost resistant to stimulatory effect. Treatment of exosomes with trypsin resulted in considerable decrease of their motility induction activity. Incubation of "nude" exosomes with fibronectin restored their activity. Further analysis revealed difference of hydrodynamic radius, surface composition and fibronectin content between the native and trypsin-treated ("nude") exosomes. **Summary:** Surface interaction of exosomes and target cells is enough to stimulate cell movement. Fibronectin attached on exosome surface may induce migration of cancer cell via activation of FAK pathway.

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PW2.15

Clinical and functional aspects of HLA-G-positive extracellular vesicles in breast cancer

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The non-classical human leukocyte antigen G (HLA-G) molecule and its soluble forms exert multiple immune suppressive functions in malignancy and in stem cells contributing to immune escape mechanisms. Soluble HLA-G (sHLA-G) can be released as free sHLA-G molecules or via extracellular vesicles (EVs). Extracellular vesicles are secreted vesicles representing important elements for intercellular signal transduction often being operative in tumour immune evasion. Our study on EVs harbouring sHLA-G (sHLA-G EVs) derived from blood plasma of locally advanced, neoadjuvant chemotherapy-treated breast cancer (BC) patients (N = 190) revealed (i) elevated sHLA-G EV levels in BC patients compared to healthy controls, (ii) an association between high sHLA-G EV levels and disease progression and (iii) the occurrence of stem cell-like circulating tumour cells. For this reason, we investigated the uptake of sHLA-G pos EVs by peripheral blood cells (PBLs), with respect to HLA-G-specific receptors ILT2 and ILT4 expression. HLA-G pos EVs were isolated from gynaecological, malignant ascites fluids by non-blocking HLA-G antibody after differential ultracentrifugation and PKH67 staining. The uptake was visualized using AMNIS Image Stream technology. HLA-G-bearing EVs were taken up by ILT2 and/or ILT4pos monocytes and B-cells in an accumulating fashion, whereas some ILT2pos and ILT2neg T cells seized HLA-G pos EVs up in a punctual manner.

Cytokine array analysis showed that HLA-G pos EVs contained a higher proportion of VEGF, uPAR, TRAIL receptors and others, which are mostly involved in tumour proliferation, metastases and angiogenesis than in contrast HLA-G neg EV, which displayed elevated levels of pro-inflammatory, chemotactically and cell adhe-

sion factors, such as Fas ligand, E-selectin, PECAM and various chemokines. In conclusion, sHLA-G EV are of prognostic relevance in BC, are taken up by ILT2pos and some ILT2neg PBLs and contain mediators known to promote tumour spread in gynaecological tumour entities.

Poster Session 3 - Analysis of EVs in body fluids

Chairs: Sergei Kruglik and Martijn van Herwijnen

PW3.02

An improved method to isolate milk-derived extracellular vesicles to study the impact of a null allele at the CSN1S1 locus on their secretion, size distribution and molecular content

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Mammary epithelial cells secrete huge quantities of proteins, lipids and other milk components such as minerals and lactose. The biosynthetic pathways responsible for the secretion of the main milk components are known in general outline but less is known about extracellular vesicles (EVs), recently identified in milk. Milk-derived extracellular vesicles (MEVs) contain proteins and RNA, which could be transferred to neighbouring cells and are recognized as mediators of intercellular communication. Goats homozygous for a null allele (naturally occurring KO) at the CSN1S1 locus encoding as1-casein display a chronic endoplasmic reticulum (ER) stress due to an accumulation of the other caseins in this compartment, thus triggering a general MEC dysfunction with a strong impact on milk composition. The objective of this study is to precisely assess the impact of as1-casein deficit on MEVs and to perform a comparative analysis of proteins, lipids and RNA content of MEVs from goats with extreme genotypes at the CSN1S1 locus. Since in our hands commercially available kits for the isolation of MEVs result in the co-precipitation of contaminants with EVs, we developed a method based on an improved conventional density gradient ultracentrifugation to purify MEVs free of contamination by other milk components, at sufficient concentrations to subsequently perform a full characterization. The quality of MEVs prepared according to the different implemented methods was analysed morphologically by transmission electron microscopy. The specific "exosome" protein markers (CD63, CD81, HSP70) were detected by western blot and the size distribution was measured by dynamic light scattering or tunable resistive pulse sensing. After validation, their proteome was acquired by liquid chromatography coupled to mass spectrometry (LC-MS/MS) approach and nucleic acid content (mRNA and miRNA) by next-generation sequencing methods. Several differences distinguish goats of extreme genotypes at the CSN1S1 locus.

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PW3.03

CD9-positive EVs isolated from size exclusion chromatography-precleared plasma from patients and healthy individuals reveal microRNA patterns

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Introduction: Size exclusion chromatography (SEC)-preclearing of plasma has been shown to reduce interfering components and thereby facilitate downstream characterization of extracellular vesi-

cles (EVs) (1,2). Our overall aim is to capture specific EVs in plasma from patients and healthy individuals, by combining SEC-preclearing and magnetic bead-based isolation, for profiling and comparing molecular patterns, here presenting an introductory microRNA (miR) study. **Methods:** Plasma (ethylenediaminetetraacetic acid or EDTA, 0.5 mL) from fasting colorectal cancer (CRC) patients and healthy individuals (n = 3) was precleared by SEC (Sephacrose CL-2B, GE Healthcare) in homemade 10 mL columns. Fractions (30, 0.5 mL) were eluted and joint fractions (8-10), harbouring the majority of EVs, concentrated 2:1 and used directly or for isolation of CD9-positive EVs (Dynabeads™, Thermo Fisher Scientific). RNA was isolated (miRNeasy Serum/Plasma, Qiagen) and selected miRs (miR-29a, miR-92a, miR-223) quantified by RT-qPCR (Vii7, TaqMan, Thermo Fisher Scientific). miR levels from CD9-positive EVs or directly from joint SEC-fractions were compared between patients and healthy individuals. Unfractionated plasmas represented the total amount of circulating miRs. Spike-in control for normalizing was ath-miR-159a (1 nM). **Results:** CD9-positive EVs, joint SEC fractions and unfractionated plasmas contained detectable levels of miR-29a, miR-92a and miR-223 for all samples. CRC patients showed higher amount of selected miRs compared to healthy individuals. miR-223 from CD9-positive EVs showed the highest level (14-fold) in patients compared to healthy individuals. Relative increase of miR-92a was 7-fold in CD9-positive EVs compared to unfractionated plasma levels and 3-fold compared to joint SEC fractions. **Conclusions:** This combined protocol of SEC-preclearing and bead-based capture of EVs allows for isolation of specific EVs and downstream quantification of miRs. Comparing miR levels derived from specific plasma EVs to unfractionated plasma may enable characterization of disease-specific sources of miRs.

PW3.04

Influence of Anticoagulation on Microvesicle Release in Stored Whole Blood

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Aim: We aimed to characterize microvesicles (MVs) released in whole blood with respect to their cellular origin and their interaction with blood cells. We further assessed the influence of storage and anticoagulation on vesicle generation. **Methods:** Freshly drawn whole blood was anticoagulated with ethylenediaminetetraacetic acid (EDTA), sodium citrate or heparin. Time-dependent release of MVs with and without gentle rolling was studied at 4°C, 21°C and 37°C. Flow cytometric analysis was performed using Gallios Flow Cytometer (Beckman Coulter) after calibration with fluorescent beads (Megamix PLUS FSC, Biocytex, France; 0.1, 0.3, 0.5 and 0.9 µm). MVs were identified as lactadherin-positive events in the MV gate. Antibodies against cell-specific markers for monocytes (CD45+CD14+), erythrocytes (CD235a+) and platelets (CD41+) were used to identify the cellular origin of MVs. **Results:** MV release was dependent on anticoagulation: EDTA contained the lowest, citrate a medium and heparin the highest concentration (3482 ± 404 vs. 6699 ± 651 MV/µL for EDTA; 2298 ± 383 vs. 23878 ± 4317 MV/µL for citrate; 6306 ± 2256 vs. 39646 ± 3350 MV/µL for heparin after rolling for 3 h at 37°C). In EDTA, blood erythrocyte-derived MVs were predominant (55%) and stayed constant during incubation. Platelet-derived MVs were mainly generated in citrate (69%) and heparin (66%) blood during storage. Heparin blood showed the fastest kinetics of MV generation. MV concentration increased with increasing temperature, and this increase was substantially higher for samples that were rolled during storage. MVs adhered to blood cells, in particular to monocytes. MV-monocyte aggregates increased significantly during storage, with 96% of all monocytes carrying MVs in heparin after 3 h. **Conclusion:**

MV generation in whole blood depends on the anticoagulation used. EDTA shows low sensitivity to preanalytical factors, while both rolling and temperature influence MV generation in citrate and heparin blood.

PW3.05

Enabling metabolomics-based biomarker discovery studies using molecular phenotyping of exosome like vesicles

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Biomarker detection and characterization from complex biological matrices, especially for markers that are low in abundance remains a challenge. Exosomes represent an information-rich matrix to discern novel disease mechanisms that are thought to contribute to pathologies such as dementia and cancer. Although proteomics and transcriptomic studies have been reported using exosomes-like vesicles (ELVs) from different sources, exosomal metabolome characterization and its modulation in health and disease remains to be elucidated. Here we describe methodologies for small molecule profiling of ELVs from human plasma and cell culture media. Our results demonstrate that ELVs represent an untapped source for metabolic and lipidomic biomarker discovery. Finally, we present an experimental pipeline that can be broadly applied for identification of low abundance biomarkers and characterization in clinical samples.

PW3.06

Advances in EV isolation from human plasma using the Vn96 synthetic peptide

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Introduction: Given the growing evidence that extracellular vesicles (EVs) may be a clinically relevant biomarker source, there is a great demand for simple and efficient EV isolation from biofluids, especially blood. The complexity of this biological material makes it a particularly challenging sample with which to work and many are still struggling to separate its constituents. We have improved our signature Vn96 synthetic peptide isolation method for optimal use in plasma. *Methods:* Plasma samples were collected from healthy volunteers and patients (consenting and ethics approved). EVs from 1 mL of human plasma were then isolated by using either the Vn96 synthetic peptide in the presence of the new PlasME buffer or the Vn96 peptide in the presence of phosphate-buffered saline (PBS). EV isolation was completed using a 1-h incubation time with Vn96. The presence of canonical EV markers was assessed by western blotting. Total RNA was extracted and yields were measured by NanoDrop and Agilent TapeStation. EV-DNA was also extracted and analysed for the KRAS mutation. *Results:* Western blot analyses revealed the presence of EV markers. The use of PlasME buffer in combination with the Vn96 peptide showed greater abundance of these EV markers compared to the PBS counterpart. From 1 mL of plasma, RNA yields for EVs isolated with Vn96 in the presence of PlasME buffer range from 20 to 25 ng when analysed with TapeStation, and as high as 140 to 240 ng when measured by NanoDrop. DNA was extracted from EVs and the KRAS mutation was detected by polymerase chain reaction from diseased samples. *Summary/conclusion:* Here we report a novel, alternative time-saving method of EV isolation that provides high RNA yields and DNA mutation detection. The Vn96 peptide in combination with PlasME buffer considerably improves the effectiveness of EV isolation and simplifies the isolation process in plasma. The efficient capture of EVs from blood with Vn96 provides a tremendous opportunity to develop clinically important liquid biopsy tools.

PW3.07

Getting insight of uterine fluid-derived extracellular vesicles

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Introduction: Uterine fluid content is worthy of investigation for both a diagnostic and a prognostic standpoint in terms of its potential role as a "liquid" biopsy. Uterine fluid contains a number of proteins differentially regulated between proliferative and secretory phases, between midsecretory samples from fertile and infertile women and between pre-receptive and receptive endometrium. Characterization of extracellular vesicles (EVs) from uterine fluid may be an indicator of endometrial status with the advantage of containing higher levels of locally secreted proteins. In our pilot study, an explorative analysis of molecular content of uterine fluid-derived EVs was performed. *Methods:* Uterine fluid samples have been collected from reproductive age patients undergoing diagnostic hysteroscopy. Uterine fluids (2–3 µL) were aspirated by an IVF transfer catheter and subjected to differential ultracentrifugation to isolate EVs. The nature of the EVs was demonstrated using transmission electron microscopy and western blot analysis. Total RNA from EVs was analysed by 2100 Bioanalyzer. Amplification of the mRNAs correlated with endometrial pathophysiology was done. Mitochondrial MT-7S D-loop region and RNase P gene were specifically amplified. *Results:* Our explorative analysis confirms that uterine fluid is extremely rich in EVs. EVs in uterine fluid contain large amounts of heterogeneous RNA species that could be used to study levels of expression of genes known in endometrial pathophysiology. The PCR amplifications indicate the presence of COX2 and WNT4 transcripts and of CYP19 transcript traces. gDNA and mtDNA were detectable in all the samples tested. *Conclusion:* The state of the art in this research area tends to support the possibility to isolate consistent amount of EV-derived biological material from few microliters of uterine fluid. This aspect together with the potential value of uterine fluid as predictor of endometrial normal functions and dysfunctions represents the focus of this study.

PW3.08

Characterization of residual urinary extracellular vesicles in ultracentrifugation supernatants

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Background: Urinary extracellular vesicles (UEVs) appear as ideal source of biomarkers for kidney and urogenital diseases. The majority of protocols designed for their isolation are based on differential centrifugation steps. Independent of the method, however, vesicles may still be present in the supernatant in considerable quantities. *Method:* Here we used an isolation protocol for UEVs, which uses hydrostatic filtration dialysis (HFD) as first pre-enrichment step, followed by differential centrifugation. Transmission electron microscopy (TEM), mass spectrometry (MS), western blot (WB) and tunable resistive pulse sensing (TRPS) were used to characterize UEVs left in the final ultracentrifugation supernatant waste. These residual EVs were subjected to RNA extraction, quantification, electrophoresis profiling and TEM. *Results:* TEM showed the presence of a variety of small size vesicles while protein identification by mass spectrometry

fully matched with the protein list available in the Vesiclepedia repository. Screening and relative quantification for specific vesicle markers showed that the waste still contained 51% of TSG101, 36% of ALIX, 97% of CD9, 38% of CD63 and 6.1% of CD81 positivity, respectively, of the signal recorded for the ultracentrifugation pellet ("catch"). TRPS quantification of the particles before and after ultracentrifugation showed that 26% of vesicles are left in the waste. Finally, extraction of the small RNA (snRNA) species from vesicles revealed that an equal amount of snRNA is present in both catch and waste with a closely similar electrophoresis profile. *Conclusion:* Here we show, for the first time, the full characterization of exosomes and other small size urinary vesicles that are normally discarded in the final supernatant of the differential centrifugation protocol. These results unequivocally call for re-evaluation of the efficiency and loss of potentially valuable material with the presently used isolation workflows.

PW3.09

Different extracting methods affected the quantity and quality of exosomes and exo-RNA from cell culture medium and serum

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Background: Exosomes are cell-derived vesicles and abundant in biological fluids, RNA in which could be served as potential diagnostic biomarkers in precision medicine. To promote clinical application of exoRNA, many isolation methods in existence should be compared to obtain exoRNA with high quality and concentration. *Methods:* Exosome in cell culture medium (CCM) or serum isolated by ultracentrifugation (Ultra), ExoQuick, Total Exosome Isolation Reagent (TEI) and hydrostatic filtration dialysis system (HFD) was compared by NanoSight and protein analysis. ExoRNA extracted by Trizol-LS, SeraMir, Total Exosome RNA Isolation (TER), HiPure Liquid RNA/miRNA Kit (HLR), miReasy and exoRNeasy were assessed by Bioanalyzer 2100, NanoDrop, Qubit, qPCR and high-throughput sequencing. *Results:* TEI showed the highest extraction efficiency and ExoQuick presented the most homogeneous size distribution. HFD revealed minimal loss and protein contaminants meanwhile. For CCM, combinations of ExoQuick and SeraMir, and for serum, ExoQuick and HLR and TEI and TER methods have more stable and higher extraction efficiency of exoRNA. Filtration, ultrafiltration, freezing and thawing may reduce the recovery of exosome. High yield and narrow size distribution pattern of small RNA presented in exoRNA isolated by Ultra and SeraMir, TEI and TER and ExoQuick and HLR from CCM, and exoRNeasy from serum. In RNA profile analysis, variation of RNA fragments size, small RNA constituent ratio, miRNA contents and amount can result from methodological variability. Profile of exoRNA isolated by kits (ExoQuick and TEI) methods was more similar. *Discussion:* Different isolation methods may account for the concentration, purity and size discrepancies of exosome or exoRNA, so the same isolation methods should be used in one research. Considering advantages and disadvantages of each method, the most appropriate one should be flexibility to choose according to research design.

PW3.10

Comparison and miRNA profiling of extracellular vesicles isolated from human urine with five different methods compatible with clinical settings

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Introduction: Urine sample analysis is irreplaceable as a non-invasive method for disease diagnosis and follow-up. However, handling of samples in clinical environment is troublesome, due to the requirement of ultracentrifuges and availability of space to storage samples of large volumes. In recent years, various methods of urinary extracellular vesicles (uEVs) enrichment using low volumes of urine and unsophisticated equipment have been developed, with variable success. *Methods:* We have characterized the cargo of uEVs obtained by 5 different methods of isolation. Protein markers were detected by western blot, and miRNA profiling was performed by a commercial service platform. The isolation methods compared include differential ultracentrifugation, lectin-based purification and three commercial kits based on EV agglutination. The study was approved by the ethical committee under the reference P-CBG-CBBA-1410. *Results:* The analysis of selected gene transcripts and protein markers of uEVs revealed that each method isolates a different mixture of uEV protein markers. In addition, the isolated uEVs contain miRNA within a panel of miRNA commercialized for liver damage studies, opening a door to miRNA profiling in urine as a source of biomarkers of miRNAs. *Summary/conclusion:* The isolation of uEVs is feasible from small volumes of urine and avoiding ultracentrifugation, making easier the analysis in a clinical facility. However, caution should be taken in the selection of the enrichment method since they have a differential affinity for protein uEVs markers. Interestingly, we also detect in urine miRNA belonging to a commercial panel of reference for liver diseases, suggesting that urine is indeed a source of biomarkers for liver diseases.

PW3.11

Detection of microRNA in plasma extracellular vesicles

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Introduction: Circulating extracellular vesicles (EVs) act as mediators of immune suppression and tumour progression in cancer. EVs contain miRNA, potent regulators of immune functions that are dysregulated in tumour cells. Plasma EVs offer non-invasive access to molecules conditioning tumour immunity that can be explored to gain prognostic information. We compared different protocols of isolation, RNA extraction and miRNA detection in plasma EVs from melanoma patients. *Methods:* EVs were isolated from 250/500 µL of plasma of melanoma patients and healthy donors (HD) by using ExoQuick or differential ultracentrifugation (UC). The isolated EVs were characterized for size by NanoSight and electronmicroscopy, and for exosome markers by western blot. RNA was extracted, quantified and tested for a set of miRNA selected in previous studies (miR146, miR155, miR125, miR100) by two different qPCR approaches using Taqman technology. Normalization of results by endogenous or exogenous spike-in RNA was considered. *Results:* EVs isolated by ExoQuick and UC were similar in size and markers. The RNA yield was not always measurable by fluorimetric analysis (1ng). miRNAs were detectable in EVs isolated by both methods. The Taqman technique including specific RT and preamplification showed a higher sensitivity compared to universal RT technique (Advanced Assays). For quantitative analysis, the exogenous synthetic RNA (ath-miR159a) was used to monitor the efficiency of RNA extraction and normalize the expression levels of tested miRNA. miRNA expression was then tested in EV samples from melanoma patients in comparison to HD (n = 40) by the set protocol. *Conclusions:* The EVs isolated from plasma of melanoma patients and HD showed different miRNA content: the number of samples resulting positive for the expression of each miRNA was higher in EVs of patients compared to HD, and a significantly higher expression level of miR146 was detected in patients' EVs.

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PW3.12

ExRNA virtual biorepository

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The exRNA virtual biorepository (EVB), developed by the Resource Sharing Working Group and the Data Management and Resource Repository (DMRR) of the exRNA Communication (ERC) Consortium, facilitates tracking and sharing of biofluids and their genetic preparations. The shared EVB is key to discovery and validation of biomarkers of diagnosis, prognosis, stratification and toxicity while providing normative and experimental controls for specificity assessments. The first “use case” for the EVB involves the evaluation of cerebrospinal fluid (CSF) for the diagnosis of primary tumours of brain (UCSD); subarachnoid hemorrhage (TGen); Alzheimer’s and Parkinson’s diseases (OHSU) and extracellular vesicle (EV) profiles of normal CSF. Each institution possesses a “node” within the system. EVB, built on the existing GenboreeKB framework, offers distributed and extensible infrastructure maintained at the DMRR at Baylor College of Medicine. The central repository hub is the “repo services broker” of web services for data query, annotation, sample request and tracking. The hub provides an EVB portal to browse and generate summaries of stored specimens. Convenient web interfaces simplify entry of specimen metadata. Genboree REST APIs provide extensibility and enable controlled data sharing across physically distributed nodes using Linked Data technologies. Clinical information, encoded using ontologies and controlled “pull-down” vocabularies, includes specimen source and handlers, diagnostic categories, specimen quantities, preparative standard operating procedures and EV quantitative and qualitative data. The EVB will devise specimen tracking request policies, voting rules and prioritization rules for sample requests. The EVB as a model for collaborative EV studies will be expanded to hepatobiliary diseases and oropharyngeal fluids as the next series of “use cases.”

PW3.13

A highly multiplexed real-time PCR demonstrated with quantitative profiling of miRNAs and DNAs from extracellular vesicles

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We have previously reported that quasi-liquid particles for real-time polymerase chain reaction (RT-PCR) enabled multiplex quantification of miRNAs in 10 µL of sample. Each particle contained specific primers for a miRNA and marked with a specific pattern. Since extracellular vesicles are very tricky to collect and analyse in traditional ways, multiplex detection of tens of genetic targets is critical to catch holistic view of the sample (1). The particle-based qPCR was improved in stability and reliability. Ten particles of individual primers were mixed in master mix and simply analysed according to the general work flow of RT-PCR. Single strike of the assay provided quantitative information of the ten target miRNAs. The reliability of the multiplex assay was validated through comparison with single-plex assays. The multiplicity of the assay is 20 target at a time in the present platform. Extracellular vesicles contain other interesting genetic targets such as DNAs and RNAs. PIN with carbon nano-tubes store a pair of specific

primers, resulting in very effective amplification of the target gene. Thus, as in miRNAs, DNAs were independently amplified in each particle and signified their quantity in parallel. To demonstrate the highly multiplexed assay, sequences of 6 anti-microbial resistant bacteria were targeted in each particle of PIN. Specificity of the assay with PIN particles is better than that of the conventional solution-phase qPCR, suppressing non-specific random dimers. Since plasma or urine often carries extracellular vesicles of pathogens containing their unique genetic fingerprints, extracellular vesicle (EV) is one of non-invasive samples for facile diagnosis. However, EVs from body fluids are challenging specimens for diagnosis due to their very limited volume. The present multiplex RT-PCR could analyse the EVs diagnosing bacterial infection in a rule-in manner, with only one action of qPCR.

Reference

1. Kim, S.K., ISEV2015, ABSSUB-281.

PW3.14

Urinary extracellular vesicles: RNA isolation shows gender-related yield profiles

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Urinary extracellular vesicles (UEVs) carry an abundance of proteins and nucleic acid species characteristic for their cells of origin along the kidney and urogenital tract. Thus, many lines of evidence highlight urine as an ideal biofluid to discover early biomarkers for kidney damage as well as for systemic diseases. Although urine is an easy to obtain biofluid, efficient enrichment of vesicles, sufficient RNA yields and quality issues remain unresolved. Accordingly, clear guidelines to reach optimized quality and quantity of RNA together with improved methods for urine collection itself and UEV isolation in particular are required. Recently, we have established a method for UEV enrichment based on hydrostatic filtration dialysis (HFD). This method concentrates and standardizes the physicochemical properties of samples and allows to process large volume of urine to provide sufficient amounts of urinary EV RNA. Using HFD for EV enrichment, we assessed the quality and quantity of RNA yield extracted with multiple approaches to identify the vesicle RNA profiles. The results showed that the yield and RNA quality are remarkably dependent on the competing extraction methods in the market. The individual RNA profiles from HFD isolated EVs were then obtained. Although enrichment of 40–80 nucleotide fractions remained similar for both genders, surprising differences were repeatedly revealed in the fraction of higher than 80 nucleotides. Notably, enrichment of this fraction occurs only in samples from females. Our results show that careful attention should be paid on selection of RNA isolation method but also on urine collection and the UEV enrichment procedure itself. All these aspects have considerable impact on quality of RNA yield. Use of HFD to UEV enrichment allowed us to establish the characteristic RNA profiles that demonstrate small but significant differences related to the gender of the donor.

PW3.15

Influence of menstrual cycle and retroviral infection on cervicovaginal extracellular vesicles in primates

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Introduction: Extracellular vesicles (EVs) have been found in every body fluid examined, but little is known about their abundance, cargo or function in cervicovaginal secretions (CVS). Previous work in our lab

demonstrated decreased particle concentrations in CVS of a rhesus macaque with endometriosis compared with secretions of reproductively normal rhesus and pigtailed macaques. For reliable interpretation of these data, more information is needed on the influence of factors, including the menstrual cycle (MC). In this study, we aimed to characterize changes in particle count and RNA expression changes in EVs during MC in simian immunodeficiency virus (SIV)-infected and uninfected rhesus macaques. *Methods:* Cervicovaginal lavage (CVL) and blood collection were performed weekly for 5 weeks. Nanoparticle tracking analysis (NTA) was performed on whole CVL and EV fractions enriched by stepped centrifugation. Plasma was analysed for levels of oestradiol and progesterone to correlate particle counts with phase of MC. Total RNA was obtained from fractions using an optimized method for biofluids RNA extraction. miRNAs were profiled

with a medium-throughput stem-loop/hydrolysis probe qPCR platform and confirmed by individual qPCR assays. *Results:* NTA showed an increase in EV concentration in the week following the progesterone peak in uninfected macaques. SIV-infected macaques did not show any significant changes in hormone levels across the 5-week period, suggesting MC irregularities. Similar findings for HIV have been sporadically reported in human, but are unreported in the nonhuman primate literature. *Conclusions:* Our preliminary results suggest that MC may affect EV concentration in CVS and should be taken into account in future studies. Additional studies of cervicovaginal EV and their RNA cargo are merited to characterize the potential role of EV and specific small RNAs as markers for any manifestation of reproductive tract disease.

Poster Session 4 – EVs as drug delivery system for antitumoral therapies and vaccination

Chairs: *Veronica Huber and David Carter*

PW4.01

siRNA delivery by exosome-mimetic nanovesicles – implications for targeting c-Myc in cancer

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Introduction: To develop RNA-based therapeutics, it is crucial to develop delivery vehicles that help the siRNA reach the cell cytoplasm. Naturally released vesicles, for example, exosomes, have been proposed as possible RNAi carriers, but the yield of exosomes is relatively small. We have previously generated exosome-mimetic nanovesicles (NVs) by serial extrusions of cells through nano-sized filters, which increases yield 100-times versus exosomes. Here, we hypothesize that NVs can function as efficient siRNA delivery systems. **Methods:** We here take two approaches to load siRNA into NVs. Firstly, we overexpress shRNA against human c-Myc to generate an NV carrying this siRNA. Secondly, we produced an NV from the cell and loaded these vesicles with a siRNA against green fluorescent protein (GFP) by electroporation. We examined whether these siRNA-loaded NVs can be taken up by recipient cells and whether a targeted gene knock-down can be achieved. **Results:** The siRNA was efficiently loaded into NVs both by endogenous expression of shRNA and by exogenous loading by electroporation. The c-Myc siRNA containing NVs were taken up by mouse lymphoma cells, downregulated their human c-Myc expression and finally induced cell death. In addition, siRNA against GFP were delivered to GFP expressing cells and reduced GFP expression. **Conclusions:** NVs have several advantages as delivery vehicles of RNAi molecules, including high yield during production compared to exosomes. This study provides two platforms for delivering siRNA by NVs, which possibly can be applied to different types of diseases.

PW4.02

Comparing the loading efficiency of exosomal protein chimeras

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Introduction: Due to the innate ability of extracellular vesicles (EVs) to deliver biological cargos and mediate changes in target cells, there is growing interest in developing bioengineered EVs as new therapeutic tools. However, one of the major obstacles in pursuing this interest is the lack of knowledge about mechanisms/factors that control the loading of proteins into EVs. Here, we use a green fluorescent protein (GFP) reporter system to screen the efficacy and specificity of different EV proteins for loading molecules into EVs. **Methods:** We selected a panel of both membrane and cytosolic proteins, which were highly ranked on our EV proteomics data set across 17 cell types. Each of these proteins was then fused with enhanced green fluorescent protein as a read-out. The chimeric proteins were overexpressed in HEK293T cells and EVs were purified

using ultracentrifugation, including a phosphate-buffered saline wash. Samples were characterized by nanoparticle tracking analysis (NTA), western blot (WB), fluorometry and electron microscopy. **Results:** We observed heterogeneous loading of different chimeric products: vesicular loading of proteins seems to be more efficient when coupling to membranous as opposed to luminal proteins. Any of the overexpressed proteins do not seem to significantly change the size of isolated EVs. Moreover, we observed that using NTA in fluorescence mode is less sensitive than WB for the analysis of EV protein loading. **Summary/conclusion:** In this study, we compare the loading efficiency of different EV chimeric proteins, using different methods. Although specific sorting mechanisms still need to be elucidated, we believe that our work will help developing new strategies for loading compounds into EVs, towards therapeutic applications.

PW4.03

Effect of exosome isolation methods on their yield, physicochemical and pharmacokinetic properties

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Introduction: Exosomes, which are expected to be used for therapeutics and drug delivery, can be collected by several methods. Isolation methods of exosomes may affect exosome yield and properties of exosomes such as physicochemical, pharmacokinetic and pharmaceutical properties. In this study, we compared the yield and these properties of exosomes collected by three typical methods. **Methods:** We collected exosomes from the culture medium of murine melanoma B16BL6 cells by simple ultracentrifugation (pelleting method), ultracentrifugation with an iodixanol cushion (cushion method) and ultracentrifugation with an iodixanol density gradient (gradient method). Exosome yield was evaluated by measuring the protein amount and particle number of the collected exosomes. Size distribution was analysed by transmission electron microscopy (TEM) and tunable resistive pulse sensing (TRPS). Clearance of intravenously injected exosomes from blood circulation was examined by using exosomes labelled with Gaussia luciferase. Recovery rate after sterile filtration using a 0.2-µm filter was evaluated by western blotting analysis of an exosome marker protein, Alix, and by measuring luciferase activity of labelled exosomes. **Results:** There was no significant difference in the exosome yield among the three methods. TEM and TRPS showed that the exosomes collected by the gradient method were the most dispersed. Irrespective of the collection methods, exosomes immediately disappeared from the blood circulation after intravenous injection into mice. Recovery rate after filtration was about 80% for the exosomes collected by the gradient method and about 40% for those collected by the other methods. **Summary:** The three ultracentrifugation-based methods collected almost same amount of exosomes. The exosomes collected by the gradient method were the most dispersed and suitable to sterile filtration, whereas no differences were observed in blood clearance after intravenous injection.

PW4.04

Extracellular vesicles as biological theranostic nano-vectors for tumour therapy: comparison of production and purification methods

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Introduction: Extracellular vesicles (EVs) are subcellular entities released by cells in a constitutive manner or in response to stress. We compared different production and loading methods for the design magnetic drug-loaded EVs as theranostic nano-vectors for tumour therapy. **Methods:** EVs were produced from human umbilical vein endothelial cells (HUVEC) previously loaded with a drug (mTHPC photosensitizer) and iron oxide nanoparticles. We compared 3 production methods: exosome preparation, serum deprivation protocol and a mechanical method (a new microfluidic set-up using shear stress in microchannels). We investigated the effect of the vesiculation method on the secreting cell, either on its morphology, cell state (living, necrotic, apoptotic), debris production or on its loading. Ultracentrifugation and magnetic sorting purification methods were investigated. We compared the samples using powerful image-based tools, as ImageStreamX flow cytometer and nanoparticle tracking analysis (NTA) to determine the number of vesicles, purity, size, nanoparticle and drug loading, and annexin V labelling. **Results:** Regarding the effect of the vesiculation method on parent cell, we demonstrated that the microfluidic method significantly affected the cell viability, morphology and debris formation compared to other methods. Although classical exosome preparation seemed apparently an efficient method according to NTA yield, the purity (p/μg) was very low probably due to serum protein contaminants. Microfluidic chip and serum deprivation enabled EV production at similar high yield and loading. Serum starvation enabled the production of the purest EVs, while microfluidics enabled EV production at high speed. Ultracentrifugation was more efficient for EV purification than magnetic sorting, but magnetically sorted EVs displayed higher magnetic load. EVs were able to transfer their cargo to cells and to induce phototoxicity. **Conclusion:** This study sets the basis for future investigations in the field of EVs as theranostic vectors.

PW4.05

Recombinant phosphatidylserine-binding proteins for targeting of extracellular vesicles to tumour cells: a plug-and-play approach

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Introduction: Extracellular vesicles (EVs) are increasingly being recognized as candidate drug delivery systems. However, manipulation of targeting properties of EVs can be challenging and time-consuming. As a novel approach to confer tumour targeting properties to isolated EVs, we generated recombinant fusion proteins of nanobodies against the epidermal growth factor receptor (EGFR) fused to phosphatidylserine (PS)-binding domains of lactadherin (C1C2). Here, we evaluated the effects of these proteins on EV tumour cell targeting. **Methods:** C1C2-nanobody fusion proteins were expressed in HEK293 cells and purified. Binding to PS and EGFR was tested in protein–lipid overlay assays and ELISAs. EVs isolated from red blood cells (RBCs) and Neuro2A cells were mixed with C1C2-nanobodies and analysed by western blotting, electron microscopy and nanoparticle tracking analysis. EGFR-dependent binding and uptake of C1C2-nanobody-decorated EVs by tumour cells were determined using flow cytometry. **Results:** C1C2-nanobody fusion proteins were obtained with near-complete purity as determined by SDS-PAGE. Proteins specifically bound PS and showed no affinity for other common EV membrane lipids. Furthermore, C1C2 fused to anti-EGFR nanobodies (EGa1-C1C2) bound EGFR with high affinity, as opposed to C1C2 fused to non-targeting control nanobodies (R2-C1C2). Both proteins readily self-associated with membranes of isolated RBC and Neuro2A EVs without affecting EV size and integrity. EV-bound R2-C1C2 did not influence EV-cell interactions, while EV-bound EGa1-C1C2 dose-dependently enhanced specific binding and uptake of EVs by EGFR-overexpressing

tumour cells. **Conclusions:** We developed a novel strategy to efficiently and universally confer tumour targeting properties to PS-exposing EVs after their isolation without affecting EV characteristics, circumventing the need to modify EV-secreting cells. This strategy may also be employed to decorate EVs with other moieties, including imaging probes or therapeutic proteins.

PW4.06

Suppression of breast cancer metastasis by targeting cancer-derived extracellular vesicles with antibodies

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Please see OPW3.6

PW4.07

Exosomal doxorubicin demonstrates improved tumour cell potency, but reduced cardiotoxicity compared to other doxorubicin formulations

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Introduction: The cytotoxic small molecule drug doxorubicin is frequently used in chemotherapy. As cardiotoxicity of the free drug has been reported, liposome-formulated doxorubicin with reduced cardiotoxicity is currently more often used in the clinic. Additionally, exosomes loaded with small molecule drugs, for example, doxorubicin, are promising new therapeutic agents as they are well tolerated by the immune system and can be targeted to specific cell types using surface motifs. However, the potency and cardiotoxicity of exosomal doxorubicin has not yet been determined and compared to the marketed pharmaceutical forms. **Methods:** Exosomes were loaded with doxorubicin (exo-Dox) by electroporation. After removal of the non-incorporated drug, doxorubicin loading was determined by the drug's fluorescence. Uptake of exo-Dox into HEK293 cells was analysed by FACS and microscopy and compared to free doxorubicin and liposomal formulations. Furthermore, toxicity of exo-Dox and other formulations of doxorubicin was tested in various cell lines and human cardiomyocytes (hiPS-CM or hESC-CM) in cell viability assays as well as assays testing the function of cardiomyocytes such as ATP depletion, calcium mobilization and beat rate. **Results:** We were able to demonstrate that exo-doxorubicin is taken up into HEK293 more efficiently compared to other doxorubicin formulations. Furthermore, Exo-Dox showed a higher toxicity than free doxorubicin and liposomal doxorubicin in all cell lines tested. Most importantly, cardiotoxicity was dramatically reduced compared to the pharmaceutical forms currently used in the clinic. **Summary:** We show that exosomal doxorubicin is efficiently taken up into cells, and exo-Dox is more potent compared to other doxorubicin forms, but shows reduced cardiotoxicity. These data provide compelling evidence that the delivery of therapeutics via exosomes could offer advantages over existing options.

PW4.09

Repurposing glioblastoma exosomes as personalized multi-antigenic antitumour vaccine

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Please see OPW3.5

PW4.10

Exosome-based cancer immunotherapy is independent of exosomal MHC I

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Peptide-loaded exosomes are promising cancer treatment vehicles; however, low T-cell responses in human clinical trials indicate a need to further understand exosome-induced immunity. We previously demonstrated that antigen-loaded exosomes carry whole protein antigens and require B cells for inducing antigen-specific T cells. Therefore, we investigated the importance of exosomal major histocompatibility complex (MHC) I for the induction of antigen-specific T-cell responses and tumour protection. We show that ovalbumin-loaded dendritic cell-derived exosomes from MHC I^{-/-} and allogeneic mice induce antigen-specific T cells to the same extent as syngeneic exosomes. Furthermore, lack of or mismatched MHC on exosomes induces tumour infiltrating T cells and increases overall survival similarly as syngeneic exosomes in a B16 melanoma. In conclusion, T-cell responses are independent of exosomal MHC/peptide complexes if whole antigen is present. This demonstrates the potential of using unpersonalized exosomes, which greatly increase the feasibility of exosome-based vaccines or therapeutic approaches.

PW4.11

Inhibition of in vitro and in vivo chronic myeloid leukaemia cell growth by IL3R-targeted exosomes

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Introduction: Chronic myeloid leukemia (CML) is a myeloproliferative disorder characterized by the expression of the constitutive tyrosine kinase BCR-ABL. The prognosis of CML patients has been improved by imatinib, a selective inhibitor of BCR-ABL. Despite this remarkable progress, acquired resistance may occur; thus, there is an urgent need to develop alternative strategies to overcome drug resistance. Exosomes have a specific cell tropism, according to the molecules expressed on their surface, which can be used to target them to specific cells. Moreover, exosomes can be loaded with a variety of molecules (drugs or siRNA), serving for the delivery of therapeutic agents. **Methods:** We use IL-3 receptor subunits, overexpressed on CML blasts compared with normal hematopoietic cells, as receptor target in our cancer drug delivery system. HEK293T cells have been engineered to express the exosome protein Lamp2b, fused to a fragment of IL-3. Transfected or un-transfected cells were treated with imatinib in order to load the drug in exosomes. Furthermore, we transfected cells with Bcr-Abl siRNA and we assayed the ability to load exosomes and to inhibit imatinib-resistant CML cells. **Results:** The therapeutic potential of exosome-mediated imatinib delivery

was demonstrated by the reduction of cell growth concomitant to the inhibition of BCR-ABL phosphorylation. We found that engineered exosomes, carrying Bcr-Abl siRNA were able to specifically target imatinib-sensitive and resistant CML cells. Furthermore, by using a CML xenograft model, we showed a significant decrease of tumour growth in mice treated with IL3-expressing exosomes loaded with imatinib or siRNA. We found that engineered exosomes exert their function by reaching tumour site. **Conclusion:** In conclusion, we demonstrated that IL3-engineered exosomes specifically target CML cells thus delivering imatinib and/or siRNA for Bcr-Abl. While focused on CML, results from this study could have an impact on other types of tumours.

PW4.12

Induction of potent antitumour immune response by development of CpG DNA-conjugated tumour cell-derived exosomes

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Introduction: Since tumour cell-derived exosomes contain tumour antigens, their application to cancer vaccine is expected. To induce potent antitumour immune response, we developed immunostimulatory CpG DNA-conjugated tumour cell-derived exosomes (CpG-exo) by using streptavidin (SAV)-biotin interaction. **Methods:** Exosomes were collected from mouse melanoma B16BL6 cells transfected with a plasmid encoding a fusion protein of SAV and lactadherin (LA). CpG-exo were obtained by mixing SAV-LA-modified exosomes (SAV-exo) with biotinylated CpG DNA. Conjugation was confirmed by fluorescent microscopic observation of CpG-exo prepared with fluorescein isothiocyanate-labelled CpG DNA and PKH26-labelled SAV-exo. Mouse dendritic DC2.4 cells were treated with CpG-exo, and the levels of TNF- α were determined by ELISA. Antigen presentation capacity was evaluated by measuring the levels of IL-2 released from BUSA14, a mouse T-cell hybridoma specific for melanoma antigen gp100, after co-culture with DC2.4 cells treated with CpG-exo. Mice were intradermally immunized 3 times with CpG-exo with 3-day intervals and subcutaneously inoculated with B16BL6 cells 7 days after the last immunization. Separately, mice with established B16BL6 tumour were intratumourally injected with CpG-exo 3 times with 3-day intervals. Tumour volume and survival of mice were evaluated. **Results:** Fluorescent microscopy showed colocalization of biotinylated CpG DNA and SAV-exo, indicating the successful preparation of CpG-exo. Addition of CpG-exo produced a large amount of TNF- α from DC2.4 cells and increased antigen presentation. In both the preventive and therapeutic models, CpG-exo efficiently retarded the B16BL6 tumour growth compared to co-administration of the unmodified B16BL6 exosomes and CpG DNA. **Summary:** CpG-exo were prepared by SAV-biotin interaction and showed high immunostimulatory activity as well as tumour antigen-specific immune response in vitro. Moreover, CpG-exo were the most effective in inhibiting tumour growth in tumour mouse models.

PW4.13

Recombinant exosomes carrying CD40L and herpesviral proteins redirect antiviral immune responses towards malignant B-CLL cells: an immunotherapeutic approach

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Introduction: Chronic lymphocytic leukemia (CLL) continues to be an incurable disease that is mainly characterized by accumulation of malignant B cells that possess a poor antigen-presenting ability.

However, this dysfunction can be restored by CD40 stimulation with its ligand, CD40L. Our works aim at the generation of recombinant exosomes in order to transfer functional CD40L together with immunodominant Epstein–Barr virus (EBV) and cytomegalovirus (CMV) proteins to B-CLL cells, redirecting the strong preexisting herpesviral immunity to these cells. *Methods*: Recombinant exosomes were generated in HEK293 cells overexpressing the proteins of interest and isolated from conditioned media by serial centrifugation and density gradient fractionation. Purified exosomes were characterized by western blot, electron microscopy and nanoparticle tracking analysis. Interaction of exosomes and B-CLL cells was visualized by flow cytometry and fluorescent microscopy. The immunostimulating effects of the exosomes were studied in T-cell assays using virus-specific T-cell clones as well as autologous peripheral blood T cells derived from CLL patients. *Results*: Our experiments showed that CD40L, the EBV protein gp350 and the CMV protein pp65 are efficiently packed into HEK293-derived exosomes and remain functional. Gp350 conferred a B-cell tropism, while exosomal CD40L increased the antigen-presenting capacity by inducing the expression of costimulatory surface molecules not only in directly bound B-CLL cells but also in bystander cells. B-CLL cells treated with CD40L+/gp350+/pp65+ exosomes induced increased IFN γ secretion in virus-specific T-cell clones and subsequently became targets for autologous cytolytic T cells. *Summary/conclusion*: We have shown that recombinant exosomes are efficient tools for the transfer of functional proteins to specific target cells and can trigger virus- and tumour-specific immune responses. This might facilitate new strategies for treatment of B-cell malignancies, such as CLL.

PW4.14

Meningococcal outer membrane vesicles as vaccine platform candidate

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Outer membrane vesicles (OMVs) have great potential as vaccine candidates. OMVs have been successfully used to elicit immune responses against the bacteria from which they are derived. However, OMVs are a complex product especially when compared to conventional subunit vaccines making the development of robust production processes demanding. By expressing heterologous antigens on the OMVs, a vesicle is created, which acts as a carrier platform that retains its intrinsic adjuvant properties. The expression of one or more proteins in this carrier platform is not expected to actively change the production process. We used a *Neisseria meningitidis* strain (MenB) expressing the borrelial outer surface protein (OspA). OspA has been bioengineered to the outside of the outer membrane by combining the protein to a meningococcal surface-exposed lipoprotein. We have tested the production of platform OMVs in our production process for neisserial OMVs. Production of both spontaneous and detergent-free extracted OspA OMVs showed to be highly similar to neisserial vesicle production. These preliminary results show a glimpse of the potential of using heterologous expressed lipoproteins on OMVs in the development of a vaccine platform.

PW4.15

Role of degS and nlpI in outer membrane vesicle production and cell-to-cell interactions in *Actinobacillus pleuropneumoniae*

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Introduction: *Actinobacillus pleuropneumoniae* (App) is a Gram-negative bacterium, which infects pigs as its sole host and reservoir. The most widely used vaccines (bacterins) confer protection against one or a limited number of App serotypes and do not prevent colonization. Thus, it is highly desirable to develop an alternative vaccine that confers broad protection against App in a serotype-independent manner. To achieve this goal, our approach is focused on the production, analysis and utilization of App outer membrane vesicles (OMVs) as vehicles to deliver a selection of conserved antigens to the host immune system. *Methods*: Deletion of degS and/or nlpI has been demonstrated to increase OMV secretion in numerous bacteria. Both genes were knocked out (separately) in App serovar 8 (MIDG2331) and OMV secretion levels were compared to that of the wild type (WT) by transmission electron microscopy (TEM) and oCelloscope analysis. Antigenicity of OMVs was determined by immunological assays using convalescent porcine serum. *Results*: TEM analysis showed that both degS and nlpI mutants secrete an increased amount of OMVs compared to the WT parental strain. The nlpI mutant produced OMVs much more variable in terms of morphology and size than the WT, thus confirming the role exerted by the nlpI gene in outer-membrane biogenesis and integrity. Furthermore, unlike the WT, degS and nlpI mutants did not aggregate during growth, suggesting a role of degS and nlpI genes in App cell-to-cell interactions that could possibly affect resistance or colonization, that is, mechanisms typically associated with aggregation. Finally, all the OMVs showed almost identical antigenic profiles when probed with immune serum, suggesting conservation of antigens in the OMVs of both mutants. *Conclusion*: In conclusion, these data support the use of App OMVs as a reliable scaffold to carry and present App antigens to the porcine immune system.

PW4.16

Novel therapeutic inhibitor cocktail suppresses extracellular vesicle-mediated inflammation induced by micro-RNA in a humanized mouse model of lupus

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Please see OPW2.7

Poster Session 5 – EVs in acute and chronic inflammation

Chairs: *Vicky Yang and Paola de Candia*

PW5.01

Redox state of exofacial molecules on leukocyte-derived EVs in inflammation

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Introduction: Exofacial thiols and redox molecules are abundantly present on all cells and they participate in inflammation, cellular activation and extracellular vesicle (EV) biogenesis. Until now, exofacial thiols and redox active surface molecules have not been investigated in EV biology. In this work, we analysed both cellular and vesicular exofacial thiol levels and a redox molecule, peroxiredoxin 1 (Prdx1), in healthy and inflammatory conditions. **Methods:** Peripheral blood samples were taken from rheumatoid arthritis (RA) patients (n=43) and healthy subjects (n=42) in ACD-A and EDTA tubes for studies on EVs and peripheral blood mononuclear cells (PBMCs), respectively. Platelet-free plasma samples were snap-frozen and stored at -80°C until analysis. A new flow cytometric (FCM) assay was developed to measure exofacial thiols on CD81+, CD9+, annexin V+ and CD41+ EVs. Exofacial thiols were also measured on PBMCs using FCM. EV-, blood plasma- and cell-associated Prdx1 were investigated by FCM and western blotting. After in vitro inducement of oxidative stress in human monocytes, using lipopolysaccharide and tumour necrosis factor (confirmed by dihydrocalcein staining), Prdx1 and surface thiols were measured on cells and EVs. **Results:** The level of exofacial thiols was elevated on monocytes of RA patients ($p < 0.05$) as compared to controls, while the surface thiols on plasma EVs did not differ. In vitro activated monocytes had an increase in surface thiol levels ($p < 0.05$). In contrast, the released EVs had decreased levels of surface thiols ($p < 0.05$). We demonstrated the presence of exofacial Prdx1 on cell lines and primary monocytes by FCM and western blotting. We detected Prdx1 on the surface of plasma EVs. Prdx1+ EVs were present in an elevated number in RA patients' plasma compared to healthy subjects ($p < 0.05$). **Conclusion:** We propose that the EV-mediated release of membrane segments with reduced exofacial thiol levels and the increase in the number of Prdx1+ EVs may serve as the cells' antioxidant defence system.

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PW5.02

Mesenchymal stem cell-derived microvesicles exposed to normoxic and hypoxic culture conditions are involved in macrophage polarization

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Mesenchymal stem cells (MSCs) are considered effective therapeutic agents inducing the functional improvement in the repair of injured tissues, not only through direct engraftment and differentiation, but mainly through their paracrine activity. Increasing evidences show that besides the secretion of trophic factors, the release of extra-

cellular microvesicles (MV) constitutes an alternative paracrine mechanism adopted by MSCs. In this study, we aim to carry out a detailed characterization of MVs released by human adipose tissue-derived MSCs, to study their possible involvement in macrophage polarization from a pro-inflammatory (M1) to a pro-resolving (M2) phenotype. The MV-isolation method is based on repeated ultracentrifugation steps of the MSC-conditioned medium exposed to both normoxic and hypoxic conditions (MV-normo and MV-hypo, respectively), followed by characterization of the resulting pellet enriched in MV through electron microscopy, flow cytometry and western blot for the expression of the proteins typically enriched in MVs. Size- and Trucount beads were used in the flow cytometric analysis to define the proper size and number of isolated MVs. In addition, culturing bone marrow-derived macrophages with MV-normo and MV-hypo previously stained with the fluorescent lipophilic dye PKH67 resulted in the uptake of MVs by macrophages. The flow cytometric analysis showed that when macrophages were cultured in standard medium, they did not express the typical M2 markers, such the mannose receptor CD206. Interestingly, the percentage of CD206-expressing macrophages significantly increased after the cell treatment with MVs cultured in both normoxic and hypoxic conditions, and this increase was even greatest in hypoxic conditions. We can conclude that the presence of MSCs-derived MVs induced and enhanced macrophage polarization towards an M2 pro-resolving profile.

PW5.03

Distinguishing PMN-derived activated EVs from apoptotic vesicles

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Introduction: Neutrophils (polymorphonuclear neutrophils or PMN) produce extracellular vesicles (EVs) in resting and also in many, differentially activated states. In some cases, the amount and biological properties of PMN-derived EVs show antibacterial activity. This capacity is reduced if the activation is partial. In order to assess the importance of cell activation in the production of antibacterial EVs, in this study we characterize the EVs produced during spontaneous death of the neutrophils and test their antibacterial effect. **Methods:** PMNs were prepared from the blood of healthy volunteers and were held in Dulbecco's modified eagle's medium in cell culture incubator for 3 days. EVs were separated by two steps, centrifugation and filtration. The separated EVs were counted and analysed with EV-optimized flow cytometry. The characteristics of EVs were analysed with dynamic light scattering, fluorescent- and electron microscopy, and with lipid and protein quantity assay. The antibacterial effect of the EVs was tested in bacterium survival assay. **Results:** During the 3 days incubation of PMN, the number of produced EV was increased parallel with PMN count decrease. The flow cytometric appearance, the annexin V and CD11b positivity, and the lipid/protein ratio of apoptotic EV were similar to antibacterial EVs, whereas the protein content and electron microscopic appearance differed significantly. Unlike EVs produced after PMN activation, the apoptotic EVs were ineffective in bacterium survival assay. **Conclusion:** The apoptotic EVs of neutrophils have a right side out vesicular nature, which is similar in size and appearance to the antibacterial EVs, but apoptotic vesicles do not have antibacterial effect. These results highlight the difference between distinct vesicle populations and support the specific formation of antibacterial EVs.

PW5.04

Investigating the apoptotic cell-derived extracellular vesicles with ICAM3 on apoptotic cell clearance

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Introduction: Apoptotic cell clearance is a vital mechanism that prevents dying cells from undergoing necrosis, which may lead to inflammatory and autoimmune disorders. Apoptotic cell-derived extracellular vesicles (ACdEV) are shed during apoptosis by loss of plasma membrane, aiding apoptotic cell removal by acting as “find me” signals to attract phagocytes. Apoptotic cell binding by phagocytes is achieved by various ligands on the apoptotic cell and counter receptors on the phagocytes, which together form a phagocytic synapse. One interesting apoptotic cell-associated ligand is ICAM-3, a highly glycosylated member of the immunoglobulin super family IgSF that is expressed on human leukocytes. On viable cells, it participates in initiating immune responses, while on apoptotic cells, it participates in the clearance of the apoptotic cells by attracting phagocytes and mediating tethering and binding of AC to phagocytes. However, the precise mechanism of ICAM-3 action is unknown. We aim to characterize the role of ICAM-3 and EVs in the clearance of apoptotic cells and attempt to identify the mechanisms that underlie their function in apoptotic cell clearance. **Methods:** Human B cells were induced to apoptosis by UV, and EVs were isolated by two-stage centrifugations. The ability of ICAM3 on ACdEV to promote phagocyte recruitment between AC and macrophages (MØ) was assessed by horizontal and vertical chemotaxis assays and by an in vivo mouse tumour model. The anti-inflammatory effects of ACdEV on lipopolysaccharide-induced inflammation was assessed. **Results:** Our results show that ICAM3 on EVs has chemoattractive potential for phagocytes and suggest ACdEV may exert anti-inflammatory effects. Our in vivo assays demonstrate that the anti-ICAM3 mAb MA4 prevent MØ migration towards the AC. **Summary:** ICAM3 on ACdEV promotes migration of MØ both in vitro and in vivo. Additionally, ACdEV may exert additional anti-inflammatory effects.

PW5.05

DNA on the extracellular vesicle surface

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Introduction: Cell-free DNA in body fluids has been suggested to have biomarker potential and can harbour disease-specific genetic mutations including oncogenes. However, cell-free extracellular DNA and its relative location with respect to the extracellular vesicle (EV) membrane is unclear. **Methods:** EVs from mast cells (HMC1.2) were isolated by differential ultracentrifugation. Isolated EVs were subjected to DNase-I treatment prior to being floated on an optiprep gradient. The zeta potential and particle number of the floated EVs were determined using nanoparticle tracking technology (Zeta viewer). Functional analysis of uptake was performed on human mesenchymal stem cells (hMSC). **Results:** DNase treatment of non-lysed EVs reduces the concentration of nucleic acids in EV isolates. DNase treatment also increases the zeta-potential and particle numbers. Additionally, cytoplasmic DNA traces (DAPI) were found in recipient hMSC after EVs treatment. **Conclusion:** We here show that EV-associated DNA at least partly can be present on the surface of EVs, which may contribute to EV aggregation. We hypothesize that EV surface DNA may assist their uptake.

PW5.06

Exosomes mediate crosstalk between the cell populations that contribute to DMD progression

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Introduction: Muscle regeneration is dependent by a complex interplay of different cell types in the muscle stem cell niche. In particular, a population of interstitial fibro-adipogenic progenitors (FAPs) and muscle stem cells (MuSCs) establish a complex network of interactions to coordinate muscle regeneration in mdx mice – mouse model of Duchenne muscular dystrophy (DMD). Recent findings demonstrated that treatment of mdx with histone deacetylase inhibitors (HDACi) increases the ability of FAPs to support MuSCs-mediated muscle regeneration at early stages of disease. We are investigating the role of exosomes released by FAPs as mediators of this functional interaction. **Methods:** FAPs are isolated from untreated (control, CTR) or trichostatin A (TSA)-treated mdx mice by FACS as Sca1-positive cells. Exosomes are isolated from muscles and from FAPs using a specific exosome isolation kit. Exosomes biogenesis was blocked using GW4869 exo-budding inhibitor. Taqman miRNA microarray was used to analyse miRNA contents in exosomes released by FAPs CTR and TSA. **Results:** FAPs released exosomes improve MuSCs differentiation. This effect is further increased by the treatment with TSA. Inhibition of exosomes biogenesis in FAPs strongly reduces their ability to support MuSCs differentiation. Transplantation of exosomes from FAPs in injured muscles increases muscle regeneration and recovery. Analysis of exosome content by miRNA-microarray shows a huge increase of myogenic miRs (myomiRs) in exosomes of FAPs exposed to TSA. **Conclusions:** Our results suggest that exosomes mediate the ability of FAPs in supporting MuSCs differentiation. HDACi increase this activity enhancing the myomiR content in exosomes. All these findings suggest that exosomes could act as therapeutic agents promoting muscle regeneration in injured or dystrophic muscles.

PW5.07

Central role of the beta2 integrin Mac1 (CD18) in activated EV-generation in murine neutrophilic granulocytes

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Introduction: In earlier studies, we demonstrated that human neutrophilic granulocytes (polymorphonuclear neutrophils, or PMN) were able to produce extracellular vesicles (EVs) that impaired bacterial growth. Production of antibacterial EVs occurred only upon stimulation of PMN with opsonized particles. In the present investigation, we applied genetically modified animals to identify the receptors that initiate the generation of activated EVs and to decide whether phagocytosis is required for generation of activated EVs. **Methods:** PMN were isolated from the bone marrow of wild-type mice or from mice deficient in major surface integrins (CD18, CD11a, CD11b), or Fc receptor signalling (FcR-γ chain, DAP12) or all the Src kinases (Hck, Fgr, Lin triple KO). Stimulation of PMN was carried out with opsonized yeast. Extracellular vesicles were separated by two-step centrifugation (2,000 × g followed by 33,000 × g). The separated vesicles were counted and analysed with EV optimized flow cytometry. Phagocytosis of opsonized bacteria was determined by flow cytometry. **Results:** Generation of activated EVs was seriously impaired in cells lacking either chain (CD18 or CD11b) of the β2 integrin Mac1, but it was not significantly influenced in the absence of CD11a (LFA), FcR signalling or Src kinases. Both in wild type and in most mutant animals PMN phagocytosis changed in parallel to EV generation, but in Src triple KO animals we observed a clear divergence: EV generation did not require Src kinase activity, whereas phagocytosis did. **Conclusion:** The β2 integrin Mac1 plays a central role in the generation of activated EVs in murine neutrophils, whereas the role of Fc receptors seems to be

moderate. However, phagocytosis is not a prerequisite of activated EV formation.

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PW5.08

Immuno-modulatory role of exosomes in tuberculosis

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Introduction: Tuberculosis (TB) ranks as a leading cause of death worldwide (WHO, 2015). Immuno-pathology of TB is characterized with increased T-regulatory cells and suppressed immunity. But the role of exosomes in modulating the function of Tregs in TB remains to be studied. Loss of T-cell polyfunctionality to oligofunctionality has been observed among TB patients. Here, we studied the role of exosomes in the immune regulation of poly-functional T cells. **Materials and methods:** Exosomes were purified by differential centrifugation followed by ultracentrifugation at 110,000 × g. The pelleted exosomes were washed with phosphate-buffered saline and stored. Further, exosomes were analysed by NanoSight, stained for surface markers and acquired in FACS Calibur, and the data were analysed using FloJo software. The impact of exosomes isolated from plasma of TB patients was studied in *in vitro* peripheral blood mononuclear cell culture. The change in effector T-cell function was monitored by flow cytometry. **Results:** We had isolated exosomes from plasma of TB patients and identified their cellular source by cell-specific surface markers. Exosomes derived from T cells showed CD3+CD4/CD8; monocyte- and B-cell-derived exosomes contained CD14 and CD19, respectively. Monocyte-derived exosomes from TB patients were significantly increased, whereas T-cell- and B-cell-derived exosomes were similar as compared to healthy controls. Next, the effect of TB-patient-derived exosomes was studied in terms of effector functions of T cells. Our preliminary results suggest an increase in polyfunctional T cells (IFN γ + TNF α +). **Conclusion:** Our preliminary study indicates that monocyte-derived exosomes are the most abundant in plasma of TB patients suggesting their role in modulation of immune functions. This was demonstrated by increased frequency of effector T-cells producing IFN γ +TNF α , suggesting a role of exosomes in controlling immune response. Further studies are in progress to define their diagnostic and immune-modulatory potential.

PW5.09

Microparticles derived from cultured macrophages and cerebrospinal fluid of patients with schizophrenic and affective disorders

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Inflammatory antigen presenting cells (APC, dendritic cells and macrophages) are characteristic for patients with affective disorders and can be enriched from Ficoll-isolated mononuclear cells by 3–4 weeks of *in vitro* culture. Flow cytometry, electron microscopy, western blots and cytokine quantification were applied to characterize the cell type and the inflammatory response in blood and cerebrospinal fluid (CSF). These analyses demonstrate that the enriched APC can be classified into either dendritic cells (iDCs), M1 or M2 macrophages. All cell isolates display high macropinocytotic activity and in addition form prominent autophagous vacuoles. The M1 type occurred more often in affective disorders than in schizophrenics. M1 cells expressed the ATP-sensitive P2X7 ion channel. ATP stimulation of the P2X7 ion channel caused the release of plasma membrane vesicles and microparticles (MPs). MPs enriched from blood-derived APC cultures

and from native CSF were subjected to protein chemical as well as miRNA analysis. All MP preparations were β -actin positive and in part stained for the autophagy marker LC3-II. When compared to the cultured APC-target cells, the microparticles were stronger positive for arginase 1, CD95, S100 and CD178, whereas CD36 and the TNF-R type I (CD120a) were decreased. miRNA analysis revealed inflammatory signatures in the majority of affective disorders. These results indicate that microparticles may transport protein and miRNA species to target tissues. The current protocol proved to be valid to simultaneously study MP generation *in vitro* using cultured APC and to isolate and characterize MP from the CSF. Electron microscopical examinations support the distinction of microparticles from exosomes.

PW5.10

The miRNAs are selectively enriched in retinal pigment epithelium cell-derived microparticles and mediate oxidative stress-induced retinal cells dysfunction

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Purpose: A normally functioning retinal pigment epithelium (RPE) is indispensable for vision. The cumulative oxidative injury induces RPE membrane microparticles (RMPs) production and RPE cell degeneration. This study was designed to investigate how RMPs participate in the retinal cells dysfunction and to investigate the role of RMPs containing miRNAs in the oxidative stress-induced RPE degeneration. **Methods:** RMPs were isolated from ARPE-19 cells under oxidative stress. The size distribution of RMPs in culture supernatants was determined using a NanoSight Tracking Analysis System. RMPs-treated RPE cells were subjected to cell viability assay, cellular senescent, apoptotic assay and FACS cell cycle analysis. RNAs were isolated from RMPs and subjected to miRNAs sequencing analysis. The miRNA (let-7f) mimic was transfected into RPE cells and cell viability was determined and compared with miRNA control. **Results:** Oxidative stress induced extracellular microvesicles (EVs) production from RPE cells. These EVs had a size distribution with diameters of 50 to over 600 nm including both exosomes and ectosomes. In addition, the majority of EVs are ectosomes which presented in RMPs. RMPs significantly reduced RPE cell viability. RMPs at low dose (5 μ g/mL) significantly induced RPE cell-cycle arrest at G0/G1 phase; RMPs-treated cells exhibited higher senescence-associated β -galactosidase activity, whereas RMPs at concentrations over 20 μ g/mL induced cell apoptosis. The miRNA sequencing revealed that miRNAs are selectively enriched in RMPs in which members of hsa-let-7 family are the most abundant ones. Enforced expression of let-7f dose-dependently reduced cell proliferation and induced cell senescence of RPE cells. **Conclusions:** We demonstrated for the first time that RMPs-containing let-7f causes RPE cell degeneration, which suggest RMPs may function as mediators to exacerbate the oxidative damages to RPE cells and let-7 may be the key active components of RMPs.

PW5.11

Identification and proteomic profiling of extracellular vesicles in peritoneal dialysis fluid

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Introduction: Dialysis treatment for chronic kidney disease has increased markedly worldwide. Increased understanding of peritoneal dialysis pathophysiology, peritoneal dialysate composition and biomarkers for patient management is needed. Obtaining samples directly from the peritoneum is invasive. Extracellular vesicles may be a useful approach to analyse changes to the peritoneum, yet there are no known reports of their presence or composition in peritoneal dialysate. **Methods:** We used a differential centrifugation technique to concentrate vesicle-associated proteins from relatively dilute peritoneal fluid, reducing abundant extravesicular proteins.

The presence and number of vesicles was demonstrated using transmission electron microscopy, nanoparticle tracking analysis (NTA), and dynamic light scattering (DLS). Finally using a high-throughput tandem mass spectrometry approach, we investigated proteins of potential interest. Proteins commonly used as identifying proteins for apoptotic bodies, ectosomes and exosomes were ranked. **Results:** The average particle size using NTA and DLS was 210 nm (47–820 nm) and 280 nm (56–1100 nm), respectively. Forty-three protein markers were identified which are used to identify different types of vesicles in extracellular fluids, consistent with the size range as being representative of a heterogeneous population of vesicle types. In total, 1142 proteins were identified, including many proteins with known roles in peritoneal pathophysiology, and other transmembrane proteins. Proteins of interest for disease characterization included proteins of epithelial to mesenchymal transition, and the TGF pathway. Other peptides of interest included markers of ultrafiltration ability (aquaporin-1), peritonitis (C-reactive protein, acute phase protein). **Conclusion:** We characterized extracellular vesicles in peritoneal dialysis fluid, and showed as proof of concept that this approach enriches for potential proteins of interest.

PW5.12

The extravesicular miRNome of senescent human fibroblasts and its impact on keratinocyte functionality

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The senescence-associated secretory phenotype (SASP) is one hallmark of senescent cells, whereby pro-inflammatory factors are secreted and alter the tissue microenvironment. Recently, miRNAs packaged into extracellular vesicles (EVs) have been found as part of SASP (miR-SASP). Here, we report the identification of EV-miRNAs as part of the human dermal skin fibroblasts SASP. For this purpose, stress-induced premature senescence was triggered by repeated low doses of H₂O₂ in cells of 3 donors and EVs of senescent and control cells were harvested by ultracentrifugation for miRNomics and functional biological studies. We identified differentially secreted miRNAs by qPCR arrays and correlated their extracellular and intracellular abundance. Based on that, we have confirmed selected prominent, highly secreted miRNAs including miR-23a, miR-29a and miR-31 to be more abundant in EVs of senescent cells. Furthermore, by nanoparticle tracking analysis we observed that senescent fibroblasts secrete more vesicles than controls. In order to test if EV-miRNAs might be part of a paracrine crosstalk between dermal fibroblasts and epidermal keratinocytes, we confirmed uptake of cel-miR-39 enclosed in fibroblast-derived EVs by keratinocytes in monolayers and in full thickness skin equivalents. To summarize, our data indicate that extravesicular miRNAs of senescent fibroblasts are bonafide members of the SASP and that they contribute to the communication between fibroblasts and keratinocytes in 2D and 3D model human skin models.

PW5.13

Lupus nephritis was associated with a high increase in the urinary amount of miR-146a via exosomes compared to microvesicles or cellular pellet

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Please see OPW2.5

PW5.14

Exposure of lung epithelial cells to man-made mineral fibres elicits the release of extracellular vesicles with pro-inflammatory properties

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Introduction: Glass wool (GW) is a man-made mineral fiber that is widely used as thermic, acoustic and flame retardant insulation of buildings, vehicles and high-tech products. Exposure to GW has been linked to interstitial lung diseases (ILD). However, sensitive markers for the early detection of GW-induced pulmonary damage are still lacking. Extracellular vesicles (EVs) are released by cells upon various stressors and are involved in intercellular communication. Hence, we hypothesized that GW induces lung epithelial cells EVs release and elicit an inflammatory response in immune cells. **Methods:** Bronchial epithelial (BEAS-2B) cells were exposed to several concentrations (10–100 µg/mL) of commercially available GW (Isover(r)) for 48 h and cell viability was assessed. EVs in the cell supernatant and after isolation were quantified by flow cytometry and further characterized by transmission electron microscopy (TEM) and tunable resistive pulse sensing (TRPS). EVs-mediated production of interleukin (IL)-8 was determined over 48 h in human macrophage-like (THP-1) cells. **Results:** Whereas 48 h GW exposures did not affect bronchial epithelial cells' viability (80–99% of unexposed cells), 100 µg/mL GW resulted in a 6.5-fold increase in EVs in the cell supernatant compared to unexposed cells. TEM revealed membrane-surrounded vesicles whose mode diameters (GW: 109 ± 6 nm (78–629 nm), control: 115 ± 8 nm (77–578 nm)) did not differ between the exposure conditions. Flow cytometry confirmed the presence of the exosomal proteins CD63 and CD81. EVs from GW exposed but not from unexposed cells stimulated the production of IL-8 in macrophage-like cells. **Summary/conclusion:** GW-induced release of EVs from bronchial epithelial cells prior to a reduction in cell viability suggests EVs as biomarkers of early cytotoxicity. Moreover, the EV-induced inflammatory response in immune cells may contribute to the early onset of pulmonary inflammation upon GW exposure.

PW5.15

MicroRNA cargo of extracellular vesicles from alcohol-exposed monocytes signals naïve monocytes to differentiate into M2 macrophages

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Introduction: Extracellular vesicles (EVs) released by immune cells can regulate immune responses via transmission of biological signals. Recently, we demonstrated that alcohol-treated hepatocytes crosstalk with immune cells via exosomes containing miRNAs. Here we hypothesized that alcohol-exposed monocytes (MØ) can communicate with naïve MØ via EVs. **Methods:** THP-1 cells or human MØ were exposed to alcohol (25–100 mM) and EVs were assessed in the supernatants with NanoSight or isolated for microRNA profiling. M1 and M2 MØ markers were assessed by FACS. **Results:** We found that alcohol affects monocyte EV secretion and contents, which signals naïve monocytes to differentiate into macrophages. We observed increased number of EVs, mostly exosomes, secreted by primary human monocytes and THP-1 monocytic cells in the presence of alcohol in a concentration- and time-dependent manner. EVs derived from alcohol-treated monocytes stimulated naïve monocytes to polarize into M2-macrophages as indicated by increased surface

expression of CD68 (macrophage marker), M2 markers [CD206 (mannose receptor), CD163 (scavenger receptor)], secretion of IL-10 and TGF β , and increased phagocytic activity. miRNA profiling of the EVs derived from alcohol-treated THP-1 monocytes revealed high expression of the M2 polarizing miRNA, miR-27a. Treatment of naive monocytes with control EVs overexpressing miR-27a reproduced the effect of EVs from alcohol-treated monocytes on naive monocytes and induced M2 polarization, suggesting that the effect of alcohol EVs was mediated by miR-27a. We found that miR-27a modulated the process of phagocytosis by targeting CD206 expression on monocytes. Importantly, circulating EVs from plasma of alcoholic hepatitis patients had increased numbers of EVs that contained high levels of miR-27a compared to healthy controls. **Summary/conclusion:** Alcohol-EVs mediate their effects via miR-27a on monocytes and induce macrophage polarization.

PW5.16

Hypoxia and TNF α co-treatment affects the number and size of adipocyte-derived EVs

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Adipocytes release extracellular vesicles (EVs) (1). In addition, adipocytes in obesity are subject to an extracellular milieu that is both hypoxic and inflammatory (2). As such, this study questioned whether a hypoxic and inflammatory environment influences the release of adipocyte EVs. Mature 3T3-L1 adipocytes were incubated for 24 h in one of four conditions: "control" (serum-free medium (SFM), 95% air/5% CO₂), "TNF α " (SFM supplemented with 30 ng/mL TNF α , 95% air/5% CO₂), "hypoxia" (SFM, 1% O₂), or "TNF α and hypoxia" (SFM supplemented with 30 ng/mL TNF α , 1% O₂). EVs were isolated from media by ultracentrifugation (500g for 5 min >15,000g for 15min >100,000g for 60 min). EVs were quantified using nanoparticle tracking analysis and results expressed as mode size and mean yield of EVs/viable cell (assessed using trypan blue). Data were analysed using one-way ANOVA and post-hoc Tukey testing, and differences deemed significant when $p < 0.05$ (N=3, n=9). No differences in cell viability were identified ($50 \pm 0.96\%$, $51 \pm 1.3\%$, $52 \pm 1.1\%$ and $53 \pm 1.2\%$ (mean \pm SD)). Compared to control (2128 ± 131 (mean \pm SD)), TNF α (3197 ± 386 ; $p < 0.05$), hypoxia (4073 ± 444 ; $p < 0.001$) and TNF α & hypoxia combined (5157 ± 331 ; $p < 0.001$), increased the EVs/viable cell yield. TNF α & hypoxia combined yielded more EVs than that of TNF α ($p < 0.001$) and hypoxia ($p < 0.05$) alone. Compared to control (188 ± 1.4 nm (mode \pm SD)), TNF α (175 ± 2.5 nm; $p < 0.01$), hypoxia (173 ± 5.3 nm; $p < 0.01$) and TNF α & hypoxia combined (160 ± 1.5 nm; $p < 0.001$), decreased EV size. TNF α & hypoxia combined yielded smaller EVs than that of TNF α ($p < 0.01$) and hypoxia ($p < 0.01$) alone. TNF α and hypoxia affect the release and size profile of adipocyte EVs, and when combined, their affect is enhanced. Future studies aim to determine the functional relevance of this observation within the field of obesity, and given its strong association (3), cardiovascular disease.

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PW5.17

Can blood–brain barrier disruption and active inflammation in multiple sclerosis be monitored by EVs in plasma?

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Introduction: Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS). Disruption of the blood–brain barrier (BBB) plays a major role in disease activity and damage of the BBB may be initiated by systemic or CNS inflammation and contribute to escalation of pro-inflammatory responses within the CNS. We hypothesized that damage of the BBB is reflected in the appearance of endothelium-derived extracellular vesicles (EVs) in the plasma and correlate with soluble biomarkers of endothelial stress and regulators of systemic inflammation. **Methods:** Five patients with untreated MS were followed by weekly blood sampling for 7 weeks. Plasma samples were analysed for 41 soluble biomarkers by Mesoscale V-PLEX, whereas EVs were analysed by the EV array. The array was customized by known EV markers and endothelial stress-induced EV markers as determined by quantitative proteomics to include 33 different antibodies against EV markers involved in inflammation and endothelial stress. For detection of EVs, a cocktail of antibodies against CD9, CD63 and CD81 was used. **Results:** In this study, the plasma contents of EVs was compared to soluble biomarkers indicating systemic inflammation and endothelial stress. Each patient revealed an individual phenotype of plasma EVs, which changed over time. Pearson correlations between the EV phenotypes, and soluble biomarkers were made. Hierarchical clustering of biomarkers and patients, and time-series clustering were generated. **Summary/conclusion:** Further data analysis has been performed in order to conclude whether any of the EV markers correlates with the soluble biomarkers and their changes over time.

PW5.18

Inflammation- and thrombin-induced endothelium-derived microvesicles and modulation of endothelial barrier function in human brain microvasculature

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Introduction: Mechanisms contributing to changes in the blood–brain barrier associated with brain pathologies are incompletely understood. We investigated the phenotype of microvesicles (MVs) released from cultured human brain microvascular endothelial cells (HBMECs) stimulated with proinflammatory, prothrombotic or oxidative stress agents, and changes in endothelial barrier function associated with their release. **Methods:** Male donor HBMECs were cultured to higher than 95% confluency, then treated for 24 h with medium alone (control) or with medium plus either tumour necrosis factor (TNF α , 20 ng/mL), thrombin (THR, 2 U/mL), angiotensin II (AngII, 10 nM) or hydrogen peroxide (H₂O₂, 0.8 mM). MVs released into the medium were isolated by centrifugation and labelled with annexin V in combination with an antibody against either PECAM-1, ICAM-1, E-selectin, integrin α V or MCAM and then analysed by digital flow cytometer. HBMECs were also cultured on microporous supports and then treated for 24 h with the above-described agents; then paracellular permeability was quantified by measurement of FITC-dextran passage across each cell monolayer during a 90-min period. **Results:** Total MVs released (MVs/ μ L) with TNF α , (615.8 ± 13.9), THR (113.8 ± 4.8) and H₂O₂ (152.8 ± 23.5) were significantly greater ($p < 0.05$) compared to control (82.2 ± 5.4). Each treatment released a distinct array of MV subtypes. Permeability (% control) was increased by 199.5%, 234.1%, 126.8% and 588.3% for TNF α , THR, AngII and H₂O₂, respectively. Also, control MVs pre-labelled with the fluorescent dye PKH67 were endocytosed into HBMECs within 60 min and trafficked to punctate peri-nuclear structures, identified by confocal microscopy. **Conclusions:** Molecules associated with inflammation, thrombosis and oxidative stress increase both paracellular permeability and MV shedding from HBMECs in vitro. Pre-labelled MVs are rapidly endocytosed supporting a contribution to modulating dysfunction of microvascular endothelium.

Poster Session 6 – EV proteomics and lipidomics

Chairs: Kwang Po Kim and Amal Al-Dossary

PW6.01

Peptide-mediated “miniprep” isolation of extracellular vesicles for high-throughput proteomics: method evaluation and application in colon cancer

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Introduction: Extracellular vesicles (EVs) are released into body fluids and their cargo represents a rich source for disease biomarkers. However, standard ultracentrifugation methods for EV isolation (abbr. UC-EV) are laborious, time-consuming and require high inputs. Recently, a novel HSP-targeted isolation method, which can be performed at small scale, was described (Ghosh et al., PLoS ONE 9:e110443). Using cancer secretome and biofluid samples, the authors showed enrichment of exosome markers with their method (abbr. HSP-EV) but a detailed description of the captured HSP-EV versus UC-EV proteomes and application to small tumour proximal fluid samples is lacking. **Methods:** Here we used label-free proteomics of replicate EV isolations from HT-29 cancer cell-conditioned medium to compare EV fractions captured using either HSP-EV or UC-EV. Subsequently, we applied the HSP-EV method to profile EVs released from fresh human colorectal (CRC) tumours (n=17) and colon adenoma tissue (n=4) as well as patient-matched normal colon tissue (n=21). **Results:** Despite a 30-fold different input scale (UC-EV: 60 mL vs. HSP-EV: 2 mL), both methods yielded comparable numbers of identified proteins (3115 vs. 3085), with reproducible identifications (72.5% vs. 75.5%) and spectral count-based quantification (average CV 31% vs. 27%). EVs obtained by either method contained established EV markers and proteins linked to vesicle-related gene ontologies. In the EV fraction of CRC tissue secretomes, 6390 proteins were identified, of which 471 proteins were at least 5-fold more abundant in CRC EVs than in normal tissue EVs. Gene ontology analysis revealed enrichment of nuclear proteins involved in DNA damage response, chromosome organization and RNA processing in CRC EVs. **Conclusion:** The HSP-EV method provides an advantageous, simple and rapid approach for EV isolation from small amounts of biological samples, enabling high-throughput analysis in a biomarker discovery setting.

PW6.02

Simple and effective isolation of exosomes by polyethylene glycol from cell culture supernatant for in-depth proteome profiling

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Exosomes are secreted nanovesicles shed by most types of cells. Recently, increasing interest has been focused on these small membrane vesicles (30–150 nm) containing functional biomolecules (i.e. proteins, lipids, RNA and DNA) that can be horizontally transferred to recipient cells and play critical role in intercellular communications. In the past few years, many different approaches to obtain exosomes with high quality are available. However, the current

isolation techniques are to some extent not convenient and often require expensive kits or specialized equipment. Herein, we introduced a very simple, polyethylene glycol (PEG)-based approach, which could permit a fast and effective isolation of exosomes from cell culture supernatant. The size and morphology of isolated exosome aggregates were visualized by high-resolution electron microscopes. All exosomes had a spherical shape with a diameter of about 100–200 nm and coated with silk-like PEG films, implying the mechanism of PEG-based precipitation may be attribute to the enhancement of sedimentation coefficient of these wrapped exosomes. The size distribution of isolated exosomes was also presented by nanoparticle tracking analysis (NTA) and showed a narrow peak with a maximum at 100 nm. After combining with tandem mass spectrometry, 4932 protein groups encoded by 4478 genes were successfully characterized from 30 mL of HeLa cell culture supernatant, including numerous exosome proteins which could overlap 95% of the top 100 exosome proteins recorded in ExoCarta, as well as a series of low-abundance cytokines and biomarkers. Furthermore, we found a higher ratio of neo-cleavage sites in exosome samples compared with cellular proteins, revealing significant roles of exosome in accumulation and transport of protein degradation intermediates.

PW6.03

Simplified method for proteome analysis of platelet-induced membrane vesicles

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Background: Activated platelets release two classes of membrane vesicles: most dominant microvesicles produced by surface shedding, which are larger ranging in size from 200 to 500 nm, and much smaller exosomes derived from exocytosis process (less than 200 nm). They are supposed to have important role in haemostasis and thrombus formation. **Aim:** Developing a method to assess the protein content of platelet organelles and microvesicles. **Methods:** Generation of platelet derived vesicles (PDVs) – platelets were washed from platelet-rich plasma (PRP) collected from healthy donors. PDVs were induced by thrombin activation (1IU). Activated platelets and released PDVs were subjected with analysis. Nano-LC-MALDI-TOF-MS/MS analysis was performed using nano-liquid chromatography method. Tryptic peptides were separated with the reverse phase column. Peptide identifications were performed using BioTools together with the MASCOT server for searching against the Swiss-Prot human database. The precision tolerance was 100 ppm for peptide masses and 0.7 Da for fragment ion masses. Individual peptide matches with score above 28 were considered statistically significant. **Results:** In total, 412 proteins were identified in PDVs and 450 proteins in activated platelets. Among them 307 proteins were identified in both types of samples. PDVs contained exosome- and microvesicle-specific sets of markers, for example, glycoproteins, transmembrane and adhesive proteins as well as transcription machinery proteins. **Conclusions:** In this study, we applied a new approach to the analysis of microvesicles based on bottom-up proteomic analysis. All preparation steps were carried in solution instead of gel.

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PW6.04

Lipid profiling of normal and tumourigenic cell-derived extracellular vesicles reveals differences in composition

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Introduction: Extracellular vesicles (EVs) are important for cell-cell communication via the transfer of protein, nucleic acid and lipid cargo. It has been extensively reported that tumour-derived EVs transport a cargo of oncogenic material, including functional oncogenes and oncoproteins that aid in the malignant transformation of target cells. Whilst the precise mechanisms controlling which cells receive this cargo are still unknown, it is proposed that the lipid composition will play a key role and that tumour-derived EV lipid profile will facilitate cargo delivery to a greater distribution of cells. **Methods:** EVs from the RWPE1 cell line, an immortalized normal prostate line, and the WPE1-NB26 cell line, a chemically modified and tumourigenic derivative of the RWPE1 line, were collected using an ultrafiltration procedure. Lipids were extracted using chloroform:methanol (2:1), freeze dried and resuspended in LCMS loading buffer and detected by LCMS. Quantification of 265 lipid metabolites, including phospholipids (PC, PE, PI, PS), ceramides, sphingolipids (SM), cholesterol esters (CE) and di- and tri-acyl-glycerides (DG, TG), was conducted in a lipid-targeted approach to identify differences in EVs from normal and tumourigenic prostate cells. **Results:** All 265 lipid metabolites targeted were detected in EVs from at least one sample. Differences were identified using a Student's t-test with Benjamini-Hochberg (BH) correction after the data was median normalized and log transformed. Of the 265 metabolites detected, 71 WPE1-NB26 EV metabolites including various CE, PC, PE and SM species were significantly different from RWPE1 metabolites based on the BH-adjusted p-value ($p < 0.05$). **Summary/Conclusion:** These results indicate that there is a selective and identifiable difference in lipid metabolites between EVs derived from normal and tumourigenic cells. Future studies will look at whether these differences in metabolites enhance the uptake of these EVs by surrounding cells.

PW6.06

Proteomics of subpopulations of extracellular vesicles: clues of subcellular origin

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Introduction: Extracellular vesicles (EVs) can be separated on density gradients from other components such as soluble proteins, but may also be utilized to spatially separate different subpopulations of EVs with different density. We herein present a proteomics analysis of two vesicle subsets with slightly different density, derived from the human mast cell line HMC1. **Methods:** Vesicles from human mast cells, HMC1, were isolated with differential ultracentrifugation followed by bottom-loaded separation on an iodixanol gradient. After separation with ultracentrifugation, 1 mL fractions were collected and analysed based on their contents. Two visually distinct fractions, found above and below 22% iodixanol, were further analysed with mass spectrometry and evaluated based on their proteomic contents. **Results:** There is a wide distribution of materials throughout the gradient after separation but a clear accumulation of markers of EVs in the low-density fractions.

Proteomics data suggest that two subpopulations of EVs not only differ slightly in density but also in protein content. Although sharing common markers for EVs, GO term analysis suggests that the slightly higher density band is enriched in organellar components, while the lower density band harbours more components characteristic of the plasma membrane. **Conclusion:** We here present proteomics analysis of two EV bands separated slightly by iodixanol density gradient, supporting the presence of several types of EVs with different origin, one possibly from the plasma membrane and the other from intracellular organelles.

PW6.07

Proteomic analysis of *Mycobacterium tuberculosis* membrane vesicles

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Introduction: A deeply rooted phylogenetic lineage of *Mycobacterium tuberculosis* termed lineage 7 was recently discovered. In order to explain its recent evolution, we performed vesicle isolation and whole cell proteomics analysis of 30 lineage 7 strains. We defined the intra-lineage proteome variation and identified unique characteristics associated with this lineage, focusing on proteins involved in DNA repair, recombination and replication (3R genes). **Methods:** *M. tuberculosis* lineage 7 strains were cultured in minimal media (MM) at 37°C. Membrane vesicles were purified using density gradient ultra-centrifugation. The purified membrane vesicles and cellular pellets were loaded on Bis-Tris Protein Gels (4–12%) for in-gel digestion and cleaned up using c-18 zip-tip columns before injecting in to the mass spectrometer. MS analysis was performed by the Q-Exactive mass spectrometry (Thermo Fischer, Waltham, MA, USA) to achieve high-resolution protein identification and quantification. MaxQuant software was used to define the amounts of the various peptides present, while post-translational modifications (PTMs) were identified by Proteome Discoverer and manual inspection. **Results:** Next-generation mass spectrometry revealed that there is enriched packaging of virulence-associated mycobacterial proteins in its vesicles. The proteomic constituent of membrane vesicles changed through different growth phases of the bacteria. Furthermore, some of the proteins identified in the vesicles were reported to be highly immunogenic. **Summary/conclusion:** This study generated novel knowledge regarding the proteomic profile of *M. tuberculosis* membrane vesicles and their surface-exposed antigens. Further in silico and in vitro/in vivo experiments will reveal the potential of *M. tuberculosis* vaccine and drug target discovery and potential.

PW6.08

Proteomic analysis of human reticulocyte-derived exosomes

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Introduction: Reticulocyte-derived exosomes (rex) are nanovesicles of endocytic origin released during the maturation of reticulocytes to erythrocytes, thus playing a role in erythropoiesis. Remarkably, *Plasmodium vivax*, the most widespread human malaria parasite has a unique tropism for reticulocytes. Using a rodent malaria model, it was shown that rex from infections contain parasite proteins capable of inducing protective immune responses (Martin-Jaular et al., 2011 PLoS One). Therefore, rex can be explored as a new vaccine against

reticulocyte-prone malarial parasites. Most of the studies on rex, however, have been performed in non-human species. Here, we present the first proteomic analysis of human rex. **Methods:** rex were isolated from cultures of human cord blood using two different culture conditions: absence and presence of exosome-depleted serum, and two different isolation techniques: size-exclusion chromatography (SEC) and ultracentrifugation. Human rex preparations were finally subjected to liquid chromatography followed by mass spectrometry. Only proteins identified with 2 or more peptides were considered for analyses. **Results:** Proteomic analyses revealed a total of 197 proteins, 53 of which were found in common in human rex purified by SEC of serum-supplemented reticulocyte cultures. Many of these proteins were also identified in rex preparations obtained by different methodologies, reinforcing this consistent core proteome. Moreover, GO analysis confirmed that these proteins have an exosomal origin, as many terms related to extracellular vesicles and exosomes were overrepresented. Of interest, we observed a core of proteins conserved in rex isolated from humans, rats and mice. **Conclusion:** To our knowledge, these results provide the first report about the proteomic profile of human rex, enabling further studies on their role in erythropoiesis and their exploration as a vaccine and platform against vivax malaria.

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PW6.09

Proteomics profiling of mouse pluripotent stem cells and their extracellular vesicles

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Introduction: Increasing evidence indicates that paracrine factors and secretory elements such as extracellular vesicles (EVs) contribute positively in mesenchymal stem cell-based therapy. Recently, it has been reported how the delivery of mRNAs and miRNAs via EVs derived from pluripotent stem cells can aid in cardiovascular disease treatment. Here, we report on our study comparing the proteomic profiles of EVs from different pluripotent stem cells and discuss how these data could help in unravelling their biological functions and lead to the development of other therapeutic applications of stem cell-derived EVs. **Methods:** Conditioned media was collected from mouse embryonic (ES) and induced pluripotent stem (iPS) cells grown on gelatin-coated plates. EVs were purified by size-exclusion liquid chromatography (LC) and characterized initially with nanoparticle tracking analysis (NTA) and western blotting. Subsequently, we applied nano-liquid chromatography (LC)-mass spectrometry (MS)/MS proteomics analysis of both ES and iPS cell lysates and their respective EVs. **Results:** Physically, EVs isolated from both ES and iPS cells have similar size distribution profiles. Interestingly, proteomic profiles were more similar among cells and among EVs, regardless of their cell type. Contrary to previous reports, we failed to detect the presence of pluripotency-associated transcription factors in the EVs. Further gene ontology (GO) analysis showed some selective enrichment of different molecular components between ES and iPS EVs. **Summary/conclusions:** Due to safety concerns in stem cell replacement therapies, there is a gradual shift to develop and characterize alternative cell-free modalities, such as EVs. Here, we demonstrate that stem cell-derived EVs express a distinct profile of selected luminal and membrane proteins as compared to their cellular origins. Importantly, this information would be useful for the future development of customized stem cell-derived EVs for treatment of various diseases.

PW6.10

Cell confluence affects the proteome of extracellular vesicles from oral cancer cells

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Tumorigenic cells produce and release extracellular vesicles (EVs) which can exert different functions on cell-to-cell communication carrying several types of molecules to recipient cells. However, how the high cell confluence, hence high cell-to-cell contact interferes in the protein repertoire of EVs has not been completely elucidated. In the present study, we used shotgun proteomics approach to compare the effects of cell confluence on the protein repertoire of EVs from oral squamous cell carcinoma. We identified differences between the proteome of EVs isolated from cells cultured until 50 and 80% confluence. The proteomics analysis revealed that proteins related to cell adhesion and cell cytoskeleton organization mechanisms are overrepresented in the EVs. Biological pathway analysis indicated that EVs from cells at 80% confluence are enriched in proteins related to vesicle fusion and metastatic potential. Functional assays demonstrated that EVs from cells cultured until 80% confluence enhanced cell migration and Erk expression in recipient tumorigenic cells, thus suggesting a possible role of cell confluence, hence cell-to-cell contact in triggering alterations on the content of EVs and confirming the role of EVs in modulating cancer-related biological pathways.

PW6.11

Targeted proteomic analysis for biomarker discovery of colorectal cancer metastasis from plasma extracellular vesicles

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Introduction: Liver metastasis is the predominant prognostic factor of colorectal cancer (CRC). However, effective biomarkers or therapeutic targets were not yet established. Recent studies indicate that cancer-derived extracellular vesicles (EVs) could contribute to cancer progression and metastasis. In this study, we aimed for a biomarker discovery from membrane fraction of clinical specimen tissues and plasma EVs by using target proteomic method. **Methods:** Membrane fraction proteins were extracted from 12 pairs of CRC primary and liver metastatic tissues. Quantitative proteomic analysis of membrane fraction proteins was performed by the isobaric tandem mass tag (TMT)-labelling method. Differentially expressed proteins between primary and metastatic tissues were verified by the selected reaction monitoring (SRM) assay, which is a high-throughput and high-resolution quantitative proteomic approach. EVs fractions were prepared from plasma samples using a differential centrifugation protocol. For detection and verification of the biomarker candidates in plasma EVs, 120 plasma (40 of each metastatic, non-metastatic and healthy sample) were prepared and subjected to SRM analysis. **Result and conclusion:** A total of 4967 proteins were identified by quantitative proteomic analysis and 342 proteins were differentially expressed between primary and metastatic tissues. Among these biomarker candidates, several dozens of proteins were detected in plasma EVs by SRM analysis and some proteins were found to

be potential biomarkers for colorectal cancer metastasis. These results suggest that target proteomics was an effective method for biomarker discovery in plasma EVs.

PW6.12

Proteomic characterization of circulating extracellular vesicles in multiple myeloma

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Multiple myeloma (MM) is the second most common hematological malignancy. Extracellular vesicles (EVs) are thought to be involved in the increased procoagulant tendency in patients with MM, contributing to increased risk of venous thrombosis. The aim of this study is to effectively isolate EVs from the plasma of MM patients and healthy volunteers and use high sensitivity label free liquid chromatography coupled to mass spectrometry (LC-MS/MS) for extensive profiling of proteome differences in the pathological processes associated with MM. EVs were isolated and purified from platelet-poor plasma from 5 healthy volunteers and 5 MM patients (approved by the local ethics committee) by repeated 18,900g centrifugation followed by 4 consecutive washing cycles in PBS-citrate. The prepared EV samples were then analysed by nanoparticle tracking analysis (NTA) for particle concentration, Bradford protein assay (BCA) for protein concentration and EV purity, western blotting (WB) for determination of vesicle-associated proteins, transmission electron microscopy (TEM) for EV morphology and quantitative proteome analysis by LC-MS/MS followed by functional annotation of the identified proteins. BCA quantification of the prepared EVs fractions showed reduced levels of plasma proteins, indicating increased sample purity. WB and TEM confirmed presence of EVs in fractions, and NTA data revealed adequate yields of particles for LC-MS/MS analysis. LC-MS/MS uncovers specific differences in the proteome of MM-EVs compared with EVs from healthy controls, displaying biological and molecular functional variation between proteins in EV samples from MM and healthy plasma. Our results accentuate that by reducing plasma proteins while still perceiving an acceptable yield of EVs is paramount for LC-MS/MS analysis of EV containing samples and that proteome profiling could possibly enable the identification of candidate biomarkers for diagnosis, disease progression and treatment in MM patients.

PW6.13

Erythrocyte cells and HRV16-infected HeLa cells derived microvesicles protease profiling

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Proteases determine the fate, localization and activity of many proteins by the hydrolysis of the unique peptide bond. Therefore, they play a key role in various physiological processes including division, differentiation, migration, ageing and death of cells. This suggests they may alter the contents of microvesicles (MVs) by proteolytic processing and assist in MV-mediated cell-cell communication. In this study, a detection panel of 34 proteases including ADAMs, cathepsins, kallikreins and matrix metalloproteinases (MMPs) was used and its presence observed in erythrocyte cells(e) and

HRV16-infected HeLa cells(HRV16iH) derived MVs. This contributes to ongoing research into the characterization and functions of MVs. eMVs and HRV16iHMVs were isolated through the ultracentrifugation method: 200 relative centrifugal force (rcf) for 5 mins to pellet cells, 4000 rcf for 1 h to remove large molecules and 25,000 rcf for 90 mins to concentrate samples. Subsequently, all samples were prepared and experiment procedures were followed as directed by R&D Systems Proteome Profiler(tm) Human Protease Array Kit protocol. The location of controls and capture antibodies identification were detected by the mean spot pixel density, created using ImageJ software. Cathepsins (A, B, D, V and X/Z/P) and MMPs (8 and 13) were observed in all samples >300 mean pixels, whereas ADAM8, ADAMTS13, cathepsins (E, S and X/Z/P), DPPIV, kallikreins (7 and 10), MMPs (2,3 and 12), presenilin and PC9 were strongly expressed on eMVs ranging >250 mean pixels with a significant expression of MM9 >550 mean pixels. In comparison, ADAM9, ADAMTS1 and MMP10 nephylisinC were highly expressed on HRV16iHMVs. This study has identified the presence of key molecules in MV biology. This is a particularly exciting prospect, given that metalloproteinases are intensively involved in MV biology. However, this concept needs further investigation and understanding of the link between MVs and proteases. This will help unravel their complex biological functions in human diseases and may open new perspectives on MV activities.

PW6.14

Proteomic analysis of redox signalling in extracellular vesicles

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Introduction: Cancer cells produce copious amounts of reactive oxygen species (ROS) owing to their high metabolic activity and increased signal transduction, but increased levels of ROS can generate aberrant oxidative stress and trigger damage events. As such, the survival, migration and proliferation of cancer cells are influenced by the delicate balance between pro- and antioxidant molecules. At the molecular level, reversible oxidation of cysteine residues is one of the key mechanisms involved in modulating the effects of ROS, but redox signalling of proteins within extracellular vesicles (EVs) remains insufficiently understood. **Methods:** The reactive cysteines in a proteome can be identified using various reactivity-based protein profiling reagents in conjunction with chemical pull-down and enrichment followed by tandem mass spectrometry, thus allowing for proteomic profiling of cell lysates and EVs from normal mammary epithelial cells and breast cancer cells. **Results:** Characterization of EVs has revealed a number of modified cysteine residues in vesicles, thereby establishing that rich redox reactivity exists within these extracellular vesicles. In addition, we are also assaying these EVs for specific ROS-generation and antioxidant machinery to better understand the contributions of EVs to redox signalling and stress pathways in cancer cells. **Summary/conclusions:** This targeted chemical biology approach for the analysis of EV proteomics will enable us to dissect the roles of ROS and their effects on protein function in extracellular vesicles during cancer progression.

PW6.15

Phospholipid profiles of red blood cells and their extracellular vesicles are not significantly changed during storage of red cell concentrates

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During storage, red blood cells (RBCs) undergo physical and chemical changes affecting their quality and function, leading to accumulation of cell waste in red cell concentrates. These changes are referred to as "storage lesion." The RBC membrane is heavily affected by storage as RBCs lose ~20% of their membrane via vesiculation.

The clinical relevance of the storage lesion, or vesiculation in relation to the quality and safety of RBC concentrates, is still unclear. However, the accumulating extracellular vesicles (EVs) might serve as potential markers of RBC quality, and EVs may also be immunogenic. Our aim was to identify the phospholipid composition of RBCs and EVs during storage. Our hypothesis was that certain lipid classes or species might accumulate in EVs. Additionally, the immunological effects of EVs were studied. Red cell concentrates (n=6), stored in standard blood banking conditions (35 days), were sampled weekly. RBCs were pelleted and resulting supernatant was ultracentrifuged to obtain EVs. Mass spectrometric analysis of phospholipids, nanoparticle tracking analysis, western blot and electron microscopy were performed. The immunological effects of EVs and supernatants were studied with in vitro assay. The most abundant phospholipid classes in RBCs were phosphatidylcholine (PC), phosphatidylethanolamine and sphingomyelin, which together constituted the majority of all phospholipids, followed by phosphatidylserine and small amounts of lysoPC and ceramides, as previously described. According to preliminary analysis, phospholipid profiles did not significantly change during storage in RBCs or EVs and no accumulation of certain lipids could be detected in EVs, even during prolonged storage, although EV amount increased considerably. The lipid profile of EVs resembled that of RBCs. Preliminary data indicate that especially red cell concentrate supernatant, but less the EVs, induced the proliferation of T cells indicating pro-inflammatory characteristics.

PW6.16

Extracellular vesicles from tumour cells display specific glycosignatures

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Introduction: Extracellular vesicles (EVs) present specific lipid and protein compositions. More recently, evidence has also shown that EVs have specific glycosylation profiles and are enriched in distinct glycoproteins. **Methods:** In this work, EVs have been purified from supernatants of tumour cells (ovarian carcinoma OVM, SKOV3, glioma Tu-2449, H4 cells) in culture. Supernatants were centrifuged at 500 × g, 10,000 × g and 100,000 × g. EVs (100,000 × g pellet) were analysed by immunoblotting with antibodies anti-Tsg101, Alix, CD63, CD9, flotillin-2 and galectin-3-binding protein. EVs were analysed by nanoparticle tracking analysis. Total membranes (MBs) were obtained by cell sonication followed by ultracentrifugation at 100,000 × g. EVs were analysed by lectin blotting with a panel of lectins (including Maackia amurensis and Sambucus nigra lectins for sialylated glycans; Phaseolus vulgaris erythroagglutinin for bisecting GlcNAc; Gomes et al., 2015). N-Glycan profiles were established by HPAEC-PAD and MALDI-TOF MS (Escrevente et al., 2013). **Results:** The 100,000xg pellet was enriched in EVs as evaluated from the detection of specific markers. Furthermore, the collected EVs had diameters compatible with those described in the literature. The glycoprotein galectin-3-binding protein was strongly enriched in EVs relatively to MBs, and it contained sialylated complex N-glycans. Lectin blotting with a panel of lectins showed that EVs had specific glycosignatures relatively to MBs. Furthermore, characteristic glycan structures have been identified in the tumour cells. **Conclusion:** EVs have characteristic glycosignatures, which could result from specific sorting mechanisms of glycoproteins into EVs. The identified glycosignatures could provide with novel cancer markers.

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Poster Session 7 – EV isolation

Chairs: Cecilia Lässer and Anirban Banerjee

PW7.01

Minimizing the loss of exosomes during their storage at 4°C

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Introduction: Extensive studies and wide applications of exosomes in clinical diagnostics require the standardization of not only isolation, but also storage conditions. However, at present there is no strictly established protocol for exosomes storage. It is a rather common practice to store the exosomes at 4°C for a couple of days to avoid freeze-thaw cycle. In this work, we analyse the influence of buffer composition and tube surface pre-treatment on kinetics of exosomes loss during the storage at 4°C. **Methods:** Exosomes from HT29 cell culture supernatant were purified by differential centrifugation, aliquoted and kept at -80°C. Further, the aliquots were consequently unfrozen, dissolved in different storage buffers, placed into the tubes with different surface treatment and kept at 4°C. The kinetics of exosomes concentration decay was followed with NanoSight LM10 HSBF instrument. TEM was used to determine the morphology of the particles. **Results:** NTA data show that the concentration of exosomes stored in PBS essentially decreases during 12 h of the storage. However, if exosomes are stored in HT29 supernatant, no essential decrease of their concentration is observed. One of the explanations may be adsorption of the exosomes on the tube wall. According to theoretical estimation, a standard 2 mL polypropylene tube could adsorb up to 10¹⁰ particles/mL. Higher stability of exosomes stored in cell culture supernatant may be due to the fast blockage of tube walls by proteins, thus preventing the adsorption of exosomes. We also demonstrate that pre-treatment of tube surface can minimize the exosomes loss during storage. **Summary/conclusion:** Exosomes may adsorb to the tube wall during storage at 4°C. Storage of non-pelleted exosomes or use of pre-treated tubes can reduce the exosomes loss. The work was partly performed at User Facilities Center of MSU.

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PW7.02

A protocol for isolation and proteomic characterization of distinct extracellular vesicle subtypes by sequential centrifugal ultrafiltration

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Please see OPW1.1

PW7.03

An ultracentrifugation-based protocol for exosome isolation in diffuse large B-cell lymphoma

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Introduction: Non-Hodgkin's lymphoma (NHL) is the eighth most common cancer in women and the seventh most common cancer in men in Turkey. Most NHL is derived from B cells and the most common type is diffuse large B-cell lymphoma (DLBCL). The studies on exosomes in DLBCL are still very limited. The aim of this study was to characterize the exosomes in the patients with DLBCL compared to healthy controls. **Methods:** Eleven patients with DLBCL (4 with germinal centre B-cell phenotype and 7 with non-germinal centre B-cell phenotype) and 13 age- and sex-matched healthy controls were included in the study. Blood samples were drawn from the right antecubital vein using a large (>21-gauge) needle and processed within 30 min. Briefly, two-step centrifugation was used to obtain the platelet-free plasma samples. After a 0.2 µm filtration, the samples were ultracentrifuged at 110,000 × g for 2 h and 100,000 × g for 1 h to pellet exosomes. The exosomes were characterized by electron microscopy, specific markers commonly used for exosome detection and protein quantity. **Results:** All exosome samples showed typical morphology and expressed exosomal markers such as CD63, CD81 and TSG101, however at different levels. Total protein content was also higher in the cancer patient exosomes compared to the controls. **Summary/conclusion:** Our results showed that the centrifugation-based procedures assessed in this study are able to isolate high-quality, analysable exosomes in DLBCL patients. We suppose that it would be important to describe the strategies for the isolation of high-quality exosomes from blood samples of DLBCL patients before focusing on further experiments.

PW7.04

Methodological comparison of CSF extracellular vesicle extraction as a novel source of biomarkers in amyotrophic lateral sclerosis

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Introduction: Biomarkers sensitive to disease activity are needed to advance therapeutic development for the adult fatal neurodegenerative disorder amyotrophic lateral sclerosis (ALS). Cerebrospinal fluid (CSF) EVs may reflect core pathogenic processes with promise for biomarker discovery, though extraction and analysis have been limited by variable, often poor yield with insufficient exclusion of contaminating protein. The aim of this study was to optimize the extraction of EVs from the CSF of ALS patients for subsequent proteomic characterization. **Methods:** EVs were purified from CSF either by ultracentrifugation (UC) or ultrafiltration/liquid chromatography (UF-LC). Purified EVs were quantified and characterized using transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA) and western blotting for known exosomal markers (ALIX and CD9). **Results:** Both methods produced purified EVs. NTA revealed more particles of the expected size distribution and improved protein exclusion following UF-LC, compared to UC. Details of comparative morphology and marker expression are presented. **Conclusions:** UF-LC is a more potent method to advance the goal of neurochemical biomarker development in ALS and central nervous system disorders more widely.

Acknowledgements: This work was funded by the Medical Research Council and Motor Neurone Disease Association UK Lady Edith Wolfson Fellowship and Oxford University John Fell Fund (both MRT).

PW7.05

Comparison of a fast column-based EV isolation method with ultracentrifugation and size-exclusion chromatography

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Introduction: The methods used for extracellular vesicle (EV) isolation are often tedious or have other limitations with respect to sample volume, aggregation, yield or purity. Therefore, EV research will benefit from the introduction of new, more simple and efficient EV isolation methods. Our aim was to compare a new column-based method to differential centrifugation and size-exclusion chromatography using plasma as the source material of EVs. **Methods:** The endpoint parameters were purity, yield, quality and integrity of EVs analysed by nanoparticle tracking analysis (NTA), electron microscopy (EM), flow cytometry, western blotting (WB) and RNA analysis. **Results:** The most notable differences between the column method and other methods were observed in EM and light scattering-based methods. Negative staining protocol for column-derived EVs showed black aggregates in addition to regular EVs that were the only population observed with other methods. The elution buffer of the column method alone showed considerable number of particles using either NTA or flow cytometry that were not detected in other buffers used as diluent. However, the purity of the EV sample was acceptable and the yield comparable or better than with the other methods. **Conclusions:** The EM and the two light scattering methods suggested the presence of larger particles in the column-derived EV preparations perhaps due to the elution buffer, which may complicate some downstream analysis.

PW7.06

Comparison of exoEasy Maxi kit (Qiagen) with size-exclusion chromatography qEV columns (Izon Science) for isolation of extracellular vesicles from human plasma

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Introduction: Identification of extracellular vesicle (EV)-associated biomarkers is crucially dependent on isolation methods that yield pure EVs without contaminating plasma proteins and lipoproteins and that are reproducible, efficient and easy to perform. Size-exclusion chromatography (SEC) has increasingly become a method of choice for isolation of uncontaminated EVs from plasma. More recently, Qiagen introduced a new membrane affinity spin column method for the isolation of highly pure EVs from biofluids named exoEasy Maxi Kit. We have compared the exoEasy Maxi kit (Qiagen) with the SEC using qEV columns (Izon Sciences) for the isolation of EVs from 2 mL of human plasma. **Methods:** EV-rich SEC fraction pools or exoEasy Maxi Kit eluates were concentrated, and the yield, size distribution and purity of EVs were determined by NTA, EM and western blot analysis. RNA was extracted by miRNeasy Micro Kit (Qiagen), and yield and size range were analysed using Agilent Bioanalyzer. **Results:** EM analysis confirmed enrichment of vesicles in SEC early fractions, which sharply contrasted with rare occurrence of vesicles in exoEasy Maxi kit eluates. NanoSight data showed smaller particle peak size of EVs isolated by SEC (127 nm) compared to exoEasy Maxi kit (196 nm). Particles per microgram protein ratio were significantly higher for SEC suggesting a protein contamination in

exoEasy Maxi kit preps. Correspondingly, enrichment of exosomal markers (syntenin, flotillin-1, CD9) was higher in SEC preps with a significant albumin contamination in exoEasy Maxi kit preps. The Bioanalyzer profile of isolated RNA indicated the presence of small RNAs without rRNA in both methods. ExoEasy Maxi kit RNA yield was higher. **Conclusion:** qEV columns (Izon Science) outperform the exoEasy Maxi kit (Qiagen) for the isolation of highly pure EVs for biomarker research.

PW7.07

Comparison of isolation methods for EVs from fresh and frozen serum and plasma samples

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Extracellular vesicles (EVs), such as exosomes and microvesicles, are increasingly considered as signalling organelles in intercellular signalling as well as biomarkers in a variety of different diseases. Despite extensive studies on their functional properties, no gold standard to isolate them from cell culture supernatants or body fluids, respectively, has been defined so far. Here, we compared several commercial and non-commercial methods to harvest EVs from plasma and serum samples. In detail, we compared 4 procedures based on ultracentrifugation, 3 on size-exclusion chromatography and 3 on polymer-based precipitation. To this end, freshly taken and frozen serum and plasma samples from 5 healthy volunteers were taken; cells, organelles and cell debris were eliminated via differential centrifugation (300 × g; 2000 × g and 10,000 × g). Aliquots of 500 μL of each serum and plasma sample were used for EV enrichment with each of the given methods. All obtained EV samples were analysed by nanoparticle tracking analysis (NTA; Zetaview(r) from Particle Metrix) to determine particle concentrations and average size distributions. In addition, protein concentrations were determined using the Pierce(tm) BCA Protein Assay Kit. In our ongoing work, we currently analyse the presence of defined EV markers, that is, CD9, CD81 and TSG101. So far, we detected severe differences in the EV and protein yield between the different methods; the average size distribution was comparable within all fractions. After obtaining the western blot data, we can finally compare and analyse the data. The obtained results will provide new insight on EV isolation methods for small serum or plasma samples, for example, to perform liquid biopsy analyses on patient-specific materials.

PW7.08

Impact of SEC, PEG and PROSPR isolation methods on Extracellular Vesicles: A Comparative Study

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Introduction: One of the major challenges in the extracellular vesicles (EVs) field is to find a consensus method for EVs isolation. Historically, ultracentrifugation has been the most widely used method though new methodologies are emerging. Lately, a cold acetone-based

protein precipitation method, Protein Organic Solvent Precipitation (PROSPR), has been reported. Here, we have compared PROSPR, PEG precipitation and size-exclusion chromatography (SEC) evaluating the workflow time, total protein content, nanoparticle tracking analysis (NTA), flow cytometry for EV markers and small RNA extraction. *Methods:* EVs from urine and serum samples were isolated by SEC, PEG and PROSPR. Protein content of purified fractions was measured by the BCA protein assay and NanoDrop analysis. Purified EVs were characterized by NTA and flow cytometry. Small RNAs were extracted from serum-derived EVs obtained from each method. *Results:* EVs were successfully purified by the 3 methods. Total protein content in the EV preparations using both precipitation methods was higher than using SEC. When characterized by NTA and flow cytometry, PROSPR and PEG preparations also showed higher EV concentration by NTA compared to SEC, but EV markers (CD9, CD63 and CD81) were uniquely detected by flow cytometry when purified by SEC. Small RNAs were positively extracted from all 3 isolation methods, being the RNA yield higher using PEG and PROSPR precipitating agents. *Conclusions:* Although precipitation strategies result in higher protein and RNA contents, SEC removes the overabundant soluble proteins and contaminants as reflected by BCA, NanoDrop and NTA analysis. Higher concentration of small RNA in PEG and PROSPR may be related to non-EV contaminants. Moreover, only SEC allowed the detection of EV markers, suggesting a putative interference of these precipitation agents in the structure and/or composition of the vesicles that should be taken into account depending on their forthcoming application.

PW7.09

Comparative analysis of urine storage, exosomes/EV purification and normalization methods for the selection of most appropriate solutions for biomarker discovery, clinical diagnostics and biobanking

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Please see OPW1.4

PW7.10

Isolating exosomes from cell culture supernatant: a comparison of commonly used protocols

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Introduction: Numerous exosome isolation methods are described including different ultracentrifugation (UC) protocols, size-exclusion chromatography (SEC) and commercial kits based on precipitation, immunoaffinity or membrane affinity capture. The most common protocol is differential (ultra)centrifugation, but even this method differs between publications, regarding pre-centrifugation treatments, speed, duration and washing. Therefore, we wanted to compare different UC-protocols among each other as well as the two most efficient UC protocols with SEC and two commercial kits. *Methods:* Cardiac cells were grown in medium (1% exosome-depleted FCS) for 48 h. Six different UC-protocols were compared: \pm sucrose cushion, filtration before or after UC and also different washing steps. UC was carried out at 118,000g for 90 min. Additionally, supernatants were concentrated and exosomes were isolated via SEC or the commercial kits ExoEasy and Exospin. Exosomes from all methods were characterized by tunable resistive pulse sensing (TRPS) and electron microscopy. Purity was assessed by protein to particle ratio and the exosomal cargo was analysed by western blot. *Results:* Different isolation methods, even the 6 UC protocols, lead to different exosomal subsets with varying marker expression. The comparison between UC, SEC, ExoEasy and Exospin showed that ExoEasy

resulted in the highest yield but also the lowest purity. UC showed fewer impurities but also resulted in less yield and was very time consuming. SEC produced the purest exosomes with the smallest yield. *Conclusion:* Isolation of exosomes with different UC protocols leads to different exosomal subpopulations, which should be carefully considered. Without the use of a sucrose cushion, the exosomes had high amounts of impurities. The kits resulted in a high exosome yield but also co-isolated contaminating proteins. Thus, SEC showed the most promising balance between yield, purity and effort.

PW7.11

Isolation of extracellular vesicles from plasma using magnetic nanoparticles functionalized with annexin-A5

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Introduction: Extracellular vesicles (EVs) are recognized to display various physio-pathological properties. Given their heterogeneity in origin, composition and function, it is critical to develop reliable methods for isolating EVs. Commonly used methods, like SEC or centrifugation, are non-specific and can lead to sample modifications (1). On the other hand, immuno-magnetic isolation is specific and has proven its efficiency to purify cells or EVs (2). We will report on the synthesis of 60 nm magnetic nanoparticles (MNPs) and their conjugation with annexin-A5 (Anx5). Anx5-MNPs were used to isolate phosphatidylserine (PS) exposing membranes, namely, lysed red blood cells (RBC) or PS-positive EVs from platelet-free plasma (PFP). Electron microscopy (EM) was used for imaging lysed RBCs and EVs, and flow cytometry (FCM) allowed us to quantify the efficiency of the magnetic isolation. *Methods:* The binding of Anx5-MNPs to PS-exposing membranes was measured by direct counting of MNPs bound to lysed RBCs, by EM. The efficiency of magnetic isolation was determined by FCM after labelling lysed RBC and PFP EVs with Anx5-Cy5. *Results:* First, we used lysed RBCs as a model system to study, by EM, the influence of the incubation time and the concentrations of Anx5-MNPs and lysed RBC on the binding of Anx5-MNPs. Then, we investigated the relationship between the number of Anx5-MNPs bound per lysed RBC and the efficiency of magnetic isolation, by FCM. Third, based on these results, the isolation of PS-positive EVs from PFP was studied. Reproducible conditions of total extraction were obtained. *Summary/conclusion:* An optimized method was developed for isolating PS-positive EVs, using Anx5-MNPs. This approach is currently applied to the isolation of erythrocyte- or platelet-derived EVs. Future work will focus on the composition analysis of isolated EVs by mass spectrometry.

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PW7.12

Improved large-scale EV production using a two-compartment bioreactor

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Introduction: Large-scale production of extracellular vesicles (EVs) requires extensive volumes of traditional 2D cell cultures, which consume media, laboratory equipment and working time. In this work, we examined the possibility to use of a two-compartment bioreactor in order to make EV production more efficient. *Methods:* PC-3 prostate cancer cells (ATCC, USA) were grown both

in T-175 cell culture flasks and in a Celline AD 1000 bioreactor (Integra). Cell culture medium for 2D culture was supplemented with FBS depleted from EVs by ultracentrifugation. The bioreactor medium was supplemented with additional glucose (4.5 g/L), and only the medium in the medium compartment (750 mL), separated by a 10 kDa cut-off membrane, was supplemented with untreated FBS. The cell conditioned medium (CM) was harvested three times per week from the 2D cultures and once per week from the bioreactor cell compartment. The EVs were collected by differential centrifugation into 20k × g and 110k × g pellets and analysed by electron microscopy, western blotting, nanoparticle tracking analysis (NTA) and Apogee flow cytometry. **Results:** The PC-3 cells in the bioreactor produced approximately $1-2 \times 10^{12}$ EVs collected in the 20k pellet and $2-4 \times 10^{12}$ in the 110k pellet from 15 mL of CM, while a single 2D cell culture flask produced approximately $1-2 \times 10^{10}$ and $8-11 \times 10^9$ EVs, respectively, from 25 mL of CM over 7 days. No FBS-derived particles penetrated the 10 kDa membrane into the cell compartment as analysed by NTA. Detailed characterization of the differently produced EVs is currently in progress. **Conclusions:** Bioreactors have the potential to improve EV production in virtually every aspect. The structure of the bioreactor allows a high concentration cell culture in a 3D matrix, improving the EV yield, reducing the workload and bringing the culture closer to in vivo conditions. To best utilize them, the produced EVs need careful comparison with the EVs produced in standard 2D cultures.

PW7.13

Rapid detection and enrichment of tumour-derived exosomes using targeted high-density lipoprotein-like gold nanoparticles

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Introduction: Exosomes produced by tumour cells (TEx) contain a wealth of information about their parent cell and could be excellent serum biomarkers for early-stage cancer or occult metastasis. However, existing protocols for exosome isolation are costly, laborious and lack the ability to differentiate between TEx and host exosomes. New detection methods would more fully reveal the diagnostic potential of TEx. Scavenger receptor type B1 (SR-B1) is present on TEx from multiple cancer cells. Because SR-B1 is a high-affinity receptor for high-density lipoprotein (HDL), we used a synthetic HDL-like gold nanoparticle (HDL NP) for targeting TEx-associated SR-B1 to detect and enrich TEx with high specificity from tissue culture media and patient sera. **Methods:** HDL NPs were labelled with a fluorophore and incubated with TEx from a variety of sources: (1) conditioned media from tumour cells, (2) TEx-spiked serum of healthy individuals and (3) sera obtained from melanoma patients. Samples were then stained with CD81-specific antibody. CD81-positive TEx labelled with the fluorescent particle(s) were visualized and quantified by flow cytometry. Low-speed centrifugation (16,000 g for 1 h) was used to isolate TEx associated with gold particles for western blot analysis. Human sample collection was covered by Northwestern University and New York University Institutional Review Board protocols, and informed consent was obtained from all patients. **Results and summary/conclusion:** Fluorescent HDL NPs bind a significant population of TEx as visualized by flow cytometry. The HDL NP-bound TEx population scaled proportionally with increasing amounts of TEx added to the sample. Precipitation of gold particles efficiently retrieved TEx, as was evidenced by western blot for CD81 and SR-B1. The yields of SR-B1 positive TEx from the sera of healthy individuals were low, while significant amounts were found in the sera from melanoma patients. In conclusion, we have developed a new HDL NP technology, which enables rapid TEx detection using small sample volumes. This technology should increase TEx utility as a biomarker, particularly for hard-to-detect cancers or occult metastasis by facilitating fast, easy and specific TEx retrieval for molecular analysis.

PW7.14

Isolation and purification of extracellular nanostructures from *Novosphingobium* sp. PP1Y: a novel example of Outer Membrane Vesicles?

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Outer membrane vesicles (OMVs) are nanoscale proteoliposomes of 20–200 nm diameter, derived from the surface of some Gram – and Gram+ bacteria as part of their growth cycle. OMVs have several roles; they can reduce levels of toxic compounds, remove stress products from the cell such as misfolded proteins, and have a role in biofilm formation (Adam K et al. *Annu Rev Microbiol.* 2010; 64: 163–84). The biotechnological use of OMVs, for example as drug delivery systems, is limited by the presence in their outer membrane of the immunogenic LPS produced by Gram – bacteria. As a consequence, much interest is currently devoted to vesiculating strains lacking LPS. In this context, an appealing source of OMVs can be found among Sphingomonadales, an order of Gram- bacteria lacking LPS. A marine microorganism belonging to this order, *Novosphingobium* sp. PP1Y, was isolated in the harbour of Pozzuoli and microbiologically characterized. This bacterium is able to grow in a very polluted environment and to use polycyclic aromatic hydrocarbon as a source of carbon (Notomista E. et al. *Microb Ecol.* 61(3) (2011) 582–594). We have successfully isolated extracellular nanostructures from *Novosphingobium* sp. PP1Y for which different growth conditions were tested. AFM analysis showed the production of circular nanostructures when PP1Y was grown in minimal medium supplemented with 0.4% glutamate. These were purified from the exhausted growth media following ultrafiltration and ultracentrifugation procedures; AFM and DLS analyses indicated for these structures a circular morphology with a diameter of ca. 200 nm, and a rough uniformity in both size and conformation. These preliminary results boost a more detailed characterization of these nanoparticles to identify both the proteomic and lipidomic profile. In addition, the complete annotation of *Novosphingobium* sp. PP1Y genome (D'Argenio V. et al. *BMC Genomics* (2014) 15, 384–397) is an advantage and gives the possibility to further engineer surface proteins of PP1Y OMVs.

PW7.15

Large-scale production of extracellular vesicles in a hollow fibre bioreactor

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Introduction: Production of extracellular vesicles (EVs) such as exosomes for clinical applications remains a challenge. Current methods can utilize large numbers of flasks and serum starvation in a batch mode process. Hollow fibre bioreactors are perhaps ideal for producing large quantities of EV at 100X higher concentrations than conventional protocols. They support the culture of large numbers of cells at high densities, $1-2 \times 10^8$ cells/mL. Cells are bound to a porous support with a 20 kDa MWCO so cell passaging is not required and EV cannot cross the fibre in either direction. **Methods:** 5×10^8 adipose-derived adult MSC were cultured in a hollow fibre bioreactor for 8 weeks. Cells did not expand (monitored by glucose uptake rate) nor did they differentiate (by multiple immunocytochemistry assays) over this time. 40 mL of conditioned medium from the extra-capillary space was harvested weekly. An HEK293 culture expressing heterodimeric interleukin-15 was similarly maintained in hollow fibre culture for over 4 months, with

3 harvests of 20 mL per week. *Results:* 4.2×10^{12} EV particles with total protein of 27.73 mg in 720 mL were harvested from the adult adipose-derived MSC culture. The HEK293 bioreactor culture yielded approximately 1×10^{12} EV/mL compared to about 1×10^9 EV/mL in flask culture. CD63 and Alix were greatly enriched from the bioreactor compared to flask culture. Additionally, EV/protein was 10-fold higher in harvests from the bioreactor suggesting higher purity as well. Purified HEK293 cell EV retained their IL-15 bioactivity. *Summary/conclusion:* Hollow fibre bioreactors have demonstrated potential for the production of EV using cGMP compliant materials and methods with a number of significant advantages compared to flask-based protocols including higher concentrations, large capacity, time and space efficiency, and perhaps EV quality. Current technology can produce gram quantities of EV, with potential use for clinical applications.

PW7.16

Development of an automated microfluidic platform for the isolation of extracellular vesicles

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Isolation of extracellular vesicles (EVs), often a tedious process, can greatly affect the clinical importance of EVs. We are developing an automated microfluidic platform that is capable of isolating EVs without user's attention. The platform consists of pneumatic valves and pumps for the transport and controlled gentle mixing of solutions, respectively. EVs were captured by the specific antibody coated on magnetic beads and then concentrated by a magnet. Total RNAs were extracted and their concentrations were quantified to assess the capture efficiency of the proposed method. The capture yield increased by 1.8 times when the duration of mixing was extended from 20 min to 22 h. Parameters affecting the mixing EVs with magnetic beads are currently under optimization to increase the capture yield.

Poster Session 8 – EVs in prostate cancer

Chairs: *Martin Van Royen and Rubina Baglio*

PW8.01

Identification of novel biomarkers by comprehensive proteomics in urinary exosomes for prostate cancer detection

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Background: Rapid and reliable diagnosis of prostate cancer (PCa) is highly desirable. Sensitivity and failure rate of current methods for diagnosis limit the success to early detect this type of cancer and, consequently, advanced disease is often encountered. Furthermore, the identification of PCa biomarkers that can classify patients into high- and low-risk groups of disease progression and therefore help in the treatment decision-making is a major area of ongoing research. **Objectives:** In this study, we aimed to identify protein biomarkers in urinary exosomes for early non-invasive detection and stratification of PCa. **Methods:** Protein biomarker candidates for PCa were initially identified from a discovery phase done in urinary exosomes from urine obtained after digital rectal examination. Specifically, label-free liquid chromatography coupled to mass spectrometry (LC-MS/MS) protein quantitation was performed on 24 samples: 8 benign samples, 8 low-risk PCa samples and 8 high-risk PCa samples (Gleason >7). Proteins significantly changing in abundance were selected for further selected reaction monitoring (SRM) validation in 53 urinary exosomes samples from PCa patients and 54 from benign counterparts. **Results and discussion:** We identified 1673 proteins including PSA, PSMA and ACP and selected a panel of 64 candidates for validation by SRM. Ultimately, we identified a profile of 2 novel urinary exosome-associated protein biomarkers after the comparison between benign and PCa patients and a promising profile of 5 proteins able to significantly distinguish between high (Gleason =7 (4+3)) and low (Gleason =7 (3+4)) risk patients. The presence of the candidates was confirmed in urinary exosomes of PCa and benign prostate pathologies patients by western blot and analysed in TMA PCa samples. In summary, our proteomic studies identified a list of markers which are very good candidates for evaluation of their clinical utility in future studies, in order to reduce PCa-related over-diagnosis and over-treatment.

PW8.02

Identification of prostate cancer protein biomarkers by proteomics of urinary extracellular vesicles

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Background: Prostate cancer (PCa) is the most common cancer in males. Extracellular vesicles (EVs) secreted by PCa cells can be found in urine. The cargo of urine-EVs therefore represents an attractive source for biomarkers to detect PCa in a minimally invasive manner.

Here we report on candidate PCa biomarker discovery using EV isolation via ultracentrifugation. Yet for clinical application, isolation should be easy and fast. Therefore, we investigated a novel EV-isolation method that captures EVs using heat shock protein (HSP)-binding peptides that aggregate HSP-decorated EVs (Ghosh, 2014). **Approach:** In this study, we determined which proteins were related to PCa using label-free proteomics in urine EVs (ultracentrifugation) from 3 control, 3 indolent and 3 aggressive PCa patients. Furthermore, the protein profile in HSP-isolated EVs was compared in urine samples of 2 control and 2 PCa patients by proteomics. **Results:** In urine EVs, > 3000 proteins were identified. Hierarchical cluster analysis separated aggressive from indolent and control patients. Candidate proteins (259) were selected based on significance ($p < 0.05$) and fold change (> 2). These proteins were functionally related to translation and cell migration. Known PCa-markers were differentially expressed, including PSA, PSMA, integrins, and CDH1. In addition, several non-reported candidates were identified. The overlap between the identified proteins isolated by HSP-kit and ultracentrifugation across all 4 patients was large (86%), with a correlation of $R=0.80$. EV fractions obtained by both methods were linked to established EV markers. In a preliminary exploration of biomarker potential, unsupervised hierarchical cluster analysis of the entire protein profile separated the control from the PCa-patients. **Conclusion:** Urine EV proteins are attractive as source for biomarkers. Furthermore, urinary EV isolation by HSP-binding peptides offers new opportunities in the identification and application of PCa biomarkers.

PW8.04

The potential of urinary exosomal miRNAs as biomarkers for prostate cancer

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Introduction: Prostate cancer is currently the most diagnosed cancer of Australian men, and the incidence is likely to keep increasing given our ageing population. Compounding this issue is the fact that the available tests are either discouragingly invasive or lacking in specificity/sensitivity and offer no prognostic information. To overcome these issues, we have been profiling the RNA content of exosomes isolated from human urine and assessing their utility as diagnostic/prognostic markers. **Methods:** Human urine was collected with consent from healthy, age-matched volunteers and prostate cancer patients. The patient urine was collected both before and after radical prostatectomy. Exosomes were then isolated from the urine samples using ultrafiltration, followed by ultracentrifugation. To ensure that exosome samples were of sufficient purity, NanoSight and DELFIA Europium assays were performed to determine particle size/concentration and to probe for exosomal markers. RNA was then extracted from the exosomes and analysed using Bioanalyzer Pico chips to ensure that only high-quality RNA was used for further analysis. Finally, the RNA samples were profiled using Taqman-based OpenArray technology. **Results:** All exosome samples isolated from urine contained particles of expected size with minimal contamination, and the markers CD9 and TSG101 were abundant in all samples, while AGO2 could not be detected. The Bioanalyzer confirmed previous results showing that exosome samples contained mostly small RNAs. The OpenArray results revealed 24 highly upregulated exosomal miRNAs, and 4 of these marker candidates became massively reduced in patient urine post radical prostatectomy. **Conclusion:** Urine exosomal RNA shows great promise as a source of diagnostic/prognostic markers for prostate cancer. Furthermore,

the non-invasive method of isolating urine is likely to fill a significant gap in the clinical care of prostate cancer.

PW8.05

A novel urinary EV-based prostate cancer assay that correctly predicts cancer grade as identified in the biopsy sample

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Introduction: Prostate cancer (PCa) remains the most commonly diagnosed cancer in men. Elevated blood PSA levels have led to unnecessary biopsies due to low specificity. Growing evidence suggests that EVs, and EV markers, are ideal candidates for liquid biopsy. Using our fast and easy Vn96 peptide EV isolation method, we are able to efficiently capture urinary EV markers that have clinical value for PCa. **Methods:** Patients scheduled for prostate biopsy agreed, per ethics approval, to provide us with post-DRE urine. Urinary EVs were isolated either by ultracentrifugation (UC) or using the Vn96 peptide (Vn) protocol. EV protein markers and prostate-specific protein markers were assessed by western blot (WB). Total and small RNA was extracted from EVs and urine sediment and analysed by qRT-PCR for prostate-specific markers. **Results:** WB revealed the presence of EV and prostate-specific protein markers. WB also shows an often greater abundance of these biomarkers with Vn-EVs compared to UC-EVs. We show improved test specificity for PCA3 when used with the Vn-EVs compared to urine sediment. Vn-based isolation increases PCA3/PSA ratio specificity by 14%. Urinary EV marker investigation led to the discovery of a 5 gene panel (5-bio) and a 10 biomarker panel (10-bio) with predictive PCa diagnosis, based on biopsy outcome. 5-bio used with Vn isolation showed better accuracy than with the UC isolation. 10-bio increased the specificity to 90% and the sensitivity to 75%. Where high-grade PCa (Gleason =7) was identified on the biopsy sample, the PCa assay using the Vn-EVs was able to predict the outcome with a sensitivity of 100%. **Summary/conclusion:** Here we have identified a Vn-isolated EV biomarker panel with strong predictive value for PCa. We have also shown that even better predictive value can be achieved as the cancer aggressiveness increases. We demonstrate here a novel urinary EV-based prostate cancer assay that is able to predict cancer grade using clinically viable tools.

PW8.06

Exosomal proteins as prostate cancer biomarkers in urine: from mass spectrometry discovery to antibody-based validation

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Introduction: Exosomes have recently appeared as a novel source of non-invasive cancer biomarkers since tumour-specific molecules can be found in exosomes isolated from biological fluids. In terms of prostate cancer, urinary exosomes may be a valuable source for biomarkers since urine is easily collected and reflects changes in the genitourinary system. We have analysed the proteome of urinary exosomes by mass spectrometry to identify proteins differentially expressed in prostate cancer patients compared to healthy male controls. Validation of potential biomarkers by methods more often used in the clinic, such as antibody-based methods, is ongoing. **Methods:** Exosomes were isolated by ultracentrifugation and subjected to several analyses to determine their concentration and quality. The proteome of urinary exosomes from 15 healthy control and 16 prostate cancer samples of urinary exosomes was analysed by mass spectrometry. Western blot and ELISA were used to validate the results of the mass spectrometry analysis. **Results:** Mass spectrometry analysis showed that 246 proteins were significantly changed in urinary exosomes from healthy controls compared to prostate cancer patients. At 100% specificity, 17 of these proteins displayed individual sensitivities above 60%. The highest sensitivity, 94%,

was observed for transmembrane protein 256. In order to validate the proteins identified by mass spectrometry as prostate cancer biomarkers, antibody-based methods have been used. We have validated several proteins by western blot and ELISA, but the validation of some proteins is challenging due to their low amounts and/or lack of good antibodies. **Summary/conclusion:** This study clearly shows the potential of using urinary exosomal proteins as prostate cancer biomarkers. Mass spectrometry is an excellent method for the biomarker discovery phase. However, since mass spectrometry is not widely used in the clinic, antibody-based methods based on the proteomic studies should also be developed.

PW8.07

Identifying extracellular vesicles-derived miRNAs in advanced prostate cancer

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The proliferation and survival of prostate cancer cells are regulated by the presence of steroid hormone androgens. We recently described the role of androgen in influencing the secretion of extracellular vesicles (EVs) in advanced prostate cancer. Here, we further investigate whether androgens also affect the secretion of small RNAs, such as micro RNAs (miRNAs), through EVs. The LNCaP prostate cancer cells were grown in the presence of foetal bovine serum or in androgen-depleted charcoal-stripped serum (CSS). Cells grown in CSS were treated with 10 nM physiological androgen dihydrotestosterone (DHT) or 10 µM anti-androgen enzalutamide. EVs were isolated from conditioned medium by differential ultracentrifugation and confirmed by EM. miRNAs were extracted by miRNeasy Mini Kit and analysed with Agilent Bioanalyzer 2100, followed by next-generation high-throughput Solexa sequencing (BG1). Data were analysed by an integrated in house tool miRDeep*. We found that growing cells in the presence and absence of androgens does not significantly alter the yield of total RNA found in isolated EVs. A total of 288 miRNAs were identified across EV samples, with 141 miRNAs (49%) are commonly found in all samples. We compared our result with miRNAs found in the corresponding parental cells (401 miRNAs), where 53.1% (213 miRNAs) were found in all cell samples. Quantitative analysis has led to the discovery of 34 candidate miRNAs which secretion in EVs was increased by > 1.5-fold upon DHT treatment, in comparison with cells grown in androgen-deprived condition. Among these, miR-454 was previously reported to regulate prostate cancer proliferation. Validation of longitudinal patient samples is ongoing. We conclude that in prostate cancer, androgens such as DHT can influence the secretion of small RNAs, including miRNAs, through EVs. Our work will help to validate EV-derived small RNAs as biomarker(s) in advanced prostate cancer.

PW8.08

Prostate cancer exosomes as molecular predictors of response to abiraterone acetate

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Prostate cancer is the second most common cancer in men worldwide. Organ-defined prostate cancer is a curable disease, but once it develops into metastatic castration-resistant prostate cancer, it is usually a matter of time until the patient develops resistance to chemotherapy, namely, abiraterone acetate, becoming an incurable disease. This evokes the need for biomarkers that can predict disease

progression and response to therapy. Cancer cell-derived exosomes, with their molecular cargo that represents the tumour cells, have been demonstrated to be a good source of such prognostic and predictive biomarkers. In this study, we have developed prostate cancer cell lines that show increased resistance to abiraterone acetate, which were then used to isolate exosomes through differential ultracentrifugation. We then characterized these exosomes by western blot, nanoparticle tracking analysis, transmission electron microscopy and linear sucrose gradient ultracentrifugation. It was followed by a molecular profiling of the exosomes based on a proteomics approach, where we compared the protein profile of these exosomes with exosomes from the parental, drug-sensitive cell lines, in order to find a predictive signature of response to therapy. This signature was then validated in exosomes isolated from human plasma samples derived from patients that were non-responsive to abiraterone acetate. One interesting finding is that cells that are resistant to abiraterone acetate secrete more exosomes than their sensitive counterparts. We have also identified a number of putative markers that may constitute a predictive signature of response to therapy. This molecular data may provide valuable information in finding predictive signatures for response to therapy, providing a more personalized medical care and stratified course of treatment, leading to improved overall survival.

PW8.09

Identifying components that regulate the secretion of stroma-activating exosomes in prostate cancer

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¹Cardiff University, Cardiff, United Kingdom; ²Division of Cancer & Genetics, School of Medicine, Cardiff University, Cardiff, United Kingdom

Introduction: Exosomes originate in multivesicular endosomes (MVEs) and are expelled into the extracellular space to mediate important effects. In cancer, local interaction of exosomes with stromal cells (fibroblasts) and angiogenic precursors serves to promote tumour growth and progression. Secreted exosomes are known to be heterogeneous, in terms of biophysical and molecular properties, and it is unclear how this heterogeneity is generated by a given homogeneous cell source. Blockade of some proteins regulating MVE trafficking (such as Rab27a) may not fully attenuate exosome secretion or may exhibit cell-type-dependent effects. Perhaps there are co-existing, parallel pathways for generating and trafficking distinct MVEs and hence provide exosome heterogeneity. To better understand this, we have investigated the roles of 6 proteins: CD9, Rab5a, Rab11b, Rab35, VAMP7 and VPS25, putatively implicated in exosome biogenesis/secretion, and their subsequent functional impact on stromal cells. **Methods:** A lentiviral-based delivery of shRNAs was used to selectively knockdown targets of interest in Du145 cells. Exosomes present in cell-conditioned media (CCM) were quantified and characterized, by nanoparticle tracking analysis and ELISA, respectively. Fibroblast activation, in response to CCM, was evaluated microscopically based on organization of alpha-smooth muscle actin fibers. **Results:** Successful stable knockdown of all targets, by at least 80%, was demonstrated at both mRNA and protein levels. Knockdown of Rab35 or VPS25 resulted in greatest attenuation of exosome secretion, with a corresponding failure to induce stromal activation compared to functionally competent control Du145 cells. **Conclusion:** These results suggest that both Rab35 and VPS25 are relevant factors for modulating exosome secretion by Du145 prostate cancer cells. Targeting these proteins may be sufficient to attenuate exosome-mediated stromal cell activation and slow tumour growth.

PW8.10

Immunosuppressive effects of tumour exosomes on dendritic cells cross-presenting tumour antigens

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Introduction: Exosomes are a distinct population of extracellular vesicles of endocytic origin, which express a protein repertoire similar to the cell of origin. Tumour-derived exosomes harbour the immunosuppressive characteristics of tumour cells. However, as tumour-derived exosomes carry tumour antigens, they may contribute to cross-presentation of antigen by DC to initiate antitumour immune responses. The aim for this study was to examine how exosomes secreted by prostate cancer cells influence tumour antigen cross-presentation. **Methods and results:** In order to investigate the effects of exosome secretion, DU145 cells were transduced with either shRNA lentiviral particles to knockdown Rab27a (DU145KD) or an irrelevant control (DU145C). Rab27a deficiency was characterized for attenuation of exosome secretion. Cross-presentation of the tumour-associated antigen 5T4 from DU145KD cells generated significantly stronger anti-5T4 T-cell responses compared to that from DU145C cells. This enhanced T-cell response was reduced when purified exogenous DU145 exosomes were added back to the cross-presentation model incorporating DU145KD cells. This suggests that knockdown of exosome secretion alleviates tumour-associated immunosuppression in this model. While exosomes did not directly inhibit CD8+ T-cell function, they exerted a negative effect on DC function. DC exposed to exosomes had an elevated expression of CD73, an ecto-5-nucleotidase responsible for AMP to adenosine hydrolysis. DC constitutively express CD39 (ectonucleotidase responsible for ATP-to-AMP hydrolysis) which, when co-expressed with CD73, resulted in ATP-dependent inhibition of TNF α and IL-12 release. Inhibition of PGE2 receptors inhibited exosome-dependent CD73 expression on DC, pointing towards a possible mechanism. **Conclusion:** Taken together, the results reveal a hitherto unknown effect of tumour exosomes on the suppression of DC function via exosomal PGE2 adding a new element to tumour-immune cell crosstalk.

PW8.11

Exosome DNA: a new promising liquid biopsy-based diagnostic tool for personalized management of aggressive prostate cancer (PCa) patients
 Chiara Foroni¹, Simona Bernardi², Natasa Zarovni³, Domenico Russo⁴, Francesca Valcamonica⁵, Alfredo Berruti⁵ and Davide Zocco³

Please see OPW3.8

PW8.12

Development of a biomarker-based extracellular vesicle assay for prostate cancer prognosis

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New, more specific biomarkers for prostate cancer (PCa) are needed to prevent unnecessary surgical biopsies. Analysis of extracellular vesicles (EVs) in biofluids is an exciting new area of investigation. A diagnostic test must differentiate EVs originating from PCa cells versus non-cancerous origins, and also define a biomarker enriched in PCa EVs. Five prostate-specific membrane antigen (PSMA) antibodies were tested. Plasma, serum, urine and semen were collected from healthy men and PCa patients. Assay conditions were optimized including sequential centrifugation of each biofluid to identify the optimal fraction for analysis. EV and biomarker levels were determined using the NanoSight LM10 and micro-flow cytometry using the Apogee A50, western blotting and TEM during optimization studies. A cohort of 500 plasma and urine samples were prospectively collected from men prior to prostate biopsy and are currently being

analysed for PSMA and other PCa biomarkers. Detection of PSMA+ EVs in biofluids and cell culture media was shown to be optimal using the J591 antibody, which detects an extracellular domain of PSMA. Detection of PSMA+ EVs was shown to be linear with concentration and assay volume. The specificity of the PSMA antibody was shown using LNCaP cells with immunofluorescence as well as standard flow cytometry. The specificity of our micro flow cytometry assay was confirmed using antibody challenge experiments. To date, we have examined ~35% of our pre-diagnosis cohort (plasma) in a blinded manner. Preliminary analysis of EVs from platelet-depleted plasma collected pre-biopsy revealed that men testing positive for PCa (+Gleason) had significantly more extracellular vesicles than men testing negative (~1.6-fold increase). PSA levels in these samples were significantly ($p=0.0021$) but weakly ($r_2=0.06$) correlated with extracellular vesicles staining positive for PSMA. Our assay holds great promise for detection of novel biomarkers in PCa.

PW8.13

Can exosome-associated heparan sulphate proteoglycans serve as potential new markers for the detection of prostate cancer?

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¹Division of Cancer and Genetics, School of Medicine, Cardiff University, Cardiff, UK; ²Cardiff School of Health Sciences, Cardiff Metropolitan University, Cardiff, UK

Introduction: Current diagnostic tests for prostate cancer lack sensitivity and specificity, resulting in underdiagnosis of men with aggressive disease. Improved means of detecting the disease are therefore urgently needed. We have previously demonstrated a role of heparan sulphate proteoglycans (HSPGs), present on the surface of exosomes, in stromal cell activation and subsequent enhancement of tumour growth. Here we explore the potential of exosomal-HSPGs, isolated from serum using size-exclusion chromatography columns, as potential biomarkers for disease. **Methods:** Exosomes were isolated from cell-conditioned media, from prostate cancer cells cultured in bioreactor flasks, using the sucrose cushion method. To aid assay development, donor serum was spiked with exosomes prior to size-exclusion chromatography-based exosome isolation. Exosome-associated and soluble proteins were identified by immunofluorescent analysis of eluted fractions. Exosome concentration was determined by nanoparticle tracking analysis, and total protein content measured by BCA-assay. The particle:protein ratio was used as a measure of exosome purity. **Results:** Our data reveal detection of several HSPGs (including glypican 1, glypican 6 and syndecan 4) on exosomes from multiple prostate cancer cell lines. Size-exclusion chromatography proved a successful method for isolation of exosomes from serum, enabling detection of HSPGs present on prostate cancer exosomes. Furthermore, we report increased levels of heparan sulphate on serum-derived exosomes from prostate cancer patients compared to healthy individuals. **Conclusion:** Defined HSPGs are present on the surface of prostate cancer exosomes. We have developed an assay capable of detecting these HSPGs on exosomes isolated from serum. Future work will explore whether expression of specific HSPGs correlates with disease progression in prostate cancer patients and will evaluate their potential as biomarkers for aggressive disease.

PW8.14

CRIPTO 1 is associated with tumour aggressiveness in prostate cancer

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Introduction: Prostate cancer (PCa) is the most common malignancy and a prevalent source of cancer-related morbidity and mortality in men. Identification of tumour biomarkers may help distinguish significant from indolent PCa and select patients at high risk of relapse for aggressive treatment. CRIPTO (CR-1), the founding member of EGF-CFC protein superfamily, plays crucial roles in embryonic development. In humans, CR-1 is expressed in a wide spectrum of tumours, but its implication in PCa has remained unexplored. Here, we investigated the expression pattern of CR-1 and the consequences of its expression in PCa setting in order to assess its potential impact on prostate malignancy. **Methods:** Prostate normal and cancerous tissue specimens were examined by immunohistochemistry. PCa cell lines were engaged in experimental studies and analysed using techniques used in biochemistry, cellular and molecular biology. **Results:** We found significant CR-1 expression in 37.9% of PCas, while being absent or marginally detected in benign conditions. Our studies demonstrated that CR-1 overexpression plays a functional role in PCa cells by promoting an epithelial-mesenchymal transition (EMT) associated with enhanced migration capacity and survival under stress conditions due to propensity to stimulate PI3K/AKT and FGFR1/ERK signalling pathways. More interestingly, cells overexpressing CR-1 excessively secreted vesicles. Among preliminary results obtained and upon treating different cell line models, CR-1-rich vesicles stimulate the aggressiveness in prostate cancer. **Summary/conclusion:** Our results highlight a new potential function of CR-1 in PCa and identify a new exosomal form of this protein. All these findings suggest CR-1 as a new promising target for PCa treatment.

PW8.15

Innovative approach of prostate cancer by the way of tumour cell-derived extracellular vesicles

Irene Tatischeff

RevinterCell, a Scientific Consulting Service about EVs, Orsay, France

Introduction: Prostate cancer represents the second most important cancer in men in the USA, with a great inconvenience but a rather low mortality. As for other cancers, an early diagnostic greatly enhances the efficiency of a subsequent therapy. Since 1986, the prostate-specific antigen (PSA) blood test brought a new tool, which, together with prostate palpation, helps the medical diagnostic. For prognosis, the aggressivity of the tumoural process is measured with both the Gleason Score and PSA. However, the specificity of PSA is now seriously questioned, as it diagnoses many false-positive patients, who may be submitted to inappropriate therapy. The need for other more reliable diagnostic tools of prostate cancer is therefore a current challenge. The aim of this study is to gather the main results obtained about "Prostate Cancer and EVs", in the 4 previous yearly ISEV International Meetings from 2012 to 2015, in order to get a leading thread for future research. **Methods:** Altogether, 107 abstracts (44 oral communications and 63 posters) dealing with "Prostate" were presented in 60 different sessions. Here, these abstracts have been classified as a function of their main respective goal: characterization of the various prostate cancer-derived EVs, search for biomarkers at the proteomics or RNAs levels, influence of prostate cancer-derived EVs from various cell lines upon normal cells, EVs clinical measurements in different human biofluids and in vivo models. New set-ups for sensitive exosome measurements, having a clinical potential for diagnostics, are also reported. **Summary/conclusion:** The main results of this scientific monitoring will be presented. Many interesting observations have already been collected about the characterization and functional properties of prostate cancer cells-derived EVs. However, a better research coordination about EVs potentials for theranostics of prostate cancer would be worth seeking.

Room: Jurriaanse

Satellite Event – Meet the National and International Societies

Chairs: *Lorraine O’Driscoll and Maria Yañez-Mo*

6:30-8:30 p.m.

This event will provide an opportunity for scientific networks and consortia focusing on extracellular vesicles to introduce themselves to the ISEV community.

Australasia Extracellular Vesicles - Representative: Prof. Andy Hill

GEIVEX - Representative: Prof. Hernando A. del Portillo

H2020 COST Action Microvesicle & Exosomes in Health and Disease (ME-HaD) - Representative: Prof. Lorraine O’Driscoll

Japanese Society for Extracellular Vesicles (JSEV) - Representative: Prof. Takahiro Ochiya

Korean Society for Extracellular Vesicles (KSEV) - Representative: Prof. Yong Song Gho

Network of Expertise on Extracellular Vesicles (NEEV) - Representative: Dr Olivier de Wever

Regional Research Network on Extracellular Vesicles (RRNEV) - Representative: Prof. Reidun Ovstebo

SOCRATES - Representative: Prof. Tan Kok Hian

UKEV Forum - Representative: Dr. Dave Carter

A French EV Society: Representatives: Dr Guillaume van Niel and Dr Clotilde Théry

Scientific Program ISEV 2016 meeting Thursday May 5, 2016

Room: Willem Burger

Meet the Experts Session 1 - EV Heterogeneity: Can we Overcome the Confusion?

Speakers: *Clotilde Thery and Dolores Di Vizio*

Moderator: *Andy Hill*

8:00-8:45

Room: Jurriaanse

Meet the Experts Session 2 - EVs in Regenerative Medicine

Speakers: *Giovanni Camussi and Sai Kiang Lim*

Moderator: *Eva Rohde*

8:00-8:45

Room: van Weelde

Meet the Experts Session 3 - EVs in Infectious Disease

Speakers: *Ana Claudia Torrecilhas and Yong Song Gho*

Moderator: *Hernando Del Portillo*

8:00-8:45

Oral Presentations

Room: Jurriaanse

Symposium Session 10 - EVs in cross-species communication

Chairs: *Yong Song Gho and Ana Claudia Torrecilhas*

9:00-10:15 a.m.

OT10.1

Non-enveloped Leishmania RNA virus 1 (LRV1) is released by Leishmania parasites within exosome-like vesicles

Vanessa Diniz Atayde and Martin Olivier
McGill University, Montreal, Québec, Canada

Leishmania are ancient eukaryotes that have retained the exosome pathway through evolution. Our work focuses on Leishmania RNA virus 1 (LRV1)-infected Leishmania species, which are associated with a particularly aggressive mucocutaneous form of leishmaniasis triggered by a TLR3-dependent response to the viral dsRNA. Thus far, how LRV1 is exposed to the host cell is unravelled. Some viruses are known to utilize the host exosome pathway for their formation and cell-to-cell spread. As a result, exosomes derived from infected cells contain viral proteins and RNAs. Herein, we propose that LRV1 exploits the Leishmania exosome pathway in order to reach the extracellular environment. To test this, exosomes derived from LRV1-infected Leishmania were characterized by biochemical approaches. Sucrose gradients revealed that most of LRV1 dsRNA co-fractionated with exosomes. Interestingly, when fractions were observed by TEM, a portion of the LRV1 particles was found surrounded by these vesicles. Exosome-trapped particles were protected from RNaseIII degradation, further confirming our findings. Mass spectrometry showed that exosome derived from infected parasites had a differed overall content compared to non-infected ones. To understand the biological potential of LRV1-containing exosomes, preparations were used in transfer assays where, remarkably, a significant amount of non-infected parasites presented an early and transient assimilation of LRV1, mechanism that may be involved in viral maintenance. Our work is one of the first reports describing exosomes as viral envelopes, which may represent an important step in the evolution of some non-enveloped virus into enveloped ones. Moreover, it revealed that Leishmania secretes LRV1 possibly to present viral material to the host as well as to transmit the virus to other parasites, significantly impacting the fate of cutaneous leishmaniasis.

OT10.2

Extracellular vesicles from milk accelerate osteoblastogenesis but impair bone matrix formation

Marina C. Oliveira¹, Adaliene V. M. Ferreira¹, Fons A. J. Van De Loo² and Onno J. Arntz²

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Introduction: Recently extracellular vesicles (EVs) that contain proteins and RNA were discovered in milk but their effect on bone formation has not yet been determined. We demonstrated previously that bovine milk-derived EVs (BMEVs) have immunoregulatory properties. Our aim was to evaluate the effect of bovine milk-derived EVs (BMEVs) on osteogenesis by mice and human mesenchymal stem cells (hMSCs). **Methods:** BMEVs were isolated from commercial cow milk by ultracentrifugation. Female 10–12-week-old DBA/1J mice received BMEVs (4.7×10^6 particles/mL or 14.3×10^6 /mL) in their drinking water for 7 weeks. Thereafter, trabecular bone area (lamellar/woven) and density of osteocytes per trabecular bone area were determined by histomorphometry. Human MSCs were cultured in osteogenic differentiating medium and BMEVs were added. Cell proliferation was determined by a crystal violet staining and the XTT assay. Osteoblast differentiation was evaluated by Alizarin red staining (mineralization), Sirius red (collagen), alkaline phosphatase

activity and by qRT-PCR. **Results:** Oral delivery of BMEVs to female DBA/1J mice during 7 weeks did not alter the tibia trabecular bone area, however increased osteocyte numbers were found. In addition, the highest dose of BMEVs markedly increased the woven bone tissue. Exposure of hMSCs to BMEVs for 21 days resulted in less mineralization but higher cell proliferation. Interestingly, BMEVs reduced the collagen production, but enhanced the expression of characteristic genes for immature osteoblasts. A kinetic study showed that BMEVs upregulated many osteogenic genes within the first 4 days. However, the production of type I collagen and its genes (COL1A1 and COL1A2) were markedly reduced at days 21 and 28. At day 28, BMEVs enhanced the expression of sclerostin, an osteocyte marker. **Conclusion:** Our study adds BMEVs to the list of milk components that can affect bone formation and may shed new light on the contradictory claims of milk on bone formation.

OT10.3

Assessment of the transcriptional response of human bladder cells to uropathogenic Escherichia coli membrane vesicles

Cherie Blenkiron, Denis Simonov, Priscilla Daurios Singorenko, Cristin Print, Anthony Phillips and Simon Swift
University of Auckland, Auckland, New Zealand

Introduction: Uropathogenic *Escherichia coli* (UPEC) have been reported to use secreted membrane vesicles (MVs) to deliver toxins into human cells. While phenotypic effects of individual MV-associated toxins on human cells have been described, no study to date has evaluated the human cell transcriptional response to UPEC MVs as a whole. **Methods:** The total MV population was isolated from UPEC strain 536 cultures by filtration and ultracentrifugation. MV uptake into bladder cells was investigated with confocal microscopy by labelling vesicle membranes with a fluorescent dye PKH67. Effects of MVs on bladder cell viability were measured using the multi-table tournament (MTT) assay. The transcriptional response of bladder cells to UPEC 536 vesicles was investigated using Affymetrix GeneChip(r) PrimeView(tm) Human Gene Expression Arrays. Quantitative real-time PCR was used to validate changes in expression of bladder cells genes identified from the microarray analysis. **Results:** MVs were delivered into cultured human bladder cells and caused reduced cellular viability. Analysis of gene expression in treated cells found that there was a rapid response after just 4 h that triggered the expression of genes responsible for an early response to infection and pathways responsible for xenobiotic metabolism. These findings will be presented and discussed. **Ongoing work:** Based on the initial data generated using a total MV preparation, we are now in the process of using density gradient based purification techniques to elucidate the component MV fraction(s) that trigger the xenobiotic metabolism pathway and associated reduction of human bladder cell viability. **Conclusions:** Our study programme has provided insights into the complex events underlying uropathogenesis by identifying pathways involved in the host bladder cell response to cytotoxic MVs produced by UPEC.

OT10.4

Investigation of extracellular small RNAs of Heligmosomoides polygyrus: scope for RNAi mediated cross-species communication

Franklin Wang-Ngai Chow¹, Cesare Ovando-Vazquez², Georgios Koutsovolos³, Tuhin Maity⁴, Mark Blaxter³, Julie Claycomb⁴, Cei Abreu-Goodger² and Amy Buck¹

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Introduction: Extracellular small RNA has been proposed as a means of cell-to-cell communication within an organism and a mechanism of cross-species communication. We previously showed that an argonaute protein (HpWAGO) and small RNAs including miRNAs and Y-RNAs are secreted in exosome-like vesicles produced by *Heligomosomoides polygyrus*, a gastrointestinal nematode that infects mice. Intranasal administration of the *H. polygyrus* vesicles to mice suppressed a Type 2 innate response in an airway allergy model and our work suggests that some of the nematodes-derived miRNAs can suppress host gene expression based on in vitro reporter assays. We implicated miRNAs as one of the functionally important classes of exRNAs. Here we examine the other classes of exRNAs that could be important for host–pathogen interactions, many of which are more abundant than miRNAs and could operate by distinct RNAi pathways. We further characterize the secreted argonaute protein and aim to identify additional protein binding partners of exRNA. **Results and discussion:** *H. polygyrus* excretory and secretory products (HES) were purified by size exclusion chromatography to examine co-fractionation of proteins with small RNAs. Small RNA NGS libraries revealed that large amounts of 5′-triphosphate 22G small RNAs are present in exosomes. Many 22G small RNAs were mapped to the same loci within the genome, suggesting they are originated from “22G siRNA cluster,” which could be features of RdRp amplified 22G siRNAs. Differential expression levels of Y-RNA, miRNAs and fragments of tRNA were also found inside and outside exosomes and several appear to co-fractionate with HpWAGO, suggesting potential RNA-protein complexes. **Conclusion:** Together these results suggest further diversity in the exRNA that is secreted by parasitic nematodes

and lays a foundation for further interrogation of the role of exRNA–protein complexes in cross-species communication.

OT10.5

Gut bacteria generated extracellular vesicles mediate cross-kingdom dialog in the gut

Regis Stentz, Isabelle Hautefort and Simon Carding
Institute of Food Research, Norwich, England

The adult human gastrointestinal (GI-) tract accommodates a bacterial community comprising trillions of cells that is dominated by two bacterial phyla, Firmicutes and Bacteroidetes. It is becoming increasingly evident that maintaining communication between those microbial populations and the host is essential for establishing and maintaining a healthy GI-tract. Considerable effort is now being made to determine the precise nature of this dialog with much of the past work focusing on the contributions made by diffusible, small-molecule hormones and nutrients. However, more recently, we have uncovered a role for extracellular outer membrane vesicles (OMVs) produced by Gram-negative bacteria in communicating with host cells in the lower GI-tract. The OMVs produced by the prominent commensal gut bacterium *Bacteroides thetaiotaomicron* (Bt) contain an inositol polyphosphate phosphatase (BtMinpp) that is structurally similar to mammalian Minpp1 and interacts with cultured intestinal epithelial cells to promote intracellular Ca²⁺ signalling. We have also shown that OMVs from Bt parental cells are normally produced in vivo in the GI-tract and associate with the intestinal epithelium. This finding identifies a novel pathway of microbe–host interaction in the GI-tract and challenges the paradigm that OMVs are associated with bacterial pathogens and the delivery of virulence factors and toxins.

Room: Willem Burger

Symposium Session 11 - EV characterization: flow cytometry

Chairs: *Marca Wauben and Alain Brisson*

9:00-10:15 a.m.

OT11.1**Optimization of imaging flow cytometry for exosome analysis using a biological calibrator**

André Görgens¹, Michel Bremer¹, Florian Murke¹, Coralie L. Guerin², Sebastian Seth³, Chantal M. Boulanger⁴, Peter A. Horn¹ and Bernd Giebel¹
¹Institute for Transfusion Medicine, University Hospital Essen, Germany; ²Luxembourg Institute of Health, Department of Infection and Immunity, Luxembourg; ³Merck Chemicals GmbH, Merck KGaA, Darmstadt, Germany; ⁴Cardiovascular Research Center at HEGP, Institut National de la Santé et de la Recherche Médicale, Paris, France

Extracellular vesicles (EVs) such as exosomes (70–160 nm) and microvesicles (100–1000 nm) can be harvested from cell culture supernatants and from all body fluids. Current standard techniques to visualize, quantify and characterize EVs are electron microscopy, nanoparticle tracking analysis (NTA) and dynamic light scattering. To further characterize and discriminate EVs, more exact high-throughput technologies to analyse the surface of EVs are highly desired. While conventional flow cytometry is limited to measure particles down to approx. 300–500 nm, a new flow cytometric method called imaging flow cytometry potentially allows for the analysis of EVs smaller than 300 nm. Here, we prepared eGFP-positive EVs from cell lines expressing CD63-eGFP fusion proteins. Using these biological calibrators, which can be clearly separated from background due to their fluorescence, we optimized different machine/acquisition parameters and the analysis protocol on the Amnis ImageStreamX MkII imaging flow cytometry platform. Obtained samples were also analysed by NTA. The main advantages of imaging flow cytometry over conventional flow cytometry is the low signal-to-noise ratio, the automatic simultaneous triggering on all channels and an improved sensitivity to detect low fluorescence signals. Using the optimized protocol, we were able to visualize and enumerate different types of individual EVs (i.e. apoptotic bodies, microvesicles and exosomes) simultaneously in unprocessed cell culture supernatants and samples enriched for exosomes. We currently compare EVs derived from different cell types and use the optimized platform to evaluate and optimize protocols used for exosome isolation. Furthermore, we compare different protocols using antibodies or membrane dyes for EV labelling. We propose that imaging flow cytometry provides an improved and sensitive high-resolution method for the field of EV research to perform high-throughput, multiparametric EV surface analyses.

OT11.2**Optimization of bulk extracellular vesicle labels for nanoFACS**

Aizea Morales-Kastresana, Katherine McKinnon, Jennifer Jones, Jay Berzofsky, Masaki Terabe, Thomas Musich and Thorsten Demberg Vaccine Branch, NCI, NIH, Bethesda, MD, USA

Introduction: The source and characteristics of extracellular vesicles (EVs) are associated with distinct functional roles, suggesting the existence of different EV subsets with relevance in immunity and cancer. Most high-resolution flow cytometry methods use a fluorescent threshold, but this approach does not detect EVs that are too dimly labelled to be detected above the threshold of the instrument. To circumvent this problem, we used a high-resolution SSC light scattering-based threshold to quantitate the proportion of the EV population that is labelled and to examine the presence of non-specific by-products of certain labelling methods. EVs isolated from DC2.4 cell line culture supernatants by ultracentrifugation were

characterized by NanoSight. NanoFACS was performed with an AstriosEQ flow cytometer, triggered with a high sensitivity SSC detector for EV analyses based on light scattering, in addition to standard FSC and fluorescent parameters. We tested several widely used lipid (PKH26, CMDil), RNA (SYTO RNA select) and protein-binding (CFSE, CellTrace Violet, CellTrace FarRed) dyes. Incorporation of CFSE/fluorescein was quantitated relative to MESF standards. **Results:** We found that lipid and RNA dyes form aggregates that confound EV flow cytometric analysis. On the other hand, protein-binding CFSE and CellTrace dyes did not produce these artefacts, and unbound dye was readily removed with size exclusion chromatography. We refined the staining protocol for protein-binding dyes to maximize EV staining efficiency and identified optimal timing, temperature and wash conditions for bulk vesicle labelling. **Conclusions:** This optimized protocol provides a robust means of labelling purified EV populations for single vesicle, high-resolution flow cytometric analysis. Our data also demonstrate the limitations of lipid- and RNA-based dyes for individual vesicular cytometric analysis and demonstrate the importance of robust controls and parallel methods of analysis for EV subset studies.

OT11.3**Diversity of extracellular vesicles in biofluids measured by vesicle flow cytometry**

John Nolan¹, Erika Duggan¹, Julie Saugstad², Ji Phillips², Jf Quinn², Kendall Van Keuren-Jensen³, R. Reiman³, Matthew Huentelman³, Doug Galasko⁴, Bob Carter⁴ and Fred Hochberg⁴
¹Scintillon Institute, San Diego, California, USA; ²Oregon Health & Science University, Portland, Oregon, USA; ³Translational Genomics Research Institute, Phoenix, Arizona, USA; ⁴University of California, San Diego, San Diego, California, USA

Background: Plasma and cerebrospinal fluid (CSF) offer potential biomarkers originating from cell-free nucleic acids and extracellular vesicles (EVs). Analysis of the heterogeneity of these biofluids informs our understanding of their cellular source, physiological role and clinical significance. We use a novel high sensitivity flow cytometry approach to count individual EVs in these biofluids, estimate their size and measure their molecular composition. **Methods:** Cell-free plasma was prepared from normal human blood (San Diego Blood Bank) and cell-free CSF was from patients and normals under informed consent. Cell-free biofluids were diluted and analysed (NTA) to estimate total nanoparticle concentration and size distribution and diluted and stained for analysis of membrane vesicle concentration, size distribution and surface marker abundance by vesicle flow cytometry (VFC). **Results:** Nanoparticle concentrations in normal plasma measured by NTA ranged from 1.9 to 2.4 × 10⁹/uL, and sizes ranged from 50 to > 300 nm in diameter (mean 122 nm). EV concentrations in normal plasma measured by VFC ranged from 0.9 to 1.8 × 10⁸/uL, and sizes ranged from 70 to > 300 nm. VFC of plasma EVs stained positive for annexin V, CD41, CD235, CD14, CD11b and CD86. Nanoparticle concentrations in normal CSF measured by NTA ranged from 2.6 to 31 × 10⁶/uL, and sizes ranged from 50 to > 300 nm in diameter (mean 122 nm). EV concentrations in normal CSF measured by VFC ranged from 1.1 to 2.2 × 10⁶/uL, and sizes ranged from 70 to > 300 nm. VFC revealed EVs staining for annexin V, CD41 and CD235. VFC of CSF pools from patients with 6 neurological disorders had EV concentrations ranging from 2.22 to 14 × 10⁶/uL. **Conclusions:** Quantitative analysis of the diversity of individual EVs in plasma, CSF and other biofluids using VFC provides a new window into the cellular origins, physiological roles and clinical biomarker utility of these cell-derived nanoparticles. Funded by NIH EB003824, TR000931, TR000891, and TR000903.

OT11.4

Towards EV-based biomarker detection using high-resolution flow cytometric analysis of rare events

Sten Libregts¹, Ger Arkesteijn¹, Andrea Németh², Esther Nolte-t Hoen¹ and Marca Wauben¹

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Introduction: Extracellular vesicles (EVs) are promising biomarkers, since their molecular content may contain important information regarding disease state and efficiency of medical intervention. Isolation, detection and characterization of EVs are, however, hampered by their size and heterogeneity, and the complexity of body fluids. Moreover, disease-related EV may appear as rare events and could be obscured by the bulk of EVs and other nano-sized particles present in body fluids. **Methods:** We have recently developed a high-resolution flow cytometry-based method (Hi-Res FCM) that allows multi-parameter analysis of EVs. To see whether Hi-Res FCM also allows for detection of rare populations of nano-sized particles, we performed spike-in experiments using fluosphere beads or EVs stained with PKH67 and fluorochrome-conjugated antibodies. Particles were spiked into either PKH67-stained EV samples or body fluids that were subsequently run over a SEC-column. Samples were then analysed using Hi-Res FCM. **Results:** Events that occur at a frequency of 1:1000 (0.1%) or less are considered rare events. Based on fluorescence and light scattering, spiked 200 nm fluospheres could readily be detected in PKH67-stained EV samples up until a frequency of 0.03%. Spiked multi-parameter-stained EVs, however, could not be distinguished from other PKH67-positive EVs at low percentages due to a low signal-to-noise ratio of fluorescence. Notably, scatters of spiked EVs changed significantly when spiked into unstained body fluids, indicating that the matrix of body fluids can affect detection of EVs using Hi-Res FCM. **Conclusion:** Hi-Res FCM technically allows for the detection of rare populations of nano-sized particles. However, for EVs to serve as biomarkers for the detection of early-stage disease, residual disease or therapy response, isolation and fluorescent antibody-labelling strategies for rare-event analysis need further optimization due to low signal-to-noise ratios and the complexity of body fluids.

OT11.5

Surface plasmon resonance imaging detects lower antigen densities than flow cytometry

Elmar Gool¹, Edwin Van Der Pol¹, Rienk Nieuwland², Leon Wmm Terstappen³, Auguste Sturk², T. G. Van Leeuwen¹, Anita Böing², Richard Schasfoort³, Frank Coumans¹ and Ivan Stojanovic³

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Introduction: The flow cytometry (FCM) mean fluorescence intensity (MFI) is proportional to the mean number of fluorochrome-antigen conjugates. However, the MFI of such conjugates on extracellular vesicles (EVs) is often undistinguishable from background due to low antigen quantities. Surface plasmon resonance imaging (SPRI) is an alternative for analysing antigen exposure, which captures EVs on a surface with an antibody array. The captured EVs cause an increase in refractive index (RI) at the surface. This RI is directly proportional to the SPRI signal, which was shown to correlate with antigen exposure on cells. Here we compare antigen exposure on EVs measured with FCM and SPRI. **Methods:** To compare cell surface antigen exposure measured by FCM and SPRI, human breast cancer cell lines H5578T, MCF7 and SK-BR-3 were used. The MFI was compared with the SPRI signal for 11 antibodies on intact cells and EVs derived from each cell line. The antibodies for CD9, CD44, CD49e, CD63, CD71, CD221, CD227, EGFR, EpCAM, Her2 and Her3 recognize cell surface antigens that are expressed on these cell lines. **Results:** On intact cells, the MFI and SPRI signals obtained with the eleven antibodies correlate for H5578T ($R^2 = 0.66$), MCF7 ($R^2 = 0.82$) and SK-BR-3 ($R^2 = 0.96$). Due to the limited number of antigens with MFI above background for EVs, correlation between FCM and SPRI could only be determined for H5578T derived EVs ($R^2 = 0.65$). These EVs had 5 antigens with MFI above background, while MCF7 and SK-BR-3 derived EVs only had 1 and 2, respectively. Interestingly, SPRI signals were above background for not only these 8 antigens, but for in total, respectively, 7 (H5578T), 7 (MCF7) and 6 (SKBR3) antigens. The remaining 4, 4 and 5 antigens, respectively, had signals below background for both techniques. These undetectable antigens also had signals below background for intact cells, except for CD227 on SK-BR-3 cells. **Conclusion:** SPRI detects lower antigen densities on EVs than FCM.

Room: van Weelde

Symposium Session 12 - EVs in cardiovascular disease

Chairs: *Ramaroson Andriantsitohaina and Susmita Sahoo* 9:00-10:15 a.m.**OT12.1****An extracellular vesicle protein signature as a new diagnostic biomarker for stable coronary artery disease**Ingrid Bank¹, Dominique De Kleijn², Leo Timmers¹, Vince De Hoog¹, Crystel Gijssberts², Gerard Pasterkamp² and Arend Mosterd³¹Cardiology, University Medical Center Utrecht, Utrecht, The Netherlands;²Experimental Cardiology, University Medical Center Utrecht, Utrecht,The Netherlands; ³Cardiology, Meander Medical Center, Amersfoort, The Netherlands

Introduction: The diagnostic workup for stable coronary artery disease (SCAD) is inefficient and expensive. Exercise ECG and non-invasive imaging techniques such as cardiac CT, MRI and nuclear perfusion imaging are frequently used, but invasive coronary angiography is often needed to diagnose (or rule out) significant coronary artery stenosis. Diagnostic blood-based biomarkers do not exist. We hypothesized that a plasma extracellular vesicle (EV) protein signature can be used as diagnostic biomarker for SCAD. **Methods:** Five extracellular vesicle proteins (SerpinF2, SerpinG1, SerpinC1, CD14, and Cystatin C) were measured in the LDL, HDL, remaining (REX) and total (TEX) plasma fractions of n = 30 patients with stable coronary artery disease and n = 30 sex-, age-, history-, cardiovascular risk factor-, and medication-matched controls, using sequential precipitation and an immune-bead assay. Patients were selected from the MYOMARKER cohort, containing patients undergoing myocardial perfusion imaging (Rubidium-82 PET-CT) in the diagnostic workup for suspected stable angina in the cardiology outpatient clinic. The MYOMARKER study complies with the Declaration of Helsinki and was approved by a recognized medical ethics committee (VCMO). Informed consent was obtained from all patients. **Results:** EV levels of SerpinC1 most accurately discriminated between patients with stable coronary artery disease and matched controls. SerpinC1 with the ratio HDL/TEX and an AUC of 0.842 (95% CI 0.741–0.944); SerpinC1 with the ratio HDL/REX and an AUC of 0.828 (95% CI 0.718–0.938) and SerpinC1 in the HDL fraction with an AUC of 0.812 (95% CI 0.700–0.924) were the three best individual markers. The best combination of markers consisted of SerpinC1-HDL, SerpinG1-TEX and SerpinC1-REX, and showed an AUC of 0.861 (95% CI 0.758–0.965). Results are being validated in the MYOMARKER cohort of 850 patients. **Summary/conclusion:** These data show that an EV protein signature can be used as diagnostic biomarker for SCAD.

OT12.2**Coronary artery calcification associates with specific cell-derived blood-borne microvesicles in middle-aged women with and without history of preeclampsia**Muthuvel Jayachandran^{1,2}, Vesna D. Garovic³, Brian Lahr⁴, Kent R. Bailey⁴, Michelle M. Mielke⁵, Wendy M. White⁶ and Virginia M. Miller^{1,2}¹Departments of Surgery, Mayo Clinic, Rochester, USA; ²Physiology and Biomedical Engineering, Mayo Clinic, Rochester, USA; ³Division of Biomedical Statistics and Informatics, Mayo Clinic, Rochester, USA; ⁴Division of Nephrology and Hypertension, Mayo Clinic, Rochester, USA; ⁵Departments of Health Science Research and Neurology, Mayo Clinic, Rochester, USA; ⁶Division of Maternal Fetal Medicine, Mayo Clinic, Rochester, USA

Background: Coronary arterial calcification (CAC), a risk factor for myocardial infarction and stroke, occurs in about 14% of women who are classified as low risk by conventional cardiovascular (CV) risk factors. This study aimed to determine if a panel of specific cell-derived blood microvesicles (MV) would associate with CAC in middle-aged

women. **Methods:** This study was approved by the Institutional Review Board at Mayo Clinic. Included in the study was a stratified sample of age-matched women (median = 60 years) free of CV events: n = 40 with and n = 40 without a history of pre-eclampsia (PE). The MV positive for phosphatidylserine, tissue factor (TF), ICAM-1, VCAM-1, CD45 (leukocytes), CD42a (platelets), CD62E (endothelium), SM22a (smooth muscle cells), CD117 (stem/progenitor cells), Pref-1 (adipocytes) and p16set (senescent cells) were evaluated by digital flow cytometry. CAC was determined by computed tomography. MV variables (both individually and combined as principal components [PC]) were analysed for an association with CAC within each strata using Spearman's correlation, and across both strata using an ordinal logistic model that adjusted for history of PE. **Results:** Of the MV tested, only those derived from smooth muscle cell correlated (p = 0.008) with CAC in women without a history PE, whereas MV positive for ICAM-1 correlated (p = 0.042) with CAC in women with history of PE. Using PC analysis performed on MV data, none of the retained PC scores correlated with CAC within the two groups. However, the first PC, which consisted of TF positive MV, MV positive for VCAM-1 and MV derived from adipocytes, was associated with CAC (p = 0.034) in a multivariable model fit on the combined group after adjustment for history of PE. **Conclusions:** A specific group of MV was identified in this study of asymptomatic but at CV risk women that warrants confirmation in other populations, to identify those who might benefit from further testing for clinically significant calcification.

OT12.3**Exosomes induce and reverse monocrotaline-induced pulmonary hypertension in mice**

Jason Aliotta, Mandy Pereira, Sicheng Wen, Mark Dooner, Michael Deltatto, Elaine Papa, Laura Goldberg, Grayson Baird, Corey Ventetuolo, Peter Quesenberry and James Klingner

Department of Medicine, Rhode Island Hospital, Providence, USA

Aims: Extracellular vesicles (EVs) from mice with monocrotaline (MCT)-induced pulmonary hypertension (PH) induce PH in healthy mice, and the exosomes (EXO) fraction of EVs from mesenchymal stem cells (MSCs) can blunt the development of hypoxic PH. We sought to determine if the EXO fraction of EVs is responsible for modulating pulmonary vascular responses and if differences in EXO-miR content explain the differential effects of EXOs from MSCs and mice with MCT-PH. **Methods and results:** Plasma, lung EVs from MCT-PH and control mice were divided into EXO (exosome), microvesicle (MV) fractions and injected into healthy mice. EVs from MSCs were divided into EXO, MV fractions and injected into MCT-treated mice. PH was assessed by right ventricle-to-left ventricle + septum (RV/LV+S) ratio and pulmonary arterial wall thickness-to-diameter (WT/D) ratio. miR microarray analyses were also performed on all EXO populations. EXOs but not MVs from MCT-injured mice increased RV/LV+S and WT/D ratios in healthy mice. MSC-EXOs prevented any increase in RV/LV+S and WT/D ratios when given at the time of MCT injection and reversed the increase in these ratios when given after MCT administration. EXOs from MCT-injured mice and patients with idiopathic pulmonary arterial hypertension (IPAH) contained increased levels of miRs-19b,-20a,-20b and -145, whereas miRs isolated from MSC-EXOs had increased levels of anti-inflammatory, anti-proliferative miRs including miRs-34a,-122,-124 and -127. **Conclusions:** These findings suggest that circulating or MSC-EXOs may modulate pulmonary hypertensive effects based on their miR cargo. The ability of MSC-EXOs to reverse MCT-PH offers a promising potential target for new pulmonary arterial hypertension therapies.

OT12.4

Trafficking of STAT5 via endothelial cell-derived extracellular vesicles regulates IL-3 paracrine action

Maria Felice Brizzi, Giovanni Camussi, Patrizia Dentelli, Giusy Lombardo, Gabriele Togliatto, Arturo Rosso, Maddalena Gili, Sara Gallo and Maria Chiara Deregibus

Department of Medical Sciences, University of Turin, Italy

Soluble factors and cell-derived extracellular vesicles (EVs) control vascular cell fate during inflammation. The present study investigates the impact of Interleukin 3 (IL-3) on EV release by endothelial cells (ECs), the mechanisms involved in EV release and paracrine actions. We found that IL-3 increases EV release, which is prevented by IL-3R α blockade. EVs released upon IL-3 stimulation were able to induce pro-angiogenic signals as shown by chromatin immunoprecipitation (ChIP) assay performed on the promoter region of cyclin D1 and tridimensional tube-like structure formation. We herein demonstrate that these effects rely on the transfer of miR-126 and, more importantly, of activated signal transduction and activator of transcription 5 (pSTAT5) from IL-3-EV cargo into recipient ECs. We show, using the dominant negative form (DN)STAT5 and an activated STAT5 (1*6STAT5) constructs, that STAT5 drives IL-3-mediated EV release, miR-126 and pSTAT5 content. Finally, using EVs recovered from DNSTAT5 expressing ECs, we provide evidence that miR-126 and pSTAT5 trafficking is relevant for IL-3-mediated paracrine pro-angiogenic signals. These results indicate that IL-3 regulates EC-EV release, cargo and IL-3 angiogenic paracrine action via STAT5. Furthermore, these results provide evidence that EC-derived IL-3-EVs can serve as pro-angiogenic clinical delivery wound healing devices.

OT12.5

Human endothelial microvesicle shedding in response to simulated blast trauma

Anna Sharrock^{1,2}, Sara Rankin¹, Rory Rickard², Harsh Amin¹ and Paul Harrison³

¹Royal British Legion Centre for Blast Injury Studies, and National Heart and Lung Institute, Imperial College, London, UK; ²Academic Department of

Military Surgery and Trauma, Royal Centre for Defence Medicine, Birmingham, UK; ³Institute of Inflammation and Aging, University of Birmingham, Birmingham, UK

Introduction: Procoagulant microvesicles have been shown to rise in clinical studies following blunt and penetrating trauma compared to healthy controls. It has been established that blast injury mechanisms are associated with an increased risk of sepsis, multi-organ failure and coagulopathy, compared to those with injury severity-matched blunt mechanisms. However, the generation and role of microvesicles following blast trauma has yet to be elucidated. **Aims:** We wished to develop a human endothelial model for studying the effects of blast waves on cell viability and microvesicle generation and function. **Methods:** Confluent human umbilical vein endothelial cells (HUVECs) were exposed to a 6-bar blast (N=6), or control conditions. Cell viability (calcein, ethidium homodimer-1) and microvesicle formation (annexin V+) were assessed by confocal microscopy. Pre-filtered supernatants were removed and assessed at 1, 3, 6 and 24 h by 0.2–1.1- μ m-gated flow cytometry (using annexin V, CD31, CD51, CD54, CD62E, CD141 and CD142) and calibrated automated thrombography (CAT) (N=2). **Results:** HUVEC viability was not diminished by blast wave exposure, and microscopy showed formation of microvesicular structures. Flow cytometric analysis demonstrated a trend (NS) increase in annexin V+ microvesicles at all time-points. While no significant increase in microvesicles with any marker was seen, there was a 4.92-fold (p=0.087) and 4.63-fold (p=0.085) increase of CD142+ and CD141+ events, respectively, at 24 h in the blast group compared with the control group. Microvesicles were shown to be thrombogenic in two CAT experiments. **Conclusions:** A model has been developed to establish the effects of blast waves on human endothelial cells. The application of 6-bar blast in the model has not convincingly shown an increase in microvesicle formation. Thrombography, CD141 and CD142 shifts are yet to achieve statistical significance. The methodology is established to explore the model with a range of blast exposures.

Room: Willem Burger

Plenary Session 2 - Exosomes and viruses

Chairs: *Jennifer Jones and Esther Nolte-'t Hoen* 10:45 a.m.-12:30 p.m.

Speakers:

Robert C. Gallo (University of Maryland School of Medicine, Baltimore, USA)

Journey with Blood Cells and Viruses – Why the Global Virus Network

Leonid Margolis (NICHD, Bethesda, USA)

Extracellular Vesicles and Viruses: Cousins in Complicated Relations

Shilpa Buch (University of Nebraska Medical Center, Omaha, USA)

HIV and drug abuse go hand in HAND: blaming the messengers

Room: van Weelde

Symposium Session 13 - EVs in remodelling and repair

Chairs: Peter Quesenberry and Fabiana Geraci

1:30-3:00 p.m.

OT13.1**Hepatocyte-derived extracellular vesicles enzymatic activity modify serum metabolome**

Felix Royo, Sebastiaan Van-Liempd, Diana Cabrera, Esperanza Gonzalez and Juan Manuel Falcon Perez
Cic BioGUNE, Bizkaia, Spain

Introduction: Hepatocytes release extracellular vesicles (EVs) to the blood stream, loaded with a varied cargo. Although the most studied role of EVs is related to cell-to-cell communication, it has been proven that they could also harbour enzymatic activity. In the present work, we evaluate the impact of EVs in the serum composition. **Methods:** We had performed a metabolomic profiling of serum samples after incubation with hepatocyte-secreted EVs that were isolated by differential ultracentrifugation. The metabolic profile was performed by using ultra-performance liquid chromatography coupled to mass spectrometry (UPLC-MS). **Results:** The results obtained revealed significant changes in various metabolites related to amino acid metabolism. Importantly, the conversion of arginine into ornithine was observed after incubation, which was consistent with the presence of arginase enzyme (ARG1) in the EVs. By sucrose density gradient, we confirmed that arginase activity was present in the exosomes-enriched fraction. More importantly, the arginase activity was found in vivo in the ultracentrifugation pellet obtained from serum of animals treated with hepatotoxicans. **Summary/conclusion:** Hepatocytes-derived EVs carry active enzymes that had a significant impact in the serum metabolome what could have important consequences for different pathophysiological processes including drug-induced liver injury and regeneration.

OT13.2**Controlled enhancement of EV vascularization bioactivity by ethanol conditioning**

Steven Jay, Tek Lamichhane and Christopher Leung
University of Maryland, College Park, Maryland, USA

Introduction: Peripheral vascular disease (PVD) is a leading cause of mortality, and new strategies for therapeutic vascularization are needed. Extracellular vesicles (EVs) from many cell types have vascularization bioactivity and thus represent a potential new class of PVD therapeutics. Engineering EVs with enhanced vascularization bioactivity would further promote this approach, thus we examined numerous chemical conditioning approaches to induce angiogenic cellular responses, eventually focusing on ethanol conditioning of endothelial cells (ECs). **Methods:** EVs were isolated from human umbilical vein ECs (HUVEC) cultured with or without ethanol (0–200 mM) by differential centrifugation and characterized via nanotracking analysis, TEM and immunoblotting. Vascularization bioactivity was assessed via an EC migration assay and a human angiogenesis PCR array. EV microRNA content was evaluated by a PCR-based whole miRNome array. **Results:** Ethanol conditioning of HUVEC enhanced EV vascularization bioactivity in a dose-dependent manner; a minimum of 50 mM ethanol was required to achieve significant increases in EC migration compared to 0 mM control (migrated area of 0.18 ± 0.032 sq. mm vs. 0.243 ± 0.018), while concentrations up to 200 mM concentration induced further enhancement (0.308 ± 0.016). PCR analysis revealed that ECs stimulated by EVs isolated from 50 mM ethanol-conditioned HUVEC

had ~6-fold increase in CD34 levels compared to control cell EVs. Analysis of EV miRNA content showed significant decreases in key anti-angiogenic miRNAs (miR-137, miR-100, miR-24, miR-573, miR-320), suggesting enhanced pro-vascularization bioactivity. **Summary/conclusion:** Ethanol conditioning downregulates several anti-angiogenic miRNAs in HUVEC EVs. This promotes enhanced vascularization bioactivity of these EVs, leading to CD34 upregulation in target ECs. Ethanol conditioning may be a scalable strategy to enhance vascularization bioactivity of EVs from ECs and other cell types.

OT13.3**Human mesenchymal stem cell-derived extracellular vesicles ameliorate eye disease by reducing inflammation and neovascularisation in a corneal alkali burn rat model after topical administration**

Thomas Ritter¹, Cerine Lal², Oliver Treacy¹, Grace A. O' Malley¹, Maura Naughton¹, Serika Naicker¹, Paul Lohan¹, Hrebesh Subash², Aideen E. Ryan³, Gerry Fahy⁴, Martin J. Leahy², Matthew D. Griffin¹ and Sweta Rani^{1,5}
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Introduction: To investigate if topically applied mesenchymal stem cell (MSC)-derived extracellular vesicles (EVs) promote corneal wound repair in a rat alkali burn model. **Methods:** EVs were isolated from media conditioned by human bone marrow-derived MSCs (n = 4) using a series of centrifugation, filtration and ultracentrifugation steps followed by transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA; NanoSight) quality control. Pro-healing effects of hMSC-derived EVs (hMSC-EV) were examined in vitro by cell migration and scratch wound healing assays using human corneal epithelial (HCE) and endothelial (HCEC) cell lines. Rat corneal alkali burn injury was induced and injury was treated with or without 30 mg of hMSC-EVs. Efficacy of treatment was monitored by biomicroscopy and optical coherence tomography (OCT) followed by extensive tissue analysis. **Results:** Successful isolation of EVs was confirmed by immune-gold TEM showing expression of CD63 and TSG101. NTA showed the average size of EVs ranged between 50–130 nm. Significant increases in HCE and HCEC cell migration in the presence of MSC-EVs were observed compared to PBS control and EV-depleted media. Moreover, a significant increase in scratch wound closure of cells treated with hMSC-EVs was observed. In vivo, analysis of images taken at days 0, 1, 3 and 7 of fluorescein stained corneal injury showed significantly enhanced healing of the corneal surface at days 1 and 3 when treated with hMSC-EVs compared to PBS control. OCT analysis revealed that hMSC-EVs reduced inflammation and maintained the physiological structure of the eye. Importantly, a reduction in corneal damage and inflammatory infiltrating cells and an increase in proliferating epithelial cells (Ki67-positive) was observed following hMSC-EV treatment. **Conclusion:** Our results show that topical hMSC-EV application is a novel promising cell-free therapy with high potential for translation in ocular surface disease treatment.

OT13.4

Treatment with extracellular vesicles from adipose tissue–derived mesenchymal stromal cells in a murine model of silicosis

Elga Bandeira¹, Helena Oliveira¹, Johnatas Silva¹, Rubem Mena-Barreto³, Christina Takiya¹, Jung Soo Suk², Kenneth Witwer², Justin Hanes², Michael Paulaitis², Patricia Rocco¹ and Marcelo Morales¹

¹Federal University of Rio De Janeiro, Brazil; ²Johns Hopkins University, Baltimore, Maryland; ³Fiocruz, Rio de Janeiro, Brazil

Mesenchymal stromal cells (MSC) have been shown to exert beneficial effects in a variety of lung diseases. Recently, it has been suggested that similar effects can be achieved by the therapeutic use of extracellular vesicles (EVs) obtained from the conditioned media of these cells. The aim of this study was to investigate the therapeutic effects of adipose tissue–derived MSC (AD-MSCs) and EVs produced by them in a model of chronic silicosis. Forty C57BL/6 female mice received saline or silica (20 mg) intratracheally (IT). At day 15, silica animals received an IT instillation of saline, AD-MSCs (E05 cells), or EVs in two different doses: equivalent to E05 cells (EV5) or E06 cells (EV6). EVs were obtained through ultracentrifugation of conditioned media. Electron microscopy, flow cytometry and nanoparticle tracking analysis were used to characterize the EV samples. Their stability in various conditions, including aerosolization of the EV suspension using a microsyringe was assessed through NTA. Labelled EVs were used to observe uptake in vitro and distribution in the lungs. The RNA content of the EVs was characterized through electrophoresis, and Nanostring analysis indicated the presence of miRNA such as miR-494, miR-302d, miR-122 and let-7a. At day 30, AD-MSCs and EVs led to a reduction in collagen fibre content. AD-MSCs and the higher dose of EVs also reduced the size of granuloma and the number of macrophages inside the granuloma and in the alveolar septa. Additionally, expression of IL-1 β and TGF β was decreased in the lung of these groups. EV6 group also presented improved lung elastance when compared to untreated silicosis group. In conclusion, EVs derived from AD-MSC presented a heterogeneous EV population, capable of sustaining aerosolization without size and number alterations, and both AD-MSCs and EVs, locally delivered, ameliorated fibrosis and inflammation, but dose-optimized EVs yielded better therapeutic outcomes in this model of silicosis.

OT13.5

Mesenchymal stromal cell-derived extracellular vesicles as vehicle of therapeutic mRNA: evidences for Walker–Warburg muscular dystrophy treatment

Enrico Ragni¹, Federica Banfi¹, Valentina Parazzi¹, Cristiana Lavazza¹, Mario Barilani¹, Vincenza Dolo², Valentina Bollati³ and Lorenza Lazzari¹

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Introduction: In vitro animal models suggested that mesenchymal stem cell (MSC)-extracellular vesicles (EVs) are able to transfer proteins, lipids and nucleic acids, thereby influencing various functions of recipient cells. Notably, increase in proliferation and apoptosis inhibition were reported upon MSC-EV treatment of injured cells in various models. Therefore, we investigated EV communication mechanisms in bone marrow (BM)- and umbilical cord blood (CB)-MSC at mRNA level, with particular attention to an O-mannosylation deficiency-associated congenital muscular dystrophy model (Walker–

Warburg syndrome, WWS). **Methods:** Human MSCs were obtained from bone marrow and umbilical cord blood after informed consent. EVs were isolated by differential centrifugation and their integrity verified by CFSE staining. RNA was extracted and a panel of selected genes assayed by qRT-PCR. Ability of MSC-EV to fuse with myoblasts was tested by FACS and confocal microscopy. Recovery of O-mannosylation in a WWS patient myoblast line after EV administration was checked by FACS. **Results:** Transcripts involved in MSC functions (IL4, IL10, FGF7, CNTF, KDR) were preferentially EV-accumulated with respect to a random internalization. Their 3'UTR analysis showed potential binding to 71 miRNA in recipient cells. These miRNAs, and alteration in their cytosolic levels, were predicted in silico to modulate transcription/translation and anti-apoptotic processes. MSC-EV also contained mRNA of O-mannosylation genes directly involved in WWS pathology and were able to efficiently fuse with myoblasts. In a patient myoblast line, EV partially reverted O-mannosylation deficiency, suggesting the translation of wild-type mRNA into a functional enzyme and contributed to a morphology recovery. **Conclusions:** These results proposes a proof of concept for the use of MSC-EV as a novel therapeutic approach to reduce apoptotic processes and restore a proper glycosylation profile in WWS muscular dystrophy myoblasts.

OT13.6

Characterization of exosomes from human amnion epithelial cells

Rebecca Lim¹, Jean Tan¹, Sinnee Lau¹, Siow Chan¹, Euan Wallace² and Joanne Mockler¹

¹Hudson Institute of Medical Research, Clayton, Australia; ²Monash University, Melbourne, Australia

Background and Aims: The efficacy of stem cell therapy is largely unrelated to functional engraftment. Studies using stem cell–conditioned media indicate that this is attributed to released soluble factors and/or extracellular vesicles. Our work to date has focused on stem-like cells from gestational tissues – human amnion epithelial cells (hAECs). We have reported on the pro-reparative effects of hAECs in small and large animal models of acute and chronic lung injury. While there is now growing literature on MSC-derived exosomes, there has been no information on hAEC-derived exosomes. Here, we aimed to characterize hAEC-derived exosomes and assess their efficacy in bleomycin-induced lung injury. **Methods:** hAEC-derived exosomes were characterized by a combination of electron microscopy, FACS and western blotting. Proteomic analysis was performed using label-free LC-MS. We assessed the ability of the exosomes to suppress T-cell proliferation and increase macrophage phagocytosis. We also assess their biological effects in vivo by administering amniotic exosomes intranasally to 6–8 months old C57Bl6 mice, 7 days following bleomycin challenge (10 ug or 50 ug). **Results:** hAEC-derived exosomes inhibited CD3/CD28-induced T-cell proliferation and increased macrophage phagocytosis in a dose-dependent manner. Both doses of amniotic exosomes reduced lung fibrosis to a similar extent ($p < 0.05$) and this coincided with the reduction of activated myofibroblasts in lung tissues ($p < 0.05$). Exosome administration also increased activation of endogenous lung stem/progenitor cells. These outcomes may be associated with some specific and relatively abundant exosomal cargo including Wnt5a and HLA-G. **Conclusions:** hAEC-derived exosomes have properties similar to MSC-derived exosomes. Future studies should include comparative analyses of their biological properties and exosomal content in order to determine which common cargo is responsible for exerting these beneficial effects.

Room: Jurriaanse

Symposium Session 14 - Engineering EVs

Chairs: *Hubert Yin and Raymond Schiffelers*

1:30-3:00 p.m.

OT14.1**Genetically engineered bacterial protoplast-derived nanovesicles that mimic extracellular vesicles as targeted drug delivery system**

Oh Youn Kim, Nhung Dinh, Seng Jin Choi, Hyun Taek Park and Yong Song Gho

Life Sciences, POSTECH (Pohang University of Science and Technology), Pohang, Republic of Korea

Introduction: Over the past years, massive improvements have been made on delivery technologies to help patients undergoing chemotherapy. However, despite the advancement, the challenge to specifically deliver the chemotherapeutics to the tumour site to reduce the undesired adverse effects still remains. Therefore, in this study, we newly developed bioinspired extracellular vesicle-mimetic nanovesicles derived from genetically engineered bacterial protoplast having removed bacterial outer membrane components as targeted delivery system for chemotherapeutics. **Material and Methods:** Bacterial protoplasts expressing human epidermal growth factor (EGF) as tumour-targeting ligand on the surface were extruded serially through membrane filters of 10, 5, 1 and 0.5 μm pores to form nanovesicles. The tumour-targeting efficacy and delivery of drug were examined on EGF receptor expressing human lung carcinoma A549 cells in vitro and A549 xenografted mice in vivo. **Results:** Electron microscopy images revealed that the protoplast nanovesicles had intact vesicular structures of about 80 nm in diameter. Western blotting analyses showed that the nanovesicles did not contain toxic bacterial outer membrane components and expressed EGF on the surface. Moreover, anti-cancer drug-loaded nanovesicles effectively targeted to tumour cells in vitro and in vivo via receptor-mediated endocytosis and induced dose-dependent cytotoxicity. Furthermore, significant prevention of tumour growth was observed after the treatment of drug-loaded nanovesicles to tumour xenograft mice without adverse effects. **Summary/conclusion:** Together, the results obtained from this study strongly suggest the potential of extracellular vesicle-mimetic bacterial protoplast nanovesicles as future advanced targeted drug delivery vehicle that is both effective and safe.

OT14.2**High-affinity single-chain fragment variables (scFvs) are novel specific targeting motifs for extracellular vesicles**Christina Schindler, Andrea Longatti, Laura Fitzpatrick, Carl Matthews, Lesley Jenkinson, Tristan Vaughan and Natalie Tigue
MedImmune, Cambridge, UK

Introduction: Extracellular vesicle targeting to specific cells or tissues using peptide motifs displayed on their surface has previously been described. So far, this approach has taken advantage of a small number of endogenous peptide ligands that target a known binding partner/receptor and therefore is limited to a small set of applications. The use of antibody-derived scFv (single-chain fragment variable) motifs, which can be engineered to bind to any cell surface receptor, with a defined affinity could be considered as a promising alternative targeting strategy. **Methods:** Herein, we describe the generation of cell line-derived exosomes equipped with scFv of different affinities to the receptor Her2 on their surface. ScFv were anchored to the cell surface by fusion to the C1C2 domain of lactadherin (MFGE8) and proteinaceous exosomal content was labelled with the dye CFSE. Exosome uptake assays were performed in HEK293 lines over-expressing different amounts of Her2 on their cell surface and CFSE content in recipient cells was analysed by FACS and confocal

microscopy. **Results:** We were able to show that exosomes bearing high and intermediate affinity scFv to Her2 were efficiently taken up into cells with high surface expression of Her2 whereas a low affinity or non-targeting scFv resulted in a lower cellular uptake of exosomes. Exosome uptake relies on high surface levels of Her2 as cells expressing intermediate levels of Her2 did not take up more exosomes compared to the parental cell line. **Summary/Conclusion:** Here, we present a novel strategy to specifically target exosomes to cells based on high affinity interactions of scFv with an abundant surface protein using Her2 as a model receptor. This broadens the spectrum of interactions that can be used for specific EV targeting and high uptake into recipient cells as scFv can be readily engineered to exhibit the desired properties. Our findings may have a broader application in the design of exosome-based therapeutics.

OT14.3**Effective cellular uptake of biofunctional peptide-modified exosomes**
Ikuhiko Nakase

Osaka Prefecture University, Osaka, Japan

Introduction: Exosomes (~ 100 nm in diameter) are small secretory vesicles released from various cell types, and they represent potential next-generation biological tools for delivering therapeutic molecules. In this presentation, I will discuss novel techniques to effectively target receptors via artificial leucine-zipper peptides and enhance cytosolic release via a pH-sensitive fusogenic peptide (1). Additionally, I will provide insights into macropinocytotic-based cellular uptake of exosomes (2) modified with arginine-rich peptides. **Methods:** Secreted CD63-GFP-containing exosomes from HeLa cells were isolated by ultracentrifugation. All peptides were chemically synthesized by Fmoc solid-phase peptide synthesis. **Results and conclusion:** A 30-residue amphipathic peptide GALA was designed to mimic viral fusion protein sequences. In our system, cationic lipids were employed to bind peptides onto the surfaces of exosomes (1). Enhanced cytosolic release of the cargo from exosomes was successfully achieved by using the GALA peptide, resulting in effective biological activity of originally encapsulated cargo in exosomes (1). On the other hand, modification of the exosomal surface with artificial leucine-zipper K4 peptides allowed for recognition of E3-fused receptors on the cell membrane, leading to efficient cellular targeting. My research group recently found that induction of macropinocytosis following the stimulated cancer-related receptors (e.g. EGFR) or expression of oncogenic K-Ras significantly enhanced cellular exosome uptake (2). In addition, arginine-rich peptides can induce macropinocytosis and result in more efficient cellular uptake. Thus, we studied the effects of exosomal membrane modification with arginine-rich peptides (e.g. octaarginine) on cellular uptake of exosome and found that uptake efficacy was significantly increased.

References

1. Nakase, I., et al., *Sci. Rep.* 5, 10112 (2015).
2. Nakase, I., et al., *Sci. Rep.* 5, 10300 (2015).

OT14.4**Efficient and rapid cellular delivery of bioactive proteins using EXPLOR: exosomes engineered for protein loading via optically reversible protein-protein interaction**Chulhee Choi¹, Nambin Yim¹, Seung-Wook Ryu², Kyungsun Choi¹, Seunghee Lee¹, Hojun Choi¹, Daesoo Kim³, Wondo Heo³ and Ji Ho Park¹

¹Bio and Brain Engineering, KAIST, Daejeon, Republic of Korea; ²Cellex Life Sciences, Incorporated, Daejeon, Republic of Korea; ³Biological Sciences, KAIST, Daejeon, Republic of Korea

Introduction: Many intracellular proteins with great potential as biopharmaceutical drugs have been identified; however, many of the challenges associated with intracellular protein delivery have yet to be solved. Cell-derived exosomes have recently been highlighted as new therapeutic strategies for the in vivo delivery of nucleotides and chemical drugs; however, these methods were based on the passive loading of siRNAs or miRNAs into isolated exosomes by electrophoresis, a method poorly suited for the intracellular delivery of cellular proteins. **Methods:** We developed an opto-genetically engineered exosome system, named 'exosomes for protein loading via optically reversible protein-protein interaction' (EXPLOR) that can deliver soluble proteins into the cytosol via controlled, reversible protein-protein interactions (PPI). **Results:** By integrating a reversible PPI module controlled by blue light with the endogenous process of exosome biogenesis, we were able to successfully load cargo proteins into newly generated exosomes. Treatment with protein-loaded EXPLORs was shown to significantly increase intracellular levels of cargo proteins and their function in recipient cells in both a time- and dose-dependent manner. In the present study, we have demonstrated the intracellular delivery of mCherry, Cre enzyme, Bax, and Super repressor I κ B proteins as functional proteins in the target cells and in vivo. **Summary:** These results clearly indicate the potential of EXPLORs as a mechanism for the efficient intracellular transfer of protein-based drugs into recipient cells and tissues both in vitro and in vivo.

OT14.5

Exosome-mediated delivery of hydrophobically modified siRNA for Huntingtin mRNA silencing

Marie Didiot¹, Lauren Hall¹, Andrew Coles¹, Reka Haraszti¹, Bruno Godinho¹, Kathryn Chase¹, Ellen Sapp², Socheata Ly¹, Julia Alterman¹, Matthew Hassler¹, Lakshmi Raj³, David Morrissey³, Marian Difiglia², Neil Aronin¹ and Anastasia Khvorova¹
¹RNA Therapeutics, University of Massachusetts Medical, Worcester, MA, USA;
²Institute for Neurodegenerative Disease, Massachusetts General Hospital, Charlestown, MA, USA; ³Novartis Institutes for Biomedical Research Inc., Cambridge, MA, USA

Introduction: Delivery represents a significant barrier to the clinical advancement of oligonucleotide therapeutics for the treatment of neurological disorders such as Huntington's disease (HD). HD is caused by the autosomal dominant inheritance of the mutant Huntingtin (HTT) allele and represents an excellent monogenic disease target for oligonucleotide therapeutics. Small endogenous vesicles known as exosomes have the potential to act as oligonucleotide delivery vehicles, but robust and scalable methods for loading therapeutic cargo into exosomes are lacking. **Method:** Here we developed a novel and simple method enabling the efficient

loading of hydrophobically modified siRNAs (hsiRNAs) upon co-incubation. **Results:** Loading of hsiRNAs does not alter vesicle size distribution or integrity. Exosomes loaded with hsiRNAs targeting Huntingtin mRNA are efficiently taken up by mouse primary cortical neurons and promote dose-dependent silencing of Huntingtin mRNA and protein. Unilateral infusion of hsiRNA-loaded exosomes into mouse striatum results in wide distribution of hsiRNA promoting the silencing of Huntingtin mRNA in both treated and non-treated sides of the brain. **Summary/conclusion:** Thus, the packaging of hydrophobically modified oligonucleotides into exosomes represents a key advance in the delivery of therapeutic oligonucleotides. The broad distribution and efficacy of hsiRNA-loaded exosomes delivered to brain is expected to improve the development of advanced therapies for treatment of Huntington's disease and other neurodegenerative disorders.

OT14.6

Customizing extracellular vesicles for therapy and imaging

Amanda Silva, Nathalie Luciani, Florence Gazeau and Claire Wilhelm
 Physics department, Université Paris 7/CNRS, Paris, France

Introduction: Extracellular vesicles (EVs) are multifaceted subcellular entities that may represent a new generation of bio-camouflaged drug/ nanoparticle delivery system. **Methods:** In a top-down procedure, our group has engineered nanoparticle/drug-loaded EVs from precursor cells previously loaded with this cargo. For this purpose, parent HUVEC cells or THP1 macrophages were first incubated with the desired payload and allowed to internalize it. Starvation stress was employed to induce the release of vesicles highjacking the cargo from their precursor cell. **Results:** By this method, EVs could encapsulate a set of nanoparticles regardless their chemistry or shape, such as iron oxide nanoparticles, iron oxide nanocubes, gold/iron oxide nanodimers, gold nanoparticles, and quantum dot. These hybrid vesicles were able to generate heat when submitted to an alternating magnetic field and could be monitored by fluorescence imaging or MRI. Dual drug/nanoparticle EV loading was also feasible by this method. We demonstrated that vesicles from THP-1 cells could be loaded with iron oxide nanoparticles and different therapeutic agents regardless their molecular weight, hydrophobic, hydrophilic and amphiphilic character. Thereby, magnetic vesicles were loaded with a chemotherapeutic drug (doxorubicin), anticoagulant protein (tissue-plasminogen activator (t-PA)), or two photosensitizer (disulfonated tetraphenylchlorin (TPCS2a) or meta-tetra (hydroxyphenyl)chlorin (mTHPC)). The theranostic potential of mTHPC-loaded magnetic EVs was tested in vivo in a murine tumour model. EVs could be tracked in vivo by dual-mode imaging (fluorescence and MRI). The engineered EVs were found to induce an efficient photodynamic action, as evidenced by tumour growth curves and histological analysis. **Summary/conclusion:** In brief, we succeeded in customizing EV by engineering them to display several nanoparticle/drug cargoes featuring therapeutic and imaging properties both in vitro and in vivo.

Room: Willem Burger

Symposium Session 15 - EVs and tumor environment II

Chairs: Lorraine O'Driscoll and Hector Peinado

1:30-3:00 p.m.

OT15.1

Exosomes from non-metastatic melanoma recruit patrolling monocytes for immune clearance of metastasisOlga Volpert, Stephen D. Miller, Nicholas L. Angeloni, Michael Plebanek and C. Shad Thaxton
Northwestern University, Evanston, Illinois, USA

Introduction: Exosomes are critical in shaping tumour microenvironment, especially pre-metastatic niche, which fosters colonization, survival and proliferation of the vagrant cancer cells. Exosomes facilitate homing of the tumour cells and determine their organ tropism through integrins on their surface; they also recruit hyper-inflammatory macrophages which provide growth advantage for cancer cells. Finally, in patients/animals with immunogenic tumours, exosomes enable the escape of metastasizing cancer cells from immune surveillance. Here, we show that the opposite is also true: exosomes from non-metastatic melanoma cells potently inhibit metastasis to the lung by stimulation of immune surveillance. **Methods:** We have used 'non-metastatic' and control 'metastatic' exosomes, in two in vivo models of melanoma metastasis, lung colonization and extravasation assays. We also performed functional analysis and molecular characterization of immune cell populations ex vivo and in vitro, using knockdown analyses, time-lapse microscopy, western blotting and flow cytometry. **Results:** 'Non-metastatic' exosomes stimulated immune surveillance and clearance (lustration) by the Ly6Clow, Ly6Glow population of monocytes, which are closely related to patrolling monocytes (PMo). The exosome-dependent PMo recruitment and subsequent phagocytosis of melanoma cells at the pre-metastatic niche required the induction of NR4A1 orphan nuclear receptor and subsequent differentiation to dendritic-like cells and TRAIL-dependent killing/ phagocytosis of melanoma cells. The NR4A1 induction and downstream events were due to the display of pigment epithelium-derived factor (PEDF), on exosome surface. **Summary/conclusion:** Our studies imply that pre-metastatic tumours express triggers of immune lustration (e.g. PEDF), which are released and delivered to the cells of immune system via exosomes and that the loss of lustration triggers contributes to metastasis.

OT15.2

Exploitation of exosomes as delivery system of functional pro-apoptotic membrane TNF-related apoptosis-inducing ligandVeronica Huber¹, Claudia Chiodoni¹, Paola Squarcina¹, Monica Tortoreto¹, Laura Botti¹, Barbara Vergani², Elisabetta Vergani¹, Antonello Villa², Mario Paolo Colombo¹, Nadia Zaffaroni¹, Alessandro Massimo Gianni³ and Licia Rivoltini¹¹Department of Experimental Oncology and Molecular Medicine, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy; ²Microscopy and Image Analysis, Università degli Studi di Milano-Bicocca, Milan, Italy; ³Department of Clinical Oncology, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy

Introduction: The exploitation of exosomes as novel therapeutic tool relies on their natural function in intercellular communication and signal delivery to target cells. We here investigated the ability of membrane TNF-related apoptosis-inducing ligand (TRAIL)-expressing exosomes to deliver pro-apoptotic signals to cancer cells and mediate growth inhibition in different tumour models. **Methods:** Exosomes produced by K562 TRAIL+ cells were characterized by NanoSight, cytofluorimetry, immunoelectronmicroscopy, western blot and ELISA. TRAIL-death receptors (DR4, DR5) and apoptosis of

SUDHL4 lymphoma, INT12 melanoma and KMS11 multiple myeloma cells were detected by flow cytometry. Anti-tumour activity was tested in local and systemic treatment approaches of SUDHL4, INT12 and KMS11 bearing SCID mice. Homing of TRAIL exosomes was evaluated by confocal microscopy and near-infrared imaging. **Results:** TRAIL exosomes activated the apoptotic cascade primarily in DR5+ SUDHL4 and INT12, with respect to DR4+ KMS11 cells and intra-tumour treatments led to growth inhibition and necrosis in SUDHL4 (68%), INT12 (51%) and KMS11-bearing animals. Systemically administered TRAIL exosomes mainly accumulated in the liver but also reached tumour site leading to a significant reduction of tumour growth (58%) in the SUDHL4 tumour model. A non-statistically significant reduction of tumour volume was detectable in INT12-bearing mice accompanied by pronounced tumour necrosis. In contrast, KMS11 lesions displayed only perivascular necrosis upon i.v. injection of TRAIL exosomes. **Summary/conclusion:** TRAIL exosomes can contribute to tumour growth control, especially in intra-tumour setting. The efficacy of systemically administered TRAIL exosomes, significant only in highly TRAIL-sensitive SUDHL4 tumours, could be potentiated by ameliorating the targeting of TRAIL exosomes to tumour site. As a natural and tailorable delivery system, exosomes represent a promising therapeutic tool in cancer.

OT15.3

In vitro and in vivo evaluation of Epstein-Barr virus latency II-derived peptides as tumour vaccine for immunotherapy of nasopharyngeal carcinomaDhafer Mrizak¹, Rami Mustapha¹, Sarah Renaud¹, Nathalie Martin¹, Hayet Rafa², Pedro Roman-Puche¹, Liu Fei Fei³, Niki Toshiro⁴, Lo Kwok-Wai⁵, Véronique Pancre¹, Pierre Busson⁶, Nadira Delhem¹ and Olivier Morales¹¹Centre national de la recherche scientifique, Lille, France; ²University of Science and Technology Houari Boumediene, Bab Ezzouar, Algeria; ³Ontario Cancer Institute, Ontario, Canada; ⁴Galpharma, El Aïn, Tunisia; ⁵Faculty of Medicine, Chinese University of Hong-Kong, China; ⁶CNRS-UMR and Institut de cancérologie Gustave Roussy, Université Paris, France

Epstein-Barr virus (EBV) is present in undifferentiated nasopharyngeal carcinoma (NPC). Thus, tumour-associated viral antigens are potential targets for immunotherapy. We favoured an approach of immunotherapy based on the stimulation of CD4+ T cell specifics of EBV antigens, while taking into account that NPC cells secrete large amounts of immunosuppressive exosomes. Both CD4+ and EBV-specific CD4+ T cell lines, were generated and cultured with increasing doses of NPC exosomes, isolated from xenotransplanted NPC in severe combined immunodeficiency (SCID) mice. We performed (i) proliferation assays, (ii) cytotoxicity tests by measuring the NPC cells lysis and (iii) cytokine secretion. The efficacy of peptides vaccination was assessed in an immunodeficient SCID mice model xenotransplanted with NPC cell lines (C666.1-Luc), and reconstituted with human PBMC. The evolution of the tumour has been appreciated, both by measuring the tumour volume and the bioluminescence emitted by NPC-Luc cells. We have previously selected 6 peptides derived from the 3 EBV latency II antigens. We show here that all these peptides used in cocktail are recognized by human PBMC, after pre-incubation with NPC cell lines, inducing a broad IFN γ cytokine secretion. Furthermore, peptide-specific CD4+ T cell lines generated, and their cytotoxic potential assessed by lysing different NPC cell lines, even in the presence of autologous exosomes. We also report for the first time the ability of the cocktail to restrain tumour growth in an original NPC xenotransplanted humanized SCID mice model. Finally, this peptide cocktail had been recognized by all NPC patients

tested whatever their disease' stage and treatment status, eliciting a strong Th1 memory response. Our data clearly suggest that EBV latency II-derived peptides could be used as tumour vaccine for immunotherapy of NPC, mainly in refractory patients or to combat residual disease and prevent relapse in high risk patients after classical treatments.

OT15.4

The shuttle of extracellular vesicles between cancer and bystander cells allows dual targeting of malignant and supporter cells of the tumour microenvironment by antibody drug conjugates

Hinrich Hansen, Maria Dams, Elke Pogge Von Strandmann and Maximilian Tator
Internal Medicine I, University Cologne, Cologne, Germany

The aim of targeted immunotherapy in cancer is to damage both malignant and tumour-supporting cells of the tumour microenvironment but spare unaffected tissue. Several hematopoietic malignancies such as Hodgkin lymphoma selectively express the TNF receptor family member CD30 and the CD30 antibody drug conjugates (ADC) SGN-35 shows an impressive clinical response in CD30-expressing malignancies. Here, we investigated how cancer cell-derived extracellular vesicles (EVs) influence the SGN-35 efficacy using in vitro models of classical Hodgkin lymphoma (cHL). The malignant Hodgkin and Reed–Sternberg (HRS) cells selectively express CD30 and release this receptor on EVs for the tumour-supporting communication with CD30 ligand (CD30L)-positive bystander cells. Both, the malignant cells and the EVs express active sheddases, which cleave CD30 and release the soluble ectodomain (sCD30) into the environment. In a 3D semi-solid model to simulate the matrix of the tumour microenvironment, EVs were retained but the cleaved ectodomain penetrated readily. This resulted in an enrichment of EV-associated CD30 inside and sCD30 outside the matrix. A low percentage of EV-associated CD30 was also detected in the plasma of cHL patients, confirming the relevance of the model. The interaction of EVs with CD30-/CD30L+ mast cells and eosinophils allowed the indirect binding of SGN-35. As a consequence, SGN-35 also damaged CD30-negative cells, provided they were loaded with CD30-expressing EVs. Loading with sCD30 was not effective. The fact that EVs loose CD30 when diffusing towards the circulation might explain the powerful local SGN-35 efficacy against cancer and bystander cells within the tumour tissue and the minute side effects in the circulation. These data provide a novel mechanistic insight into the functionality of ADCs.

OT15.5

Large oncosomes reprogramme prostate fibroblasts towards an angiogenic phenotype

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Introduction: Cancer cells communicate with different cells in the microenvironment, establishing a supportive stroma that sustains tumour development and facilitates the first steps of metastasis. Extracellular vesicles (EVs) have emerged as key functional mediators of this process. **Aim:** To determine the mechanism of intercellular communication mediated by the atypically large EVs produced by highly migratory and metastatic tumour cells, referred to as large oncosomes (LO), and prostate fibroblasts (NAF). **Methods:** Filtration, differential and iodixanol gradient centrifugation; flow cytometry; confocal imaging; western blotting; kinase assay; TF array; luciferase assay; tube formation; siRNA; RT-qPCR. **Results:** Active AKT1 is significantly more expressed and functional in LO than in exosomes (Exo). Patients with metastatic disease express abundant active AKT1 in plasma LO. Uptake of LO harbouring active AKT1 by NAF results in AKT1 and c-MYC activation. Conditioned media from LO-treated NAF, but not from Exo-treated NAF, promoted endothelial morphogenesis. The dynamin (DNM) inhibitor dynasore (Dyn)-inhibited LO-uptake, as well as MYC activation and tube formation. Transient silencing of DNM2 significantly reduced LO uptake, suggesting that uptake occurs by phagocytosis. LO treatment increased levels of MYC targets in NAF, suggesting that MYC is involved in LO-induced reprogramming of NAF. Accordingly, MYC expression was higher in activated fibroblasts than NAF, and MYC overexpression in NAF induced hyperplasia in normal prostate epithelium in mice, suggesting MYC activation is an early event in cancer development. **Conclusion:** Our results indicate that tumour-derived LO induce a novel, c-MYC mediated, pro-tumorigenic reprogramming of fibroblasts that can be reverted by selectively inhibiting LO uptake. **Support:** National Institutes of Health NCI NIH R00 CA131472; NIH UCLA SPORE in Prostate Cancer award P50 CA092131; Avon Foundation Fund 02-2013-043 (to DDV).

Room: van Weelde

Symposium Session 16 - New techniques for EV characterization

Chairs: *Martijn van Herwijnen and Frank Coumans*

3:30-4:30 p.m.

OT16.1**A nanofluidic device to study of extracellular vesicles on a single vesicle basis**Marta Bally¹, Stephan Block², Fredrik Westerlund³,
Mohammadreza Alizadehheidari³, Elin Esebörner³ and Remo Friedrich¹¹Department of Physics, Chalmers University of Technology, Göteborg, Sweden; ²Chalmers University of Technology, Göteborg, Sweden;³Department of Biology and Biological Engineering, Chalmers University of Technology, Göteborg, Sweden

Introduction: Extracellular vesicle samples are characterized by an enormous degree of heterogeneity both in vesicle biomolecular composition and physical properties. This complexity calls for the development of methods allowing for the characterization of small biological vesicles on a single vesicle level allowing for the accurate description of vesicle subpopulations including the identification of rare but relevant subfractions. **Methods:** We present a nanofluidic device allowing for the fluorescence-based detection of small lipid vesicles with high-throughput and on a single particle basis. The device consists of 300 nm × 300 nm parallel nanochannels fabricated in fused silica. **Results:** In pin proof-of-principle experiments carried out with liposomes, we show that the device can be used to quantify the fluorescence intensity of individual fluorescent vesicles; vesicles carrying a few tens of fluorophores could successfully be detected. We further demonstrate that the device accurately determines the number of vesicles found in solution; vesicle quantification was reliable over more than three orders of magnitude and down to a couple of hundred femtomolar. Finally, our setup is compatible with dual-colour detection as demonstrated using liposomes carrying both fluorescent lipids and a fluorescent membrane-binding peptide. For all experiments, samples consumption was low: less than 20 microliters were required for full analysis. The nanofluidic device was shown to be well-suited to analyze biological samples. Extracellular vesicles isolated from a human mast cell line could be visualized after staining with a membrane dye and their concentration was determined. **Summary/conclusion:** These results illustrate the high potential of our nanofluidic device in the characterization and quantification of exosomes and other extracellular vesicles without ensemble averaging. It is likely to find applications in a variety of fields, ranging from diagnostics to fundamental biology.

OT16.2**Size and refractive index determination of extracellular vesicles smaller than 70 nm in a nano-fluidic optical fibre**Edwin Van Der Pol¹, Sanli Faez^{2,3}, Yoav Lahini⁴, Markus A. Schmidt⁵,
Stefan Weidlich^{5,6}, Frank Coumans¹, Rienk Nieuwland⁷,
T.G. Van Leeuwen¹ and Auguste Sturk⁷¹Department of Biomedical Engineering and Physics, Academic Medical Centre, University of Amsterdam; ²Department of Physics & Debye Institute for Nanomaterials Science, Utrecht University; ³Huygens-Kamerlingh Onnes Laboratory, Leiden University, Leiden, The Netherlands; ⁴Department of Physics, Massachusetts Institute of Technology, Harvard University, Cambridge, MA, USA; ⁵Fiber sensors, Leibniz Institute of Photonic Technology, Jena, Germany; ⁶Heraeus Quarzglas GmbH & Co. KG, Hanau, Germany; ⁷Laboratory of Clinical Chemistry, Academic Medical Centre, University of Amsterdam, The Netherlands

Introduction: State-of-the-art detection methods show that typically ~70% of the extracellular vesicles (EV) have a diameter <70 nm. Thus far, EV <70 nm have only been studied in frozen state or with

methods requiring EV to be adhered to a surface. Thus, the majority of EV have never been studied in their physiological environment. Here we present a novel label-free optical method to track single EV <70 nm in suspension. We use the method to determine the diameter and refractive index (RI) of EV. **Methods:** EV from human cell-free urine (n = 5) were contained within a single-mode light-guiding silica fibre with a 450 nm or 660 nm nano-fluidic channel. Laser light (660 nm wavelength) was coupled to the fibre, resulting in a confined optical mode in the nano-fluidic channel, which continuously illuminated the freely diffusing EV inside the channel. Elastic light scattering from the EV, in the direction orthogonal to the fibre axis, was collected using a microscope objective (NA=0.95) and imaged with a home-built microscope. Light scattering was calibrated with 35 nm and 50 nm polystyrene beads. To derive the diameter and RI of EV, we described the measured thermal diffusion by the Stokes–Einstein equation and light scattering by the Rayleigh approximation. **Results:** Using a nano-fluidic optical fibre, we could track single EV as small as 50 nm for >60 seconds by elastic light scattering. Furthermore, by combining data on thermal diffusion and light scattering, we confirmed that most EV have an RI <1.4. **Summary/conclusion:** For the first time, we have tracked single EV <70 nm freely diffusing in suspension to determine their diameter and RI. Knowledge on the diameter and RI of EV is essential to data interpretation and standardization. Potential applications of the nano-fluidic optical fibre are dimensional characterization, zeta potential analyses and particle–particle interaction analyses of the smallest EV present.

OT16.3**A biosensor study of extracellular vesicles derived from inflammation-triggered endothelial cells**Baharak Hosseinkhani¹, Sören Kuypers¹, Jan D'Haen²,
Nynke Van den Akker³, Daniel G. M. Molin³, Inge Nelissen⁴,
Jef Hooyberghs⁴ and Luc Michiels¹¹Hasselt University, BIOMED, B-3590 Diepenbeek, Belgium; ²Hasselt University, Institute for Materials Research, B-3590 Diepenbeek, Belgium; ³Maastricht University, dept. Of Physiology, Cardiovascular Research Institute Maastricht (CARIM), Universiteitssingel 50, 6200 MD Maastricht, The Netherlands; ⁴Flemish Institute for Technological Research (VITO), Boeretang 200, 2400 Mol, Belgium

One of the major challenges using extracellular vesicles (EV) as diagnostic biomarkers is the assessment of translational methodologies to profile EV in relation to their physiological perturbations. Surface plasmon resonance (SPR) offers a promising real-time, label-free and highly versatile sensor platform with high potential for EV biomarker detection. Therefore, we aim to develop a uniform SPR methodology to detect specific protein surface markers on EV. EV were purified from the culture supernatant of HUVEC, either untreated or treated with TNF- α to induce an inflammatory stress response by using different isolation procedure. Different analysis tools such as TEM and NTA were used to study the size distribution and concentration of EV. Thereafter, biomarker profiling EV was carried out using Immuno-EM, western blot, ELISA and SPR techniques. EV having a size range of approximately 100–200 nm were isolated. The mean size of EV derived from TNF- α treated cells was relatively bigger EV from untreated HUVEC. Overall, higher concentrations of EV were detected in TNF- α treated HUVEC when compared to non-stressed cells. Semi-quantitative screening of biomarkers present on EV-surface was carried out using immunogold-conjugated antibodies against ICAM-1, VCAM-1, PECAM-1, CD9, CD81, CD63 and TNF- α receptor. Collectively, ratios of ICAM-1 and CD63 biomarkers allowed to discriminate between EV derived from untreated and inflamed cells. In order to assess the specificity of antibodies towards EV

detection on SPR, the surface of a C1 chip was first functionalized with antibodies and then exposed to EV. Results showed that both anti-ICAM1 and CD63 tended to bind more EV produced by TNF- α treated cells than others. Our current findings open up novel opportunities for in-depth and label-free investigation of EV related biomarkers in a high-end SPR platform. This work was co-financed by the Interreg IV-V Flanders–the Netherlands project VaRiA and TTD.

OT16.4

EVQuant: a novel extracellular vesicle quantification and characterization assay for scientific and diagnostic use

Thomas Hartjes¹, Diederick Duijvesz¹, Roy Van Der Meel², Mirella Vredenbregt¹, Matthijs Bekkers¹, Adriaan Houtsmuller¹, Raymond Schiffelers², Guido Jenster¹ and Martin Van Royen¹
¹Erasmus MC, Rotterdam, The Netherlands; ²University Medical Center Utrecht, The Netherlands

Introduction: Extracellular vesicles (EVs) are an important source for biomarker discovery in cancer. As EVs in body fluids (i.e. urine) can be obtained without invasive biopsies, EVs could be ideal for detection or monitoring disease. Although various methods exist for quantifying EVs, these methods are often time-consuming and detection in clinical samples remains challenging. Current methods are also unable to perform immunofluorescent imaging to detect multiple

biomarkers on single vesicles. Here we provide a rapid and easy to use fluorescence-based assay (EVQuant) to quantify and characterize single EVs in research and clinical samples without the need for EV purification. *Methods:* In short, membranes of vesicles are fluorescently labelled using dyes for general cell membrane labelling (i.e. rhodamine, PKH26/67) and/or immunofluorescent antibodies. To detect low intensity signals, EVs are immobilized in a transparent medium and visualized using confocal microscopy. Fluorescent EV signals are quantified using a customized protocol. *Results:* Medium from a panel of cell lines, isolated EVs and urine samples were quantified using EVQuant, nanoparticle tracking analysis (Nanosight NS300) and a CD9/CD63 time-resolved fluorescence immunoassay (TR-FIA). We found a good correlation between EVQuant and NanoSight but observed cell line specific differences when comparing EVQuant and TR-FIA. When combining Rhodamine with fluorescent antibodies against CD9 and CD63, we were able to identify subpopulations of EVs with specific phenotypes. *Conclusion:* EVQuant is a rapid, robust, low-cost, widely accessible assay which can be of great value in research and clinical settings. As only a small sample is needed without the need for EV purification, but mainly the opportunity to perform multi-colour labelling of EVs (combination of biomarkers) makes this technique a suitable candidate for implementation in a clinical setting.

Prostate Cancer UK (G2012-36) funds this project.

Room: Jurriaanse

Symposium Session 17 - EV biomarkers for cancer II

Chairs: *Hidetoshi Tahara and Leonora Balaj*

3:30-4:30 p.m.

OT17.1**EV-mediated procoagulant activity in multiple myeloma and its precursor monoclonal gammopathy of undetermined significance**

Thøger Nielsen¹, Søren Kristensen¹, Shona Pedersen¹, Henrik Gregersen¹, Elena Manuela Theodorescu¹ and Gunna Christiansen²
¹Aalborg University Hospital, Aalborg, Denmark; ²Aarhus University, Aarhus, Denmark

Multiple myeloma (MM) and its precursor monoclonal gammopathy of undetermined significance (MGUS) have a high risk of developing thromboembolic events. Many factors are believed to contribute to this hematological imbalance. Possible contributors may be extracellular vesicles (EVs) circulating in the blood exerting procoagulant behaviour linked to tissue factor (TF) and phosphatidyl serine. Here, we investigated hypercoagulability in relation to EVs in blood from MM and MGUS patients. Platelet free plasma was collected from 15 MM and 14 MGUS patients and 10 age-related control persons. All test subjects gave written consent and the local ethics committee approved the study. EVs were visualized using transmission electron microscopy (TEM) and size and concentration were examined by nanoparticle tracking analysis (NTA). Furthermore, calibrated automated thrombogram (CAT) and procoagulant phospholipids (PPL) analyses were executed. To examine the procoagulant effect of EVs exposing TF, fibrin generation test (FGT) and commercial antigenic and activity assays for TF were conducted. By means of NTA we observe a markedly higher particle count in patient plasma with the most distinct difference in MM. Both patient groups exhibited both shorter lag time and time-to-peak and a slightly elevated peak thrombin in the CAT analysis. Faster clotting times in the PPL assay were found in both MGUS and MM patients compared to controls, thus indicating more procoagulant phospholipid activity. In addition, the levels of circulating EV-associated TF in the age-related controls differ from the patients, towards a more diseased profile. Our results indicate that there is a link between EV-phenotypes and hypercoagulability in patients with MM and demonstrates a similar premalignant pattern of hypercoagulability present in the precursor stage, MGUS. These findings may prove useful in identifying individual patients at risk of developing thrombosis and allow for an early intervention.

OT17.2**Extracellular vesicles mediate a hypercoagulable profile in small cell lung carcinoma patients**

Anne Flou Kristensen¹, Gezelius Emelie², Shona Pedersen³, Søren Risom Kristensen¹ and Mattias Belting²

¹Department of clinical biochemistry/Department of clinical medicine, Aalborg University Hospital, Aalborg, Denmark; ²Department of Clinical Sciences, Skane University Hospital, Lund, Sweden; ³Department of Clinical Biochemistry, Aalborg University Hospital, Aalborg, Denmark

Introduction: Small cell lung cancer (SCLC) is an aggressive subtype of lung cancer and patients have an increased risk of developing venous thromboembolisms (VTE). Chemotherapy administration is associated with increased shedding of extracellular vesicles (EVs) that are involved in thrombosis and metastasis. Recently, EVs have been proposed to mediate a hypercoagulable profile in cancer patients, therefore the aim of this project is to investigate the hypercoagulability mediated by EVs in SCLC patients during disease progression and treatment. **Methods:** In a randomized phase III study (NCT00717938) with 245 SCLC patients (105 with limited disease

(LD) and 140 with extensive disease (ED) receiving chemotherapy), blood samples were collected at baseline, during treatment and at follow-up 2 months after the end of chemotherapy. The hypercoagulability was determined using calibrated automated thrombography (CAT) and measuring the procoagulant phospholipids of EVs, i.e. the PPL assay. To enumerate EV concentration and size, nanoparticle tracking analysis (NTA) was used. Controls were 60 age-matched healthy donors collected at Aalborg university hospital (N-20140055). **Results:** At baseline, we observed a 40% increase in the thrombin generated (CAT) and a 50% increase in PPL activity in patients with SCLC compared to healthy controls. There was no difference in the coagulation profile of patients with LD and ED. Moreover, we find that the peak thrombin increased approx. 25% during treatment and the same at follow-up compared with baseline. In addition, PPL activity increased approx. 10% at follow-up. We find an increased mean particle concentration with a reduced particle size during treatment and follow up compared to baseline. **Conclusion:** Our results demonstrate that SCLC-derived EVs possess a procoagulant potency compared to controls and this may even be augmented during and after therapy.

OT17.3**Cerebrospinal fluid microRNA (CSF miRNA) signature as a biomarker for glioblastoma detection**

Johnny Akers¹, Valya Ramakrishnan¹, Ying Mao², Wei Hua², Bob S. Carter¹ and Clark C. Chen¹

¹Center for Theoretical and Applied Neuro-Oncology, University of California, San Diego, San Diego, California, USA; ²Department of Neurosurgery, Huashan Hospital, Fudan University, Shanghai, China

Introduction: Glioblastoma (GBM) is the most common form of primary brain neoplasm and remains one of the deadliest of human cancers. Clinical care for GBM patients is largely limited by the absence of biomarker for assessment of tumour burden or therapeutic response. **Methods:** Clinical GBM specimens and extracellular vesicles (EV) of cerebrospinal fluids (CSF) from the same patient were profiled using the TaqMan OpenArray miRNA platform. CSFs from non-oncology patients were also profiled. A miRNA signature that discriminated GBM CSF from non-oncology patients was identified and validated in independent cohorts. Secretion of miRNAs comprising this signature by GBM was studied using in vitro cell line models. **Results:** In miRNA profiles of 24 matched clinical GBM specimens and CSF, ~10% of the GBM miRNAs can be reliably detected in the matched clinical CSF. Good correlation was noted between the abundance of the miRNAs in the clinical specimens and cerebrospinal fluid. Profiling of additional GBM CSF (n=164) and non-oncology CSF (n=111) revealed a molecular signature that reliably discriminated GBM CSF from non-oncology CSF. For CSF derived from the cerebral ventricle (cisternal), the sensitivity and specificity for GBM detection were 80% and 67%, respectively. For CSF derived from lumbar puncture, the sensitivity and specificity for GBM detection were 28% and 95%, respectively. These results were confirmed in two additional patient cohorts (30 GBM CSFs and 30 non-oncology CSFs). In GBM cell lines, secretion of signature miRNAs was highly stimulated by co-culture with cell components that comprise the in vivo GBM microenvironment (including bone marrow stromal cells). **Conclusion:** The study represents the largest profiling effort of clinical GBM CSF specimens to date and provides compelling results supporting CSF miRNA signature as a biomarker platform for GBM detection. Source of CSF (cisternal vs. lumbar) significantly influences the sensitivity and specificity of this platform.

OT17.4

Identification of individual exosome like vesicles by surface enhanced Raman spectroscopy

Stephan Stremersch¹, Monica Marro², Bat-El Pinchasik³, Pieter Baatsen⁴, An Hendrix⁵, Stefaan C. De Smedt¹, Pablo Loza-Alvarez², Andre G. Skirtach^{3,6}, Koen Raemdonck¹ and Kevin Braeckmans¹

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Introduction: Exosome like vesicles (ELVs) are increasingly studied in a diagnostic context. The fact that they contain many molecules derived directly from the parent cell in combination with their easy accessibility makes them interesting biomarker candidates. To date, most approaches focus on identifying one specific molecular component by elaborate 'omics' studies on bulk ELV isolates. Here instead we tried to tackle the pending challenge to discriminate

cancerous ELVs from healthy ELVs on the single vesicle level. In this respect, we explored gold functionalization of ELVs and surface enhanced Raman spectroscopy (SERS) to obtain a molecular fingerprint of single vesicles while circumventing the long integration times of conventional Raman spectroscopy. **Methods:** ELVs were purified from B16F10 melanoma cells and RBC by density gradient UC. Raman spectra of single, gold nanoparticle (AuNP) coated ELVs were recorded with conventional confocal Raman microscopes. Spectra were analyzed by multivariate statistical analysis, i.e. PLS-DA and MCR-ALS. **Results:** To obtain a SERS signal, a nanoplasmonic material has to be in close proximity to the ELVs. To this end, ELVs were coated with a self-assembling AuNP shell, formed by charge-based interaction, while still maintaining a colloidal stable suspension of ELVs. Next, Raman spectra were obtained with a short acquisition time of 500 ms. We found that cancerous B16F10 ELVs have a different Raman fingerprint from 'healthy' RBC-derived ELVs. By means of PLS-DA analysis we could demonstrate that both types of ELVs can be discriminated from one another on the single vesicle level. Finally, we validated this approach in a mixture of ELVs derived from both cell types and were able to quantify the ratio of both ELV types. **Conclusion:** We presented a method to obtain Raman spectra from individual ELVs with a minimal acquisition time. Based on these spectra, ELVs from different origin could be identified and quantified in a mixture.

Room: Willem Burger

Symposium Session 18 - EVs in neuropathies

Chairs: Andrew Hill and Eva-Maria Krämer-Albers

3:30-4:30 p.m.

OT18.1

Exosomes and microvesicles from ALS CNS tissues contain misfolded SOD1 and transmit intracellular propagated misfoldingSarah Fernando, Leslie Grad, Catherine Cowan, Neil Cashman and Judith Silverman
University of British Columbia, Vancouver, Canada

Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease causing progressive paralysis in affected patients. ALS pathology begins focally and spreads spatiotemporally throughout the neuroaxis, reminiscent of the systematic mechanism of prion propagation. Superoxide dismutase (SOD1) gene mutations cause ~20% of inheritable ALS, and misfolded SOD1 protein is found in both sporadic and familial ALS. We have previously shown that cell-to-cell transmission of misfolded SOD1 can occur via the uptake of exosomes. However, the role of exosomes and microvesicles in protein misfolding and propagation in ALS patients is yet to be defined. Exosome and microvesicle populations (EVs) isolated from frozen whole neural tissues of ALS mouse models and human ALS patients by serial centrifugation and purification on a sucrose cushion. Misfolded SOD1 protein was identified by ELISA, immunoprecipitation, and TEM on the surface of both vesicle populations using conformation-specific antibodies. Detergent lysis of EVs showed a far greater abundance of misfolded SOD1 in the lumen of the vesicles than on the surface. Quantitative proteomic analysis suggests that exosomes from an ALS mouse model display a greater amount of EV surface Excitatory Amino Acid Transporter 2 (EAAT2) relative to control. Similarly, analysis by flow cytometry showed more ALS mouse model-derived vesicles were positive for the astrocyte marker EAAT1 than control vesicles. Finally, preliminary results suggest that applying ALS mouse model tissue-derived EVs onto fresh HEK cell cultures causes misfolding of wild-type SOD1 in those cells. In summary, we have successfully isolated EV populations from ALS patient and murine neural tissues, and shown these vesicles to carry misfolded SOD1. Our results suggest that EVs bearing misfolded protein can be detected with conformation-specific antibodies, intimating their diagnostic and therapeutic potential in ALS and other protein misfolding diseases.

OT18.2

Influence of Parkinson's pathology upon exosomal alpha-synucleinLydia Alvarez-Erviti¹, J. Mark Cooper² and Michele Di Stefano²
¹Molecular Neurobiology, Center for Biomedical Research of La Rioja (CIBIR), Logrono, Spain and Clinical Neuroscience, University College London, London; ²Clinical Neuroscience, University College London, London, UK

Introduction: Alpha-synuclein aggregation is a central theme in Parkinson's disease (PD) pathology and the transmission of the protein between cells has been suggested to mediate the spread of the pathology within the brain as the disease progresses. We have shown that alpha-synuclein can be released from cells in exosomes which can mediate the generation of aggregates in neighbouring cells. We have demonstrated that alpha-synuclein enters exosomes via an hsc70-dependent pathway. Subsequently we have investigated the impact of the pathways implicated in PD upon alpha-synuclein release and transmission. **Methods:** Using cell models we have investigated the pathway of the intracellular transfer of alpha-synuclein to exosomes to understand how factors associated with PD pathology may influence the level of alpha-synuclein release from cells in exosomes. We have modelled chaperone-mediated autophagy (CMA) dysfunction (LAMP-2A knockdown), and various

important risk factors for developing PD including: alpha-synuclein A53T, S129D mutations; LRRK2 G2019S mutation and GBA1 knock-down or inhibition. **Results:** Our experiments demonstrated that decreased alpha-synuclein degradation due to CMA and GBA1 dysfunction, or LRRK2 mutation increased exosomal and total alpha-synuclein release. Moreover we confirmed that alpha-synuclein mutation increased its release by exosomes. **Conclusion:** We have demonstrated that influencing the delivery of alpha-synuclein to lysosomes for degradation by CMA leads to elevated exosomal alpha-synuclein levels and subsequent release, highlighting a new target for therapeutic intervention.

OT18.3

PrPC on exosomes drives fibrillization of amyloid beta and ameliorates amyloid beta-mediated neurotoxicityAlexander Hartmann^{1*}, Clemens Falke^{1*}, Inga Guett¹, Frank Dohler¹, Hermann Altmeppen¹, Christian Betzel², Robin Schubert², Pooja Joshi³, Claudia Verderio³, Markus Glatzel¹, Susanne Krasemann¹

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Alzheimer's disease (AD) is the most common neurodegenerative disorder. Deposition of amyloid beta into plaques is a key step in AD pathogenesis. However, small soluble amyloid beta oligomers rather correlate with neurotoxic effects, disease initiation and progression. A couple of recent findings identified the cellular prion protein (PrPC) as receptor for amyloid beta oligomers, eliciting these neurotoxic effects. The role of exosomes in Alzheimer's disease is only poorly understood. Besides serving as disease-biomarkers, they may promote amyloid beta plaque formation, thereby decreasing amyloid beta-mediated synaptotoxicity and enhancing amyloid beta-clearance. To investigate the potential role of exosomal PrPC and its effect on the protective functions attributed to exosomes in AD, we generated a mouse neuroblastoma PrPC knockout cell line. Using these, as well as SH-SY5Y human neuroblastoma cells, we showed that PrPC is highly enriched on exosomes compared to cells. Moreover, we found that exosomes bind amyloid beta via PrPC with highest binding affinity for low molecular weight oligomeric amyloid beta species. Exosomal PrPC accelerates fibrillization of amyloid beta on exosomes, whereas exosomes devoid of PrPC did not show this effect. This leads to a significant reduction of neurotoxic effects imparted by oligomeric amyloid beta. In conclusion our data leads to further evidence for a protective role of exosomes in amyloid beta-mediated neurodegeneration. Moreover, it highlights the importance of exosomal PrPC in molecular mechanisms of Alzheimer's disease.

OT18.4

Glioblastoma extracellular vesicles drive normal astrocytes toward a tumor-enhancing phenotypeMichael Graner¹, Soliman Oushy², Justin Hellwinkel², Mary Wang¹, Ger Nguyen¹, Tessa Harland², Dicle Gunaydin¹¹University of Colorado Denver Anschutz Medical Campus, Aurora, Colorado, USA; ²University of Colorado Denver School of Medicine, Aurora, Colorado, USA

Glioblastomas (GBMs, WHO grade IV astrocytomas) are the worst of the central nervous system tumors; despite maximum (and damaging)

therapeutic intervention, median survival time for patients is <15 months, and overall quality of life becomes quite poor. These abysmal outcomes have changed little in the past 20 years. Clearly, our current therapies are inadequate, and we need innovative strides in understanding GBM biology to rectify this situation. One "hot" research area is that of the impact of tumor extracellular vesicles (EVs) on normal recipient cells. Tumor cell EVs have extraordinary abilities to manipulate their microenvironments and recipient cells both proximally and distally (eg, fibroblast differentiation, endothelial cell tubule formation, stromal cell interactions, immune cell influence). Tumor EVs prepare the "metastatic niche" for circulating tumor cells prior to colonization of a target organ. Thus, tumor EVs can impact recipient cells to support tumor growth and progression,

which undoubtedly holds true for GBMs as well. However, it appears no one has asked what effects GBM EVs have on normal astrocytes—do GBM EVs drive astrocyte phenotypic changes, potentially making the astrocytes into tumor promoters? The answers could re-shape our paradigms on gliomagenesis, particularly in the recurrent setting. Here we show that GBM EVs activate cancer-type signaling pathways in recipient astrocytes, promoting astrocyte migration towards the EVs, as well as astrocyte anchorage-independent growth in soft agar. The extracellular release of various factors by astrocytes generates a tumor-promoting milieu, which results in increased tumor cell growth. We will discuss the consequences of these phenomena in the context of our current therapies with a view towards therapeutic improvement.

Poster Presentations

Room: Willem Burger Foyer/Van Der Mandele and Arcadis Zaals
Poster Session 1 - EVs, microbes and cross-species communication

Chairs: Leonid Margolis and Oh Youn Kim

5:00-8:00 p.m.

PT1.01

Identification of extracellular vesicles in three parasitic nematodes of veterinary importance

Eline Palm Hansen, Merete Fredholm, Stig Milan Thamsborg,
Peter Nejsum and Kasper Lind Andersen
University of Copenhagen, Copenhagen, Denmark

Introduction: Parasitism has been denoted the most successful way of life and their success relates to their ability to evade or modulate the host immune response. However, the underlying mechanisms behind this are poorly understood. Parasites from all major parasitic groups have been shown to release extracellular vesicles (EVs) containing protein and RNA species and seems to be of importance in the host–parasite interplay. The aim of this study was to examine the secretion of EVs for three of the most important parasitic nematodes of pigs, *Ascaris suum*, *Trichuris suis* and *Oesophagostomum dentatum* in order to later functionally test their role in host–parasite interactions. **Methods:** Adult worms of *A. suum*, *T. suis* and *O. dentatum* were incubated in RPMI 1640 containing streptomycin, penicillin, amphotericin B and gentamycin (Gibco(r)) for 24–48 h. EVs were purified from parasite-depleted RPMI by an initial filtering step (200 nm) followed by differential centrifugations with two final ultracentrifugations at 110.000g (Beckman Optima(tm) L-70) for 70 minutes. The final pellet was analyzed with a CM 100 BioTWIN transmission electron microscope (TEM) and RNA was purified using a miRCURY(tm) RNA Isolation Kit (Exiqon A/S). The RNA concentration was measured using a NanoDrop 2000 spectrophotometer. **Results:** The TEM analysis revealed that all three species produce EVs in the size of ~ 100 nm and the NanoDrop measurements show that the purification products contain RNA. **Conclusion:** These results suggest that adult worms of the three porcine parasite species, *A. suum*, *T. suis* and *O. dentatum* secrete RNA-containing EVs. Future studies should focus on an in-depth characterization of the EVs and their contents through RNA sequencing and proteomic analysis. With this, we may unravel important aspects of the complex interplay between parasites and their hosts and potentially discover novel targets for diagnostics and therapy.

PT1.02

Examining the exosome cargo of *Theileria annulata*-infected cells

Victoria Gillan, Jane Kinnaird, Brian Sheils and Eileen Devaney
University of Glasgow, Glasgow, UK

The protozoan parasite, *Theileria annulata* causes Tropical Theileriosis, a disease that is often severe and can be fatal. One of the most interesting features of the *T. annulata* infection cycle is its phase of neoplastic growth. The parasite can transform its host leukocyte resulting in an immortalized phenotype in vitro. In vivo these cells become highly metastatic, forming tumours in multiple organs of the host with subsequent destruction and disorganisation of the lymphoid system. As evidence from cancer biology has shown, metastasis is a highly complex process and although the pathogenic events have been defined, the mechanisms that initiate and drive metastasis are still largely unresolved. The field of microvesicle biology has significantly expanded to reveal that intercellular communication is critical for metastatic progression, and that the release of

exosomes from tumour-infected cells into the micro-environment induces pre-metastatic niche formation. It is intriguing to consider that exosomes derived from *T. annulata*-infected cells may transfer proteins, soluble factors and nucleic acids to recipient cells and therefore may be involved in altering adaptive immune responses of the host or have a role in migration/invasion of infected cells. To investigate the role of exosomes in this system we carried out proteomics on exosome samples from a bovine lymphosarcoma cell line (BL20) and the same cells infected with *T. annulata* (TBL20). Differentially regulated proteins were analysed using Ingenuity Pathway Analysis, revealing that infection associated proteins and molecules essential to migration and extracellular matrix digestion are up-regulated in TBL20 exosome samples compared with controls. miRNA sequencing of these samples is also underway. Exosome transfer experiments will be carried out to observe if exosomes from TBL20 cells can increase the migratory or invasive potential of control cells, or disrupt the cellular immune response.

PT1.03

Mechanics of extracellular vesicles derived from malaria parasite-infected Red Blood Cells

Raya Sorokin¹, Daan Vorselen¹, Yifat Ofir-Birin², Wouter H. Roos¹, Fred C. MacKintosh¹, Neta Regev-Rudzki² and Gijs J. L. Wuite¹
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Malaria is a life-threatening disease caused by parasites that are transmitted through the bites of infected mosquitoes, with *Plasmodium falciparum* (Pf) causing the most severe form of malaria (1). Very recently it was discovered that Pf infected red blood cells (iRBC) directly transfer information between parasites within a population using exosome like-vesicles that are capable of delivering genes (2). This communication promotes parasite differentiation to sexual forms and is critical for its survival in the host and transmission to mosquitoes. Efficient DNA transfer via extracellular vesicles (EVs) occurs mainly at the early ring stage within the blood-stage asexual cycle, and it can be inhibited by the addition of actin polymerization inhibitors. This suggests that actin polymerization is required for cell–cell communication (2). We expect, therefore, that mechanical properties of vesicles at different stages of the life cycle will be optimized for their gene-delivery function. With the aim to understand how mechanical properties of EVs effect their efficiency of cargo delivery, we use Atomic Force Microscopy for mechanical characterization of extracellular vesicles secreted from Pf infected RBCs. We compare bending modulus values of these vesicles and those secreted from healthy RBCs, as well as compare their size, morphology and surface charge. We also prepare and characterize synthetic vesicles (SVs) with varying mechanical strength to determine how stiffness of SVs affects their adhesion to cells and cellular uptake rate, to gain a broader understanding of the link between mechanical properties and efficient cellular uptake.

References

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PT1.04

The immunomodulatory role of human macrophages exosomes during *Trypanosoma cruzi* infection

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Introduction: EVs isolated from *Trypanosoma cruzi*, the etiologic agent of Chagas disease, modulate innate immune response via TLR2 and inflammatory responses in host cells and during in vitro and in vivo infection. Macrophages are major targets of *T. cruzi* infection however, whether infected macrophages also release specific EVs that could modulate the inflammatory response is completely unknown. Therefore, we aimed to characterize EVs from human macrophage (THP-1) infected with trypomastigote from *T. cruzi*. **Methods and Results:** THP-1 cells were differentiated into macrophages by using phorbol 12-myristate 13-acetate and incubated with LPS, parasite antigens, *T. cruzi* EVs, or infected with the trypomastigotes. The macrophage culture supernatants were centrifuged several times (twice at 1,500xg for 10 minutes and once at 8,000xg for 5 minutes) to remove residual cells and debris, and EVs were collected in the pellets of 1 h centrifugation at 100,000xg. EVs were resuspended in saline buffer and analyzed by NTA, scanning electronic microscopy and western blotting to CD63, CD9, MHC class II, TLR2, TLR4 receptors, and anti-*T. cruzi* antigens. We found that 24-h treatments with LPS, *T. cruzi* EV or infection with trypomastigotes induced the secretion of larger amounts of EVs by the macrophages than control untreated macrophages. The EVs ranged from 20 to 100 nm in size and they contained CD63, CD9, MHC class II which are known markers from macrophages-derived exosomes, but they lack parasite antigens. Remarkable, trypomastigotes EVs or trypomastigote infection also increased TLR2 and TLR4 expression by THP-1 macrophages. **Conclusion:** As *T. cruzi* EVs have a regulatory role through macrophage cells, we propose that macrophage-derived exosomes could transmit signals to alert the immune system during *T. cruzi* infection.

PT1.05

Characterization of extracellular vesicles from stressed pathogenic fungal cells

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In pathogenic fungi, extracellular vesicles (EVs) carry molecules potentially relevant for pathogenicity and host interaction, as suggested by the protein, lipid, carbohydrate, and RNA contents described in some species. Interestingly, a high percentage of EV proteins have been described in the fungal cell wall, suggesting that EV transportation is responsible for the finding of cytosolic proteins there. Little is known about modulation of fungal EV production or fungal cell signalling by EVs. We aimed to characterize EVs from pathogenic *Paracoccidioides brasiliensis* and *Candida albicans* submitted to oxidative, nitrosative, farnesol, and tyrosol stimuli; we also evaluated the fungal cells ability to uptake EVs. EVs were isolated from cell-free culture supernatants by standard concentration/centrifugation procedures. EV proteins were precipitated and analyzed by liquid chromatography coupled to mass spectrometry (Synapt G2 HDMS). EVs morphology/concentration were measured by NanoSight (Malvern). We identified 153 *P. brasiliensis* EV proteins in the control, versus about 280 at oxidative or nitrosative stresses. In both conditions, the amount of EVs isolated was two-fold larger than in controls. In *C. albicans*, we identified 233 proteins in control EVs, versus 222–250 in EVs from stimulated cultures. The amount of EVs increased eight

times under farnesol stimulus, but decreased under tyrosol and oxidative stress. *P. brasiliensis* showed 40 exclusive proteins to each stress condition, but not *C. albicans* EVs. Differences in EV protein amounts were observed in both species. Cell uptake was observed for EVs stained with PKh67 or PKh26 dye (Sigma) co-incubated for 1–4 h with *C. albicans* and analyzed by fluorescence and confocal microscopy. We observed interaction after the first hour of incubation, with apparent uptake. Our results suggest that EVs production/contents can be modulated by stress conditions and that EVs can potentially play a role in fungal cell communication.

PT1.06

Characterisation of extracellular vesicles secreted by HPV+ and HPV- oropharyngeal carcinoma cells

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Introduction: Viruses are capable of manipulating host endosomal-exosomal pathways which can aid in tumourigenesis. Studies have shown that human papilloma virus (HPV) encoded proteins can alter the production and contents of extracellular vesicles (EVs) secreted by cervical cancer cells. However, the extent of HPV's oncogenic properties relating to EV release in oropharyngeal carcinoma (OPC) is not well understood. This study aims to evaluate differences in size, quantity, and molecular contents of EVs released by two HPV+ and two HPV- OPC cell lines. **Methods:** EVs were purified from the conditioned medium of OPC cell lines by size exclusion chromatography (SEC). EV size and concentration was measured by Tuneable Resistive Pulse Sensing (TRPS). Transmission electron microscopy was used to validate size measurements made by TRPS. Vesicular protein and RNA were extracted and measured by BCA and Agilent Bioanalyzer, respectively, for subsequent iTRAQ proteomics and next generation sequencing. Vesicular impact on fibroblast and endothelial cell phenotype was assessed by MTT assay and/or tubule formation assay. **Results:** The number of EVs released per cell did not vary significantly between cell lines. The modal diameter of vesicles from HPV+ cell lines was noticeably smaller than that of HPV- lines, although this was not statistically significant. The yield of EVs purified by SEC that were deemed pure enough for downstream analysis was approximately 30%. Vesicular RNA isolated from ~50 million cells over a 72 h incubation period ranged between 500 and 1500 ng across the cell lines, with approximately 50% of RNA being miRNA, and vesicular protein ranged between ~25 and 50 µg. **Conclusion:** TRPS analysis detected size differences between EVs produced by HPV+ and HPV- OPC cell lines. SEC appears to be a valid technique for purification of EVs suitable for further downstream analysis by iTRAQ and RNA-seq.

PT1.07

Functional analysis of packaged viral RNAs from Epstein-Barr virus (EBV) by using recombinant exosomes

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Introduction: EBV is a widespread human herpesvirus that persists in an infected host lifelong by establishing latent infection in B cells. Viral particles do not only contain the DNA-genome, but additional viral RNAs. These RNAs are transduced to target cells during infection, where they can be translated immediately. By using recombinant exosomes, carrying selected viral transcripts, we are aiming to investigate the function of these RNAs. We are focussing on transcripts which are important for the initiation of pre-latent phase of the viral transcription (e.g. BZLF1) and for latent establishment of the genome. **Methods:** Exosomes were generated by transfecting producer cells with plasmids encoding for the viral transactivator

BZLF1, and the glycoprotein gp350. Purification was done via differential centrifugation, including a filtration step and exosomes were characterized using dot blot and flow cytometry, followed by RNA isolation to control successful packaging of BZLF1-RNA. Next, primary B cells were incubated with BZLF1-containing and non-containing exosomes and performed a subsequent RNA-isolation. RNA-sequencing was used, to see if the transduced BZLF1-RNA has an impact on the expression pattern of the target cells compared to the control. **Results:** We were successful in generating exosomes which carry gp350 (which mediates the binding to B cells), and mRNA encoding BZLF1, which is an important trigger in the early phase of infection. The purified exosomes were positive for CD63 and gp350 in dot blot and flow cytometry. Moreover, we were able to determine the concentration of our preparation by nano particle tracking analysis (NTA). Successful packaging of BZLF1-RNA into exosomes could be shown by RNA isolation and subsequent qRT-PCR. **Summary:** Recombinant exosomes containing viral transcripts could be a suitable tool for studying the impact of individual transduced viral RNAs on the early phase of infection and establishment of latency and thus for the biology of EBV.

PT1.08

Cellular survival and response to HIV infection in the absence of extracellular vesicles

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Introduction: Extracellular vesicles (EVs) are involved in intercellular communication and affect disease processes, e.g. by promoting tumour progression or by shuttling messenger molecules between cells. Furthermore, EVs are important in immune and antiviral responses. Given that cells are normally exposed to high concentrations of EVs, cultured cells may be affected by surrounding concentration of EVs from serum. These effects may include response to viral infection. We examined the effects of varying EV levels in different media on viral production and cell viability. **Methods:** T-cell and myeloid-lineage cell lines and primary cells were grown in 10% serum medium depleted of EVs using ultracentrifugation or a proprietary method (Life Technologies/Gibco) and in 10% FBS serum medium with no depletion of EVs. Cells were subsequently infected with HIV-1. Cell counts, viability and activity (MTT and WST assays) and HIV production (p24) were assessed. **Results:** In both T-cell and macrophage cell cultures, significant increases in HIV-1 viral production (p24 assay) were noted when EVs were depleted from cell culture medium. Changes in cell morphology and viability/activity were also observed in EV-depleted media. In contrast, primary CD4+ T-cells showed no significant changes in viability post infection, although a slight increase in viral production was observed. Increased infectivity of virus released under depleted EV culture conditions was also observed, correlating with increased expression of several cell surface receptors. **Conclusions:** The concentration of EVs around HIV-1 susceptible cells affects viral production and infectivity, as well as cell viability. These effects appear to be especially pronounced for cell lines. EV dependence of cell cultures should be tested carefully prior to including additional experimental variables, particularly in infection experiments.

PT1.09

The role of exosomes in the development of *Chlamydia*-induced upper genital tract pathology

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It is estimated that 70% of chlamydial infections in women are asymptomatic and, if left untreated, can lead to severe irreversible

complications including ectopic pregnancy and pelvic inflammatory disease. *Chlamydia trachomatis* infects cells in the cervix of the female genital tract and ascends to the upper genital tract (UGT) where it can cause diverse pathologies. It has been shown that *Chlamydia*-induced UGT pathology occurs even in the absence of live *Chlamydia* in the lower genital tract. The mechanism of ascending infection and disease is largely unknown. We hypothesize that the ascending infection that leads to UGT pathology is mediated by exosomes. Exosomes are vesicles that can package intracellular molecules from donor cells and release their cargo into recipient cells thereby manipulating gene expression and cellular activity. To test our hypothesis, HeLa cells were infected with *C. trachomatis* serovar D and exosomes isolated from culture supernatants were characterized. Cytokines and chemokines from exosomes were assessed by a multiplex cytokine ELISA assay. Furthermore, purified exosomes were co-cultured with uninfected cells and the protein content of the lysed cells was examined. The results showed infected cells released higher concentrations of exosomes during early infection compared to uninfected cells and significantly higher levels of inflammatory cytokines were associated with these exosomes. Moreover, exosome recipient cells were found to contain chlamydial proteins. These results suggest exosomes could transport pathogenic chlamydial proteins from infected to uninfected cells with potential to stimulate inflammation in recipient cells and suggest a possible mechanism for development of UGT pathology.

PT1.10

Identification of outer membrane vesicles derived from *Orientia tsutsugamushi*

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Orientia tsutsugamushi, a causative pathogen of Scrub typhus, is a gram-negative intracellular bacterium. Outer membrane vesicles (OMVs) are produced from the membrane of bacteria and play many roles related to the survival of the pathogen. However, there have been no reports confirming whether *O. tsutsugamushi* indeed produce OMVs. *O. tsutsugamushi* boryong was cultured in ECV-304 cells for the purification of OMVs. Western blot analysis and immunoenrichment using anti-*O. tsutsugamushi* monoclonal antibody and electron microscopy were employed for identification and characterization of OMVs. We confirm the presence of OMVs derived from *O. tsutsugamushi*, and also found that those OMVs contain a major surface antigen of 56-kDa protein and variant immunogenic antigens.

PT1.11

Cytotoxicity of host cells induced by *Staphylococcus aureus* membrane vesicles is dependent on proteomes of membrane vesicles

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Staphylococcus aureus secretes membrane-derived vesicles (MVs), which can deliver virulence factors to host cells and induce cytopathology. However, cytopathology of host cells induced by MVs derived from different *S. aureus* strains has not been characterized yet. The present study compared the cytotoxic activity of MVs from different clinical *S. aureus* isolates on host cells and analyzed the proteomes of *S. aureus* MVs. MVs purified from *S. aureus* M060 showed a high cytotoxic activity towards host cells, compared with MVs from three other clinical *S. aureus* isolates. *S. aureus* M060 MVs induced apoptosis of HEP-2 cells in a dose-dependent manner, but cytotoxic activity of MVs was completely abolished by treatment with proteinase K. Proteomic analysis revealed that a total of 153 and 143 proteins were identified in the MVs from *S. aureus* M060 and 03ST17, respectively. Comparative proteomic analysis showed that *S. aureus* MVs contained =60 strain-specific proteins. d-hemolysin

(Hld), γ -hemolysin, lukocidin D, and exfoliative toxin C were commonly identified in *S. aureus* MVs, but exfoliative toxin A (ETA) was specifically identified in *S. aureus* M060 MVs. ETA was delivered to HEp-2 cells via *S. aureus* MVs. MVs from clinical *S. aureus* isolates have different cytotoxic activity in host cells, which may result from difference in proteomes of MVs. Further proteogenomic analysis can be useful for identification of virulence factors in MVs from clinical *S. aureus* isolates.

PT1.12

Extracellular vesicles from gram-positive bacteria *Staphylococcus aureus* and *Staphylococcus epidermidis* induce a strong inflammatory response and cell death in vitro

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Introduction: The majority of biomaterial-associated infections (BAI) are caused by *S. aureus* and *S. epidermidis*. Bacterial extracellular vesicles (EVs) are involved in the delivery of toxins and bacterial components to host cells. It is therefore of relevance to investigate if the EVs from these bacterial species have a role in BAI. The aim of the present study was: i) to investigate whether clinical strains from *S. aureus* and *S. epidermidis*, isolated from osteomyelitis associated with bone-anchored amputation prosthesis, release EVs, ii) to study the biological effects of these EVs on monocytes and iii) to evaluate the effect of these EVs on the secretion of pro-inflammatory substances by monocytes. **Material and methods:** EVs were isolated from *S. aureus* 64516 and *S. epidermidis* 64518 cultures. The collected EVs were characterized by western blot. Human primary monocytes were stimulated by different concentrations of EVs (0, 5, 10 and 50 $\mu\text{g/ml}$). After 24 h, cell death was evaluated using propidium iodide in a nucleocounter and further, levels of IL-8, IL-6, TNF- α , MCP-1 and MMP-9 were determined by ELISA. **Results:** The two staphylococcal strains secreted EVs in vitro. Both types of EVs contained d-toxin whereas protein A and SCP-A were only detected in *S. aureus* EVs. The monocyte viability was reduced in a dose-dependent manner after incubation with EVs. More than 50% of monocytes died with 5 μg *S. aureus* EVs. The *S. epidermidis* EVs had relatively less toxic effects. Stimulation of monocytes with *S. aureus* and *S. epidermidis* EVs significantly increased the release of IL-8, IL-6, TNF- α , MCP-1 and MMP-9. **Conclusion:** The results from this study show that clinical pathogens causing BAI have the ability to secrete EVs in vitro. The EVs promote pro-inflammatory cytokine release from human monocytes and subsequent cell death. It is suggested that EVs contribute to the inflammation and injury associated with biomaterial-associated infection.

PT1.13

Membrane vesicles released during macrophage-infection with airway pathogens modulate innate immune responses

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Introduction: During infection a plethora of messenger molecules are released by both host and bacterial cells. These molecules serve to confer information and can be released in a soluble form as well as in association with membrane vesicles (MV). In this respect it has been demonstrated that host-cell vesicles are involved in the immune response to bacteria. Moreover, bacteria also shed membrane vesicles which are able to affect immunity by serving as transport

vehicles of several virulence factors. Hence, the objective of this study was to assess side by side vesicles released following infection of macrophages by the airway pathogens *Pseudomonas aeruginosa*, non-typeable *Haemophilus influenzae*, *Moraxella catarrhalis* and *Streptococcus pneumoniae*. **Methods:** MV-release kinetics were determined by flow cytometry following infection of Thp1 macrophages (MFs). Thereafter MVs released by infected MFs or by bacteria only were isolated by ultrafiltration and purified by size exclusion chromatography to assess their functional activity. **Results:** Flow cytometric analyses confirmed the release of MVs during macrophage infection. Functional experiments showed that MVs released upon infection as well as bacterial MVs, induced the release of TNF- α from naive MFs. The amount of TNF- α released varied markedly in response to MVs from different bacterial species. Moreover, pre-incubation of MFs with MVs dampened the TNF- α release in response to several TLR-agonists (LPS and PAM3CSK4). Finally, pre-incubation of MFs with MVs led to an enhanced bacterial adhesion and invasion. **Summary:** Overall, we found that the activity of MVs was highly species dependent with a high resemblance in the activity between vesicles released by infected MFs to vesicles released by bacteria only. Furthermore, our findings indicate that MVs released during infection may be beneficial to some bacteria by their ability to suppress pro-inflammatory responses and to enhance bacterial adhesion and invasion.

PT1.14

Bacterial membrane vesicle RNA in host-pathogen interactions: on the road towards specific functional studies

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Introduction: We have previously reported that MVs from Uropathogenic *Escherichia coli* (UPEC) can deliver bacterial RNA into human cells. This observation raises questions about the function of bacterial MV RNA in cross-species and cross-kingdom communication. However, the isolation of bacterial MV RNA and demonstration of its effects on target cells poses a number of unique challenges. This includes the presence of bacterial toxins in MVs and co-isolation of some of them with MV RNA. Here we report on our study investigating the effects of RNA from UPEC MVs on human bladder cells. **Methods:** MVs were isolated from UPEC strain 536 cultures by filtration and ultracentrifugation followed by density gradient centrifugation. RNA from each fraction was extracted with a mirVana RNA kit. RNA purification was performed with Norgen CleanAll DNA/RNA kit. Fractions and extracted MV RNA were analysed for protein and endotoxin levels. Bladder cell viability was quantified using MTT reagent. **Results:** Culture growth conditions and MV isolation methods determined levels of contaminants present in both MV and MV RNA preparations. Density gradient centrifugation of MV preparations allowed for isolation of distinct fractions characterised by differences in MV particle number, protein, endotoxin, and nucleic acids levels, as well as human bladder cell toxicity profiles. Endotoxin was identified as a common contaminant of RNA isolated from UPEC MVs. The effect of the contaminating endotoxin on bladder cell viability was found to be dependent on the method of delivery of isolated MV RNA into human cells. Methods for separating effects of MV RNA on bladder cell phenotype from those of contaminants were investigated. **Conclusions:** Our study addresses the challenges associated with isolating RNA from membrane vesicles of Gram-negative bacteria. Methodological and experimental approaches to studying the role of vesicle-delivered bacterial RNA in host-pathogen interactions are considered.

PT1.15

Extracellular vesicles from milk promote the formation of small osteoclasts but reduce bone resorption

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Introduction: Recently, extracellular vesicles present in milk have emerged as biologically active components. In this study we investigated the effect of bovine-derived milk extracellular-vesicles (BMEVs) on osteoclast differentiation and bone resorption. **Methods:** BMEVs were isolated from commercial cow milk by ultracentrifugation. Bone marrow cells (100,000 cells/well) isolated from femurs of male C57BL/6 mice were cultured in a-MEM containing 5% exosome-free FBS, 2 or 20 ng/ml RANKL and +/- BMEVs (200 µg/ml) added at day 1 or day 4. Osteoclast differentiation was determined by TRAP-staining and stratified accordingly to their nuclei. Osteoclast resorption was determined on calcium phosphate-coated plates. After, 7–10 days of culture with BMEVs added at day 1 or day 4, the cells were removed and resorption pits were visualized by von Kossa staining. On bovine cortical bone slices, cells were lysed after 9 days and resorption pits were stained with Coomassie brilliant blue. Cell proliferation was assessed by XTT assay, acidification of osteoclasts by acridine orange staining and gene expression by qRT-PCR. **Results:** BMEVs treatment until day 4 increased the number of TRAP-positive mononuclear cells and small (≤ 5 nuclei) osteoclasts. The number of large (≥ 6 nuclei) osteoclasts remained the same. These alterations were associated with increased expression of TRAP, NFATc1 and c-Fos. Cells seeded in a calcium-phosphate coated plate or bone slices showed reduced resorption area when exposed to BMEVs during the differentiation phase and even after osteoclast formation. Interestingly, BMEVs treatment enhanced Cathepsin K expression but reduced Carbonic Anhydrase 2 gene expression. Moreover, intracellular acid production was also decreased by BMEVs treatment. **Conclusion:** We showed for the first time that BMEVs increased the formation of small osteoclasts but this does not lead to more bone resorption probably due to reduced acid secretion.

Introduction: Recent studies suggest that human consumption of fruit/vegetable products, e.g. watermelon juice, leads to increased circulating levels of plant-specific microRNA (miRs). Here we investigate whether these function-regulating miRs could be transferred between species via uptake and export of miR-containing exosomes by the small intestine. **Methods:** Exosomes were isolated from watermelon via centrifugation, labelled fluorescently and characterised by Nanoparticle Tracking Analysis. Human intestinal epithelial cells (Caco-2) were cultured for 21 days post-confluency in transwells to allow formation of a small intestine-like enterocyte monolayer. Exosomes were applied apically to the Caco-2 cell monolayer, incubated for up to 4 h (to mimic the duration of chyme in the small intestine) then cells were analysed by fluorescence microscopy and flow cytometry. Plant-specific miR-156a was quantified using qPCR. **Results:** qPCR demonstrated that watermelon exosomes are rich in miR-156a. Microscopy and flow cytometry demonstrated that these exosomes were internalised by the tight, polarised Caco-2 barrier (confirmed using Lucifer Yellow and staining for ZO-1 and β -catenin). Exosomes were selectively transferred into Ki67+ cells, suggesting uptake into a proliferative subtype. Analysis of uptake kinetics revealed maximal internalisation at 180 minutes (48.90%, IQR 7.5, $p < 0.01$). By 240 minutes, miR-156a was detected in the basal compartment ($p < 0.05$), suggesting export of plant-specific miRs by intestinal cells. **Conclusions:** Watermelon-derived exosomes containing plant-specific miRs are internalised into a proliferative subpopulation of Caco-2 cells, concurring with reports of fruit-derived exosome uptake into mouse small intestinal stem cells. Plant-specific miRs were transported from the apical to basal side of a tight Caco-2 barrier, suggesting cross-kingdom transfer of dietary miRs into human circulation is likely mediated by exosome uptake by the intestine.

PT1.16

Cross-kingdom uptake and transfer of exosomal watermelon microRNAs in human intestinal epithelial cells

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Poster Session 2 - EVs in cardiovascular disease

Chairs: *Pia Siljander and Bas van Balkom*

5:00-8:00 p.m.

PT2.01

Obligatory role of mitochondrial cross-talk with endoplasmic reticulum in the regulation of oxidative stress leading to endothelial dysfunction by human microparticles

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Elevated levels of circulating microparticles (MPs) are detected in many diseases including metabolic syndrome (MetS). We have previously shown that MPs from MetS patients (MetS MPs) and those generated from apoptotic T cells induce endothelial dysfunction. Here, we analyzed the mechanisms of this endothelial dysfunction with respect to endoplasmic reticulum (ER) stress and the mitochondria in the regulation of reactive oxygen species (ROS) production in human aortic endothelial cells and mouse aorta. MPs at their circulating levels from MetS and T lymphocyte MPs increased protein expression of ER stress such as XBP-1, p-eIF2 α and CHOP and nuclear translocation of ATF6. Both types of MPs decreased endothelial NO release and reduced endothelium-dependent relaxation of mouse aorta. All the above-mentioned effects of MPs are abrogated by pharmacological inhibition of ER stress. MPs increased expression of mitofusin-2 and decreased expression of VDAC-1. Interestingly, MPs evoked sequential increase of cytosolic and mitochondrial ROS with differential pattern of regulation between MetS MPs and T lymphocyte MPs. Silencing of neutral sphingomyelinase (SMase) expression reduced all the effects induced by both types of MPs. Finally, neutralization of Fas Ligand carried by MPs abolished the effects of the two types of MPs on ER stress, whereas neutralization of LDL receptor on endothelial cells prevented the effects of T lymphocyte MPs only. Collectively, we demonstrate that endothelial dysfunction triggered by MPs involves temporal cross talk between ER and mitochondria with respect to spatial regulation of oxidative stress via the SMase route. These events occur via interaction of MPs on endothelial cells with Fas and/or LDL receptor. These results highlight novel potential targets to fight against the pivotal role of MPs on endothelial dysfunction leading to the increase of cardiovascular complications including those associated with MetS.

PT2.02

An imbalance between circulating microparticles and annexin-A5 may promote vascular injury during sickle cell disease

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Chronic hemolytic anemia, including sickle cell disease (SCD) is characterized by painful vaso-occlusive crises (VOC), vascular injury, red blood cell (RBC) aggregation and vesiculation, and intravascular hemolysis. At steady state, RBC release haemoglobin, heme and microparticles (MP) in plasma. This increases again two-fold during VOC. MP from stressed RBC express phosphatidylserine (PS) at their surface and contain cytotoxic heme. On the other hand, annexin-A5 is an intracellular protein released in plasma during cell stress. Annexin-A5 acts as a PS inhibitor, neutralizing PS-mediated effects in stressed cells and MP. We collected plasma from cohorts of SCD patients during steady state, or VOC, and controls. We designed a novel ELISA-based assay to capture PS+ MP with an anti-annexin-A5 antibody. It quantified annexin-A5-covered MP in plasma, but also estimated ligand-free functional annexin-A5. We characterized plasma MP by FACS with labelling of ligand-free PS with annexin-

A5 and anti-CD235a, and size by NTA (50–1500 nm). We tested annexin-A5 to block ROS production induced by SCD MP in endothelial cells. In controls, FACS and ELISA revealed that MP were few and covered with endogenous annexin-A5. Some annexin-A5 remained bioavailable in plasma. Conversely, RBC MP levels were increased in steady state SCD MP, with increased mean fluorescence intensities (MFI), but similar size vs. controls. SCD MP bore more PS available to annexin-A5 at their surface and the equilibrium between PS and annexin-A5 was compromised. In VOC, MP levels were increased even further and bore cytotoxic heme and CD235a. We still found virtually no ligand-free annexin-A5. This suggested that endogenous annexin-A5 might be consumed by excess PS externalization in SCD, and insufficient to neutralize the high levels of PS+ MP produced by RBC during hemolysis. In SCD, the therapeutic use of recombinant annexin-A5 may thus help compensate the imbalance between PS+ MP and annexin-A5.

PT2.03

Exosomes released from sulforaphane-treated fibroblasts protect the cardiomyocytes from angiotensin II-induced hypertrophy

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Introduction: The communication between fibroblasts and cardiomyocytes underlies the pathological cardiac hypertrophy induced by angiotensin-II (AngII), which contributes to heart failure. Fibroblast-derived exosomes (F-Exo) have been implicated in mediating AngII-induced cardiomyocyte hypertrophy. However, how release of anti-hypertrophic F-Exo is induced, remains an unanswered issue. Sulforaphane (SFN), a naturally occurring isothiocyanate extracted from cruciferous vegetables, attenuates AngII-induced cardiomyocytes hypertrophy. We tested the effects of SFN on the release of anti-hypertrophic F-Exo in vitro. **Methods:** Murine embryo fibroblasts were treated with non-toxic dose of SFN (3 μ M/7 days). Intact F-Exo were isolated from cell culture media by differential centrifugation. F-Exo were quantified by Western blot using CD63. Hypertrophy of HL-1 cardiomyocytes was induced by AngII (100 nM/12 h). Cell viability was assessed by MTT assay. Cell surface area, an indicator of cell hypertrophy, was measured after 3 or 24 h incubation with 30 μ g exosomes isolated from SFN-treated (SFN-F-Exo) or untreated (F-Exo, control) fibroblasts. Uptake by HL-1 of DiA-labeled exosomes was measured under rest or AngII. Exosomal content of Maspin, a protease inhibitor with function of inhibitor of histone deacetylase 1, was assessed by Western blot. **Results:** Treatment with F-Exo significantly increased HL-1 viability by 53% under stress compared to control. Stressed HL-1 treated for 24 h with SFN-F-Exo displayed cell surface area similar to resting cells, but not those treated with F-Exo. Stressed HL-1 exhibited a ~3-fold increase in SFN-F-Exo uptake rather than F-Exo. SFN-F-Exo are enriched in Maspin. **Summary/conclusion:** SFN increases the uptake of F-Exo which display the ability to prevent AngII-induced cardiomyocytes hypertrophy. Higher content of Maspin in SFN-F-Exo suggests that modulation of exosomal uptake and hypertrophy in stressed cardiomyocytes may be epigenetically driven.

PT2.04

Preconditioning with isoflurane and hypoxia: a possible role of exosomes?

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Introduction: A promising technique to protect the heart from ischemia-reperfusion (IR) injury is preconditioning with brief cycles of hypoxia or volatile anaesthetics like isoflurane. Lately, exosomes emerged as possible communication mediators that may be involved in the exchanges of cardioprotective proteins. Recent publications indicate general cardioprotective properties of exosomes but it still remains to be investigated whether specific preconditioning stimuli trigger the additional release of exosomes and influence cargo composition. **Methods:** Rat cardiomyocytes and fibroblasts were preconditioned with isoflurane (1.5 Vol %) and hypoxia (<1% O₂). Supernatants were collected after 48h and exosomes were isolated by size-exclusion chromatography or the ExoEasy Kit. Particles were characterized by tunable resistive pulse sensing (TRPS) and electron microscopy. Purity was assessed by protein to particle ratio. Exosome composition was analysed by western blot. To investigate their cardioprotective role, preconditioned supernatants or isolated exosomes were transferred to unstimulated cells for 24 h prior to I/R damage. Cell viability was analysed by AlamarBlue. **Results:** Preconditioning triggers the release of exosomes from both cell types but the specific release is more pronounced in fibroblasts. Interestingly, protein composition (Alix, CD63, HSC70, Flot-1) differed depending not only on the cell type and stimulus but also on the isolation method. Preliminary data from still on-going transfer experiments indicate a protective role from fibroblast-derived, preconditioned supernatants and exosomes on cardiomyocytes. **Conclusion:** Primary cardiac cells actively secrete exosomes after different preconditioning stimuli. The protein composition of these exosomes differs, depending on cell type, stimulus and purification method. Current on-going exosomes-transfer studies will give further insight whether these differences result in differential roles during cardiac preconditioning.

PT2.05

Matrix vesicle-like particles are associated with aortic valve calcification Lin Cui¹, Dongxing Zhu², Daniel Lerman³, Gillian Milne⁴, Marc Dweck³, Alex Vesey³, David Newby³, Elspeth Milne⁵, Miep Helfrich⁴, Frank Rao⁶, Colin Farquharson¹ and Vicky Macrae¹

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Introduction: There is currently no medication to inhibit the progression of aortic valve calcification (AVC), and the underpinning mechanisms are not fully understood. This study determined whether elevated calcium (Ca) and/or phosphate (Pi) induces calcification of aortic valve interstitial cells (VICs) via the secretion of mineralisation-competent matrix vesicles (MVs). **Methods:** Sv40T rat VIC cells were treated with elevated Ca and/or Pi, and assessed by qPCR. MVs were harvested using ultracentrifugation from primary rat VICs, cultured with control or calcifying medium (2.7 mM Ca and 2.5 mM Pi) for 16h, and assessed by mass spectrometry. Immunohistochemistry and transmission electron microscopy were performed on human valve tissue obtained with appropriate ethical approval and informed consent from patients undergoing valve replacement surgery (Ethics No: 10/S1402/33). This study was done in conformation with the declaration of Helsinki. **Results:** Elevated Ca treatment (=2.7 mM Ca) induced calcium deposition in Sv40T cells (4.5-fold; P < 0.01). Ca treatment (> 3.6 mM Ca) increased mRNA expression of the osteogenic markers PiT-1, Runx2 and Msx2 (P < 0.05). While no effect of Pi treatment alone was observed, treatment with 2.7 mM Ca and 2.5 mM Pi synergistically induced calcium deposition (4.14-fold; P < 0.001). VIC-MVs isolated from calcifying medium shared similar protein composition with chondrocyte-derived MVs, including the enrichment of the calcium-binding proteins: annexin (Anx) A2 (4.8-fold), A5 (4.6-fold) and A6 (4.3-fold). Currently, the functional role of selected Anxs in VIC calcification is being examined. Increased

Anx A6 expression was noted in severely calcified human valve compared to uncalcified control. MV-like structures were observed in the extracellular matrix of the calcified valve. **Summary/conclusion:** We establish calcium as a novel trigger of VIC calcification and suggest that AVC is a cell-mediated process regulated by vesicle release.

Funding: BBSRC, UK.

PT2.06

Endoplasmic reticulum stress in vascular smooth muscle cells regulates exosome release and results in calcification

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Introduction: Vascular calcification (VC) is the process of hydroxyapatite deposition in the blood vessel wall, which leads to vascular stiffening and heart failure. It is a health problem common in ageing populations, and in patients with diabetes and chronic kidney disease. VC is a regulated process mediated by vascular smooth muscle cells (VSMCs), with similarities to developmental osteogenesis. The exact molecular events responsible for triggering VC are unknown. The endoplasmic reticulum (ER) is involved in folding of proteins. ER stress occurs as a result of unfolded protein accumulation and has been implicated in osteoblast differentiation and bone mineralization. Therefore we hypothesized that ER stress signalling regulates VC. **Methods:** Human primary VSMCs were treated with tunicamycin (TM) and thapsigargin (TG) and elevated Ca²⁺ and PO₄²⁻, to induce ER stress and calcification, respectively. Calcification was quantified with the o-cresolphthalein assay. Exosomes were captured on beads and detected with FACS. Grp78 and Grp94 expression by western blotting. SMPD3 expression was measured by qPCR. **Results:** ER stress was induced in VSMCs by TM and TG and increased their calcification. Warfarin, an anticoagulant whose known side effect is inducing calcification, induced ER stress in rat aortas as well as VSMCs in vitro. Warfarin-induced calcification of VSMCs in vitro is ER stress dependent, as blocking ER stress with the inhibitor PBA decreased calcification. Induction of ER stress in VSMCs was associated with an increase in exosome release regardless of the ER stress inducer. Increases in exosome release correlated with upregulation of SMPD3 expression. **Conclusions:** VC is partially regulated by ER stress-mediated increase of exosome release. This is the first time that a link between ER stress and exosome release has been reported. In addition, these findings provide a novel mechanism of action of warfarin on VSMCs.

PT2.07

Extracellular vesicles isolated from conditioned media from cells overexpressing osteopontin promote an increase in cardiomyoblast cell size

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Introduction: The stroke-prone spontaneously hypertensive rat (SHRSP) develops increased left ventricular mass index (LVMI) prior to the onset of hypertension. We identified a quantitative trait locus (QTL) for LVMI on chromosome 14 and by using chromosome 14 congenic strains and gene profiling have identified positional candidate genes within the chromosome 14 congenic interval, including osteopontin (Spp1). Here, we show that Spp1 may cause cardiac remodelling via extracellular vesicle (EV) signalling. **Methods:** H9c2 cells were seeded

(3.4×10^4 cells per well) 24 hrs prior to transfection with Spp1 cloned into pcDNA1 (3 ug) for 48 hrs. EVs were isolated from conditioned media (CM) via ultracentrifugation, verified by NanoSight, re-suspended in PBS and placed onto fresh H9c2 cells for 48 hrs. Crystal violet stained H9c2 cells were analysed using ImageJ. **Results:** Spp1 expression was significantly increased in H9c2 cells transfected with Spp1 (Spp1-cDNA) as determined by qRT-PCR (Spp1-cDNA 58-fold increase*, pcDNA two-fold increase, compared to cell control * $p < 0.01$). EVs isolated from CM transfected with Spp1-cDNA significantly increased H9c2 cell size (cell control 74.2 ± 0.9 vs. Spp1-cDNA 126.0 ± 2.4 *, * $p < 0.001$). Inhibition of EV biogenesis using GW4869 (20 μ M) after transfection with Spp1-cDNA prevented cellular hypertrophy of fresh H9c2 cells after treatment with CM (cell control 78.3 ± 1.09 vs. Spp1-cDNA 82.3 ± 1.22 , $p > 0.05$) or isolated EVs (cell control 80.3 ± 1.3 vs. Spp1-cDNA 80.6 ± 1.4 , $p > 0.05$). Pre-treatment of Spp1-cDNA EVs with Spp1 neutralising antibody (1 μ g) did not prevent cellular hypertrophy (cell control 80.1 ± 1.6 vs. Spp1-cDNA 118.1 ± 1.9 *, * $p < 0.001$), suggesting that Spp1 is not present on the surface of the EVs. **Summary/conclusions:** Collectively these data suggest that overexpression of Spp1 promotes an increase in cell size via EV signalling. Further studies are required to characterise the EVs and the downstream mechanisms leading to hypertrophy.

PT2.08

Neutrophil microvesicles preferentially adhere to endothelium exposed to disturbed flow

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Background: Atherosclerosis is an inflammatory disease of arteries that develops at areas exposed to disturbed or low oscillatory wall shear stress (WSS). At these sites, elevated adhesion molecule expression facilitates inflammatory cell transmigration. Although neutrophil depletion is associated with reduced lesion progression, neutrophils are seldom found within developing plaques and their role in atherosclerosis remains uncertain. Neutrophils release microvesicles (MVs) that are able to interact with and activate the endothelium. We hypothesise that neutrophil MVs preferentially adhere to endothelial cells at sites of low WSS, contributing to atherogenesis. **Methods and Results:** The spatial distribution of MVs in vivo was studied by injecting CFDA-SE labelled MVs into the tail vein of ApoE^{-/-} or C57BL/6 mice. After 2 h, en face immunohistochemistry revealed more MVs at atheroprone (low WSS) than atheroprotected (high WSS) regions of the aortic arch (MVs per field of view; atheroprotected = 1.3 ± 0.4 , atheroprone = 8.1 ± 2.4 , $P < 0.001$). Additionally, human coronary artery endothelial cells (HCAEC) were pre-conditioned under high unidirectional WSS (13 dyn/cm²) or low oscillating WSS (4 dyn/cm², 0.5 Hz) for 72 h, then incubated with PKH-26 labelled MVs for 2 h. Fluorescence microscopy revealed that low oscillating WSS induced greater adhesion of MVs to hCAECs compared to high WSS (MVs per field of view; low WSS = 20.6 ± 1.4 , high WSS = 4.7 ± 1.5 , $P < 0.05$). Enhanced binding of MVs under low WSS conditions correlated with greater expression of ICAM-1 (2.3-fold greater in hCAECs subject to low oscillatory compared to high WSS ($P < 0.01$)). **Conclusions:** Neutrophil MVs preferentially adhere to endothelial cells at atheroprone regions of the arterial tree in hypercholesterolemic mice. The mechanism involves low WSS which primes cultured HCAEC for enhanced expression of ICAM-1 and capture of MVs. These data implicate neutrophil MVs in the focal nature of atherogenesis.

PT2.09

A functional role for extracellular vesicles in heart failure and cellular electrical remodelling

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Introduction: We have previously identified plasma microRNAs that are associated with heart failure (HF) and are enclosed in extracellular vesicles (EVs). This study compares the characteristics of EVs in HF patients compared to healthy controls and investigates their functional effect on human induced-pluripotent stem cell-derived cardiomyocytes (iPSC-CMs). **Methods:** All experiments were conducted with informed consent and in accordance with the IRB of BIDMC. Coronary sinus blood was collected from patients undergoing electrophysiology study for supraventricular tachycardia (healthy controls) or implantation of cardiac resynchronization therapy for HF. Cell-free plasma was analyzed by nano flow cytometry to determine differences scatter properties of EVs. Plasma EVs were isolated by iodixanol density gradient and miRNA expression was assessed by qRT-PCR. The functionality of these EVs was tested by addition to iPSC-CMs infected with lentiviral vector carrying ArLight. Action potentials were obtained through ArLight fluorescence imaging and analyzed at 80% repolarization. **Results:** EV profiles in HF patients had an increase in the geometric mean value of side scatter compared to controls (SVT, 1.02 ± 0.19 gMFI; HF, 1.39 ± 0.064 gMFI). Increases in microRNAs previously associated with HF were observed in the EV fraction from HF patients compared to controls, including miR-151a, miR-30d and miR-660. Addition of EVs isolated from HF patients to iPSC-CMs produced a 34% increase in APD80 at 24 hrs ($p < 0.0001$), with no change observed following the addition of control patient-derived EVs. **Conclusions:** EV profiles and microRNA contents differ between patients with HF and healthy controls, and EVs in HF patients appear to play a functional role in electrical remodelling at the cellular level. EVs and their contents may reflect a novel paracrine signalling mechanism in HF that may be targeted for therapy.

PT2.10

Exosomal miRNA as liquid biomarker for canine arrhythmogenic right ventricular cardiomyopathy (ARVC) in Boxer Dogs

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Introduction: ARVC is a heritable disease that accounts for 11–22% of sudden cardiac death (SCD) in young athletes and also affects middle aged Boxer Dogs. In both species, histopathologic changes from ARVC include fibrofatty replacement of the heart muscle. The disease is often transmitted with an autosomal dominant pattern with incomplete penetrance in both people and Boxers. Mutation in desmosomal proteins have been implicated. Given the presence of phenotypic variation, epigenetics, including miRNA control, may be involved. We attempted to find plasma exosomal miRNAs in Boxers that can serve as biomarkers and as potential therapeutic targets for ARVC. **Methods:** Echocardiograph and ECG were used to diagnose normal Boxers or Boxers with ARVC. Exosomes from 1ml of plasma were isolated from each dog (normal Boxers, $n = 7$, ARVC Boxers, $n = 8$) using ultracentrifugation. The exosomal miRNAs were then isolated and pre-amplified. miRNA expression was analyzed using commercially available miRNA RT-qPCR arrays. **Results:** Eight of the 277 miRNAs tested showed statistically significant ($p = 0.05$) differences between normal dogs and dogs with ARVC, including miR-125b (fold change, FC = 13.1), miR-28 (FC = -4.1), miR-29c (FC = -8.7), miR-30b (FC = -7.2), miR-497 (FC = -7.5), miR-93 (FC = -5.3), miR-98 (FC = -88.8), and miR-133b (FC = 2.0). miR-125b showed the most significant change ($p = 0.008$). miR-125b, miR-29c, and miR-93 have been shown to modulate cardiomyocyte ion channels and electrical signaling, and upregulation of miR-125b predisposed mice to

ventricular tachycardia. In addition, upregulation of miR-125b or downregulation of miR-29c has been associated with increased fibrosis. **Summary:** Plasma exosomal miRNAs may serve as potential biomarkers and therapeutic targets for Boxer ARVC. Plasma biomarkers enable early detection so that necessary monitoring may be initiated. Prospective validation of the biomarkers is warranted, and therapeutic potential of these miRNAs requires further study.

PT2.11

Processed small hairpin RNAs are secreted from transduced cells to surrounding tissue in vivo in mouse myocardial infarction model

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We have reported that lentiviral shRNAs targeted to murine VEGF-A promoter are able to repress or induce VEGF-A expression both in vitro and in vivo by transcriptional gene regulation. In this study we focus on clarifying the in vitro and in vivo transfer of small RNAs mediating transcriptional gene activation of mouse VEGF-A. Our data demonstrates that mature shRNA-451 sense and antisense strands are efficiently secreted to cell culture medium. Still, neither C166 nor MS-1 cells were found to intake secreted shRNA-451 strands in vitro. However, when cells from transduced infarcted hearts were sorted for co-expressed GFP (positive and negative fractions), we detected mature strand of shRNA-451 also in GFP negative population by qRT-PCR analysis, suggesting that in infarcted mouse heart shRNA-451 transfer from cell to cell occurs, proposing that transfer may be cell type or tissue dependent. Further studies are required to find out if epigenetic changes are passed on to recipient cells in vivo. Mature form of shRNA-451 is also found in serum of MI mice that have received intra-myocardial injection of lentiviral vector expressing shRNA-451. Therefore, also tissue to tissue transfer of shRNAs is possible. This study explains why promoter targeted shRNAs have surprisingly good in vivo efficiency and also point out that the safety of traditional RNAi therapeutics should be reconsidered because possible biodistribution and off-target effects in chromatin.

PT2.12

Valve interstitial cell (VIC) miRNA profile and phenotype are affected by canine Wharton's Jelly mesenchymal stem cell conditioned media (WJ-MSC CM) in canine myxomatous mitral valve disease (MMVD)

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Introduction: Histological changes in canine MMVD and its functional consequences are virtually identical to human mitral valve prolapse. Changes include disarray of collagen/elastic fibres and greater prevalence of VICs with myofibroblastic phenotype, growth retardation, and lower viability. Our previous results showed that canine WJ-MSC exosomes (EX) improve cell growth and viability, and that these EXs are internalized by VICs. We further investigated effects of WJ-MSC CM on VIC miRNA profile and phenotypic expressions. **Methods:** VICs were isolated from normal (n = 3), mildly (n = 4), severely affected valves (n = 3). CM was collected from WJ-MSC cultured in aMEM + 15% FBS for 24 hrs. RT-qPCR was used to evaluate mRNA and miRNA expressions of VICs cultured with or without WJ-MSC CM and of WJ-MSC and its EXs. **Results:** VICs from diseased valves have decreased miR-29b, miR-30a, and increased miR-99a (p < 0.05) expressions. With WJ-MSC CM, increased miR-26a (1.5, SD 0.4), miR-29a (1.5, SD 0.3), miR-29b (1.4, SD 0.3), and decreased miR-21 (-1.4, SD 0.2) expressions for VICs from mildly diseased valves were seen. For VICs from severely affected valves, increased miR-29c (3.8, SD 2.6), miR-146a (1.3, SD 0.1),

and decreased miR-133a (-1.8, SD 0.2), miR-218 (-1.6, SD 0.2) expressions were seen. With WJ-MSC CM, VICs from severely diseased valves had decreased aSMA (-1.8, SD 0.4), COL4A5 (-4.9, SD 3.4), COL7A1 (-5.0, SD 1.9), HAS3 (-2.6, SD 1.2), MMP28 (-1.8, SD 0.6), p21 (-1.5, SD 0.6), and increased vimentin (1.4, SD 0.6) expression. WJ-MSC and its EXs both contain miR-29a/b/c, miR-30a/b/c. **Summary:** VICs from diseased valves have decreased expressions of miR-29b and miR-30a that are important in fibrosis control, and both are found in WJ-MSC and EXs. Co-culturing with WJ-MSC CM decreased expressions of myofibroblast-related genes in VICs from diseased valves and increased expressions of miR-29a/b/c. Our results suggest that WJ-MSC and EXs have potential therapeutic benefits for MMVD treatment.

PT2.13

Tumour necrosis factor- α carried by microparticles from apoptotic RAW 264.7 macrophage cells triggers deleterious effects on cardiomyocytes from adult mice

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Introduction: After ischaemic injury and in patients with atherosclerosis, the pool of inflammatory macrophages is enlarged in the heart and in atherosclerotic plaques. Monocyte/macrophage-derived microparticles (MPs) are part of the pathological process of unstable atherosclerotic plaques. The hypothesis that these MPs could induce deleterious effects in adult myocardial cells was evaluated. **Methods:** MPs were isolated through differential centrifugation from the supernatant of RAW 264.7 macrophages. MPs were characterized through flow cytometry and western Blot. Their effects on cardiac cell shortening were evaluated in presence or absence of specific inhibitors. Interaction between MPs and cardiomyocytes was detected through confocal microscopy. MP pro-apoptotic effects were assessed through western blot. **Results:** We showed that these MPs contained the soluble form of tumour necrosis factor alpha (TNF- α). Furthermore, cardiomyocyte sarcomere shortening amplitudes and kinetics were reduced within 5 min of exposure to MPs without any modifications in Ca²⁺ transients. The contractile effects of MPs were completely prevented after pre-treatment with nitric oxide synthase, guanylate cyclase or TNF- α inhibitors as well as blocking TNF- α receptor 1 with neutralizing antibody. Moreover, confocal microscopy showed that, after 1 h, MPs were clearly surrounding rod-shaped cardiomyocytes, and after 2 h they were internalized into cardiomyocytes undergoing apoptosis. Indeed, after 4 h of treatment with MPs, cardiomyocytes expressed increased apoptotic markers expression (caspase-3, caspase-8, Bax and cytochrome C) as demonstrated by western blot. **Summary/conclusion:** The present study showed that these MPs carry functional TNF- α and activate TNFR1 in isolated adult murine ventricular cardiomyocytes, inducing a negative inotropic effect via a mechanism sensitive to NO synthase inhibitor and that the mitochondrial intrinsic pathway was implicated in their proapoptotic effects.

PT2.14

Bone marrow endothelial progenitor cells are the cellular mediators of pulmonary hypertension in the murine monocrotaline injury model

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Aims: We sought to determine if BM-derived endothelial progenitor cells (BM-EPCs) are responsible for inducing pulmonary hypertension

(PH) and if their PH-inducing effect could be attenuated by mesenchymal stem cell (MSC)-derived extracellular vesicles (EVs). **Methods:** Three BM populations were studied: 1. BM from vehicle, monocrotaline (MCT)-treated mice (PH induction); 2. BM from mice treated as above after MSC-EV infusion into these mice (PH reversal, in vivo); 3. BM from vehicle-, MCT-treated mice cultured with MSC-EVs (PH reversal, in vitro). BM was separated into EPCs (sca-1+/cKit+/VEGFR2+) and non-EPCs (sca-1-/cKit-/VEGFR2-) and transplanted into healthy mice. Right ventricular (RV) hypertrophy was assessed by RV-to-left ventricle+septum (RV/LV+S) ratio and pulmonary vascular remodelling by blood vessel wall thickness-to-diameter (WT/D) ratio. **Results:** PH induction: Transplantation of EPCs from mice with MCT-induced PH (MCT-PH) resulted in similar RV/LV+S, WT/D ratios as treatment with MCT, whereas transplantation of non-EPCs from MCT-PH mice did not. PH reversal, in vivo: Compared to EPCs isolated from MCT-PH mice treated with vehicle, EPCs isolated from MCT-PH mice treated with MSC-EVs did not impact RV/LV+S, WT/D ratios in healthy mice. PH reversal, in vitro: Treatment of EPCs from MCT-PH mice with MSC-EVs before transplantation prevented increases in RV/LV+S, WT/D ratios. MCT-PH mice treated with MSC-EVs had more BM-EPCs, decreased EPC adhesion receptor expression in the lung and increased BMPRII and PDGFR α expression in the lung compared to MCT-PH mice not treated with MSC-EVs. **Conclusions:** Pulmonary hypertensive effects of BM may be mediated by EPCs and MSC-EVs can attenuate these effects by down regulating EPC adhesion receptors in the lung. These findings provide new insights into PH pathogenesis and offer a potential target for development of novel therapies for this disease.

PT2.15

Non-canonical WNT signalling is mediated by extracellular vesicles in pulmonary fibrosis

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Idiopathic pulmonary fibrosis is a lethal lung disease of yet unknown etiology characterized by epithelial injury, myofibroblast activation and increased extracellular matrix deposition. Reactivation of WNT/ β -catenin pathway is linked to pulmonary fibrosis (PF); however, the role of non-canonical WNT signalling has not been explored in detail. Recently, it has been shown that WNT ligands can be secreted on extracellular vesicles (EVs). We hypothesize that WNTs contribute via EVs to impaired cellular crosstalk in PF. First, we performed a comprehensive profiling of EVs in experimental and human PF (informed consent was obtained and the study was approved by the ethics committee at the Ludwig-Maximilians Universität München). EVs were isolated by ultracentrifugation from bronchoalveolar lavage fluid (BALF) and characterized by transmission electron microscopy, western blotting and nanoparticle tracking analysis. Notably, we found increased EV secretion in BALF obtained from fibrotic mice which are enriched in non-canonical WNT5A. We also detected an upregulation of WNT5A in lung homogenates of PF patients compared to donors. Moreover, we observed an increase in CD81-derived EVs in human PF BALF suggesting fibroblasts as a potential source. Indeed, WNT5A was found upregulated in EVs from TGF β -treated primary human lung fibroblasts (pHLFs). In line with the profibrotic effects, treatment of pHLFs with autocrine EVs enhances proliferation, and WNT5A-conditioned media potentiates TGF β -induced Col1a1 and MCP1 in lung epithelial cells which have been linked to PF. In conclusion, we report an increase of EVs in PF, which impacts autocrine profibrotic signalling and we found that WNT5A increases in pHLF-EVs upon fibrotic stimulus, suggesting a new role of EVs traffic of WNTs in the disease.

Poster Session 3 - EVs in cardiovascular disease and coagulation

Chairs: Rienk Nieuwland and Saara Laitinen

5:00-8:00 p.m.

PT3.01**Neutrophil-derived-microvesicles could play a role in the early stages of atherosclerosis**Ingrid Gomez¹, Ben Ward¹, Paul Hellewell², Paul Evans¹ and Victoria Ridger¹¹Immunity, Infection and Cardiovascular Disease Department, University of Sheffield, Sheffield, UK; ²Life Sciences, College of Brunel, Brunel, UK

Please see OPW2.6

PT3.02**Endogenous cardiac extracellular vesicles are transiently released during experimental myocardial infarction in mice**Xavier Loyer¹, Min Yin¹, Coralie L. Guérin¹, Jose Vilar¹, Clotilde Théry², Jean-Sébastien Silvestre¹ and Chantal M. Boulanger¹¹Inserm U970 Paris Cardiovascular Research Center (PARCC), Paris France; ²Institut Curie -Inserm U932 Paris France

Introduction: Different subsets of monocytes and macrophages have been shown to coordinate cardiac remodelling after myocardial infarction (MI), at least in part through their ability to release anti-fibrotic and pro-angiogenic factors as VEGF. Extracellular vesicles (EVs), including exosomes (Exos) and microvesicles (MVs), may play a seminal role during cardiac remodelling by transferring biological information to recipients cells. So far, no data exist on the endogenous release of EVs following MI. We hypothesized that EVs are locally released in response to MI and synchronize the monocyte-induced left ventricle remodelling. **Methods:** MI was induced by permanent left anterior descending artery ligation in C57BL/6 mice. Sham-operated mice were used as controls. Sham and MI mice were sacrificed between 0 and 14 days after the onset of ischemia. EVs from ischemic and sham left ventricles were isolated by sequential centrifugations, analyzed by Nanoparticle tracking analysis (NTA), and MVs cellular origin as well as phosphatidylserine exposure (annexinV-AV labelling) by flow cytometry. Bone marrow-derived monocytes were also stimulated for 24 h with EVs isolated from sham and MI hearts 15 h post ligation and subsequent VEGF release assessed by ELISA and qPCR. **Results:** When comparing with Sham, AV+MVs levels rapidly increased between 6 and 24 hrs in the infarcted myocardium and then returned to basal level after 3 days. NTA analysis also demonstrated greater release of MVs and Exos in infarcted myocardium 15 h post-MI, when compared to sham experiments. Local MVs in ischemic tissue originated mainly from cardiomyocytes (86% caveolin3+MVs). Both MVs and Exos isolated from ischemic myocardium or cultured cardiomyocytes stimulated the release of VEGF by bone marrow-derived monocytes. **Conclusion:** Endogenous EVs originating from the myocardium accumulate rapidly and transiently in ischemic heart to stimulate monocyte production of VEGF.

PT3.03**Role of extracellular vesicles in thrombosis in paroxysmal nocturnal hemoglobinuria patients**Adeline Wannez¹, Bérandère Devalet¹, François Mullier¹, Bernard Chatelain¹, Christian Chatelain², Jean-Michel Dogné² and Celine Bouvy²¹Chu Dinant Godinne, Ucl Namur, Namur, Belgium; ²University of Namur, Namur, Belgium

Introduction: Paroxysmal nocturnal hemoglobinuria (PNH) is a complement-mediated hemolysis caused by the lack of complement regulator GPI-anchored proteins (CD55-CD59) at cell surface. 40% of PNH patients will undergo thromboembolic events. The release of procoagulant extracellular vesicles (EV) by the cells could explain the patient's thrombosis sensitivity. Eculizumab, used in the treatment of PNH, interacts with C5 leading to an inhibition of the membrane attack complex (MAC) formation. The aim of the study is to set-up an in vitro model of PNH in order to assess the production of EV during a hemolytic crisis. The second objective is to study the procoagulant profile of PNH patient during the treatment with eculizumab. **Method:** The in vitro model is based on several cell types (endothelial cell line, leucocytes and red blood cell) from which we remove GPI-anchored proteins with an enzyme. Besides, we collect plasma of PNH patients before, after 4 and 11 weeks of Eculizumab (clinical study was approved by a recognized ethics committee and informed consent was obtained). We assess the effect of treatment on the EV procoagulant activity by thrombin generation assay. **Results:** For the in vitro model, the enzyme performance varies between cell types. It seems to be less effective on red blood cells. For the ex-vivo part, we observe a trend showing a decrease of the procoagulant properties of EV during the treatment. **Conclusion:** We succeeded in obtaining PNH-like cells for all cell types with more or less efficacy. The next step is to study the complement attack with serum and to check if procoagulant EV will be released. For the ex vivo part, we observed some trends suggesting a decrease of the EV procoagulant properties with the treatment. We found no significant difference in terms of coagulation profile for patients before and after several weeks of treatment, however, the number of studied patients was low and significant results could be reached with a wider cohort.

PT3.04**Platelet-derived extracellular vesicles exposing fibrinogen may be a potential marker for ongoing thrombus formation**Anita Böing¹, Sami Valkonen², Auguste Sturk¹, Najat Hajji¹, Chi Hau¹ and Rienk Nieuwland³¹Department of Biosciences, Division of Biochemistry and Biotechnology; Division of Pharmaceutical Biosciences, University of Helsinki; Finnish Red Cross Blood, Helsinki, Finland; ²Laboratory of Experimental Clinical Chemistry, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; ³Academic Medical Centre, Amsterdam, The Netherlands

Background: Arterial thrombosis is a major cause of human death. Patients at risk of, e.g. an acute myocardial infarction (AMI) are in a pre-thrombotic state, with increased activation of platelets and coagulation. In patients with AMI, thrombi of 10 days old can be present, indicating that thrombus growth can occur silently before becoming clinically evident. However, no biomarkers are available to identify such pre-symptomatic patients. We investigated whether in vitro generated human thrombi release extracellular vesicles (EVs) exposing unique epitopes that may be useful as biomarkers to identify patients with on-going thrombus formation. **Methods:** Citrate-anti-coagulated blood was collected from healthy subjects (n=2) and platelet-rich plasma (PRP) was prepared by centrifugation. PRP was incubated with ADP (10 µmol/L, CaCl₂ 2.5 mmol/L) under stirring conditions (37°C; Multiplate analyzer) in the absence or presence of Abciximab as glycoprotein (GP)IIb/IIIa receptor antagonist. Samples were collected at t=0, 1 and 24 h. Samples were analyzed for EVs exposing glycoprotein IIIa (CD61), P-selectin, fibrinogen (FG), or phosphatidylserine (PS) by flow cytometry (Apogee A50) and their morphology was studied by transmission electron microscopy (TEM). **Results:** The number of EVs exposing CD61, P-selectin, FG and PS increased 155-, 38-, 3- and 6- fold at 1 h after thrombus formation. After 24 h, only minor changes were observed in the numbers of

CD61-, P-selectin and PS-exposing EV compared to $t = 1$, but the number of FG-exposing EVs increased 64-fold compared to $t = 1$. All increases in EV numbers were completely abolished by Abciximab, indicating that both platelet activation and binding of FG to GPIIb/IIIa are necessary for EV release. With TEM mostly EVs were present at $t = 1$ h, whereas at $t = 24$ h not only EVs but also larger non-EV structures were present. **Conclusions:** The release of FG-exposing EVs may offer a biomarker for on-going thrombus formation.

PT3.05

Flow cytometric measurement of CD41/CD61 and CD42b major platelet receptors of infusible platelet membrane as a platelet substitute

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Introduction: Blood transfusion centres are under considerable pressure to produce platelet concentrates with a shelf life limited to 3–5 days. Many approaches have been investigated experimentally to produce new hemostatically active platelet products that are capable of long-term storage. Recent studies have shown that infusible platelet membrane may have a hemostatic effect. We investigated both major adhesion and aggregation receptors of IPM in comparison with fresh platelet concentrate by flow cytometric method. **Methods:** Infusible platelet membrane was prepared from outdated platelet concentrates. Platelet concentrates were pooled, disrupted by freeze–thaw procedure, washing steps were performed, formulated with sucrose and human serum albumin and lyophilized. Flow cytometric assay was performed to identify CD41/CD61 and CD42b for IPM after reconstitution with distilled water for injection and also for pooled fresh platelet concentrate. **Results:** Flow cytometric analysis of CD41, CD61 and CD42b showed that IPM express these receptors with 66.27%, 66.92% and 44.47% and pooled fresh platelet concentrate with 81.5%, 87.5% and 81.1% respectively. **Conclusion:** We concluded that IPM still works with major adhesion and aggregation receptors that confirms potential hemostatic effect and finally may support its clinical potential utility as a transfusion substitute for platelets.

PT3.06

Exploring the phenotypic assessment of antithrombin deficiency by means of exosomes

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Antithrombin (AT) is the most important plasma inhibitor for the activated coagulation factors. Its primary target is thrombin followed by Factor Xa, IXa and VIIa. AT deficiency is associated with an increased risk of deep vein thrombosis and pulmonary embolism, major causes of morbidity and death. When reduced activity levels are identified it is important to measure the AT antigen levels to differentiate type I (quantitative) from type II (qualitative) disorders, as type II defects have varying thrombotic risk. No functional routine diagnostic assay, however, can be assumed to detect all forms of deficiency, due to lack of specificity. In our study we first show by a nanoplasmonic assay that the exosomal molar concentration in Type I, Type II and healthy individuals is the same. Exosomal concentration normalization allowed to reveal for the first time that AT is physiologically present in plasma exosomes and Type II defect-derived vesicles harbour a higher copy number of AT compared to controls. Furthermore Type II deficient exosomal AT shows a unique pattern in 2D electrophoresis, probably related to differential glycosylation. These findings suggest plasma exosomes could complement routine diagnostic tools to unravel qualitative defects in AT deficient patients.

PT3.07

A novel thrombo-inflammatory pathway for monocyte recruitment mediated by platelet-derived microvesicles

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We have recently described a novel paradigm where platelets support monocyte recruitment to the vessel wall in a model of inflammation by acting as adhesive bridges between monocytes and endothelial cells. Given this observation, we wondered whether formation of platelet-monocyte aggregates in whole blood might also support the thrombo-inflammatory recruitment of monocytes to the vessel wall. After addition of platelet agonists to whole blood, we assessed binding of platelets to monocytes by measuring accumulation of GPIb on CD14 and CD16 labelled monocytes using flow cytometry. Platelet activation in blood resulted in monocytes acquiring GPIb in small quanta that were significantly smaller than the amount of GPIb present on a single platelet. This suggested that monocytes acquired GPIb from platelet-derived vesicles. Heterotypic aggregate formation was time-dependent and largely monocyte-specific. Provision of pre-labelled platelet-derived vesicles in whole blood also resulted in rapid accumulation of GPIb on monocytes with similar dynamics. Super-resolution microscopy demonstrated numerous GPIb positive, sub-micron particles, on the surface of monocytes. Monocyte borne GPIb was also functional. Thus, we could observe high levels of adhesion of heterotypic aggregates flowed across immobilised recombinant vWF or TGF- β -stimulated endothelial cells, which present vWF on their surface. Thus we describe a new functional consequence of platelet-derived vesicle accumulation by monocytes. This thrombo-inflammatory pathway of monocyte recruitment may be important in vascular disease, as it is likely to bypass the usual regulatory pathways that control monocyte recruitment during inflammation.

PT3.08

Citrate concentration influences platelet activation and microvesicle generation during lipid apheresis

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Introduction: We aimed to assess the influence of citrate anticoagulation on platelet activation, microvesicle (MV) generation and cell adhesion to acrylamide-polyacrylate adsorbents for low density lipoprotein (LDL) apheresis. **Methods:** Blood was drawn from healthy volunteer donors and anticoagulated with citrate (2, 4 and 13 mM final concentration). Aliquots of 50 mL were recirculated over columns (3.5 × 1.8 cm) containing adsorbents for LDL apheresis at a flow rate 1.2 mL per min for 4 h. Samples were taken every hour, and blood cells in the flow-through were quantified using a blood cell counter. MVs were determined with a Gallios Flow Cytometer (Beckman Coulter) after calibration with fluorescent beads to cover the MV (0.3 and 0.9 μ m) and the cell size (0.9 and 3 μ m) ranges. Lactadherin staining was used to identify MVs of cellular origin. The following markers were used to differentiate cell-derived MVs: CD45+, CD14+ (monocytes); CD45-, CD41+ (platelets); CD45-, CD235a+ (red blood cells). Platelet activation was monitored by expression of CD62p (p-selectin), platelet factor-4 (PF4) and PAC-1 (activated GPIIb/IIIa). **Results:** Platelet activation markers CD62p, PF4 and PAC-1 were significantly elevated for citrate anticoagulation below 4 mM, which was accompanied by significantly higher adhesion of platelets and white blood cells to the adsorbents, as demonstrated by cell counting and electron microscopy. Likewise, the release of MVs

was dependent on citrate concentration and higher amounts of MVs (red blood cell- and platelet-derived MVs) were released at lower citrate concentration. After 4 h more than 70% of all monocytes were lactadherin+, indicating their association with MVs. We analysed the cellular origin of MVs adhering to monocytes and found that nearly 80% were derived from platelets. **Conclusion:** Our data show that citrate suppresses cellular activation and generation of MVs during contact of whole blood with adsorbents for lipid apheresis.

PT3.09

Extracellular vesicles characterization and possible pathogenic role in β -thalassemia major

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Background: Patients with β -thalassemia major (TM) require blood transfusion for life and develop severe dysfunctions in major organs and systems; however not all complications can be explained by iron overload. **Aims:** Characterization of extracellular vesicles (EV) in TM and evaluation of their role in the pathogenesis of TM complications. **Methods:** 30 transfusion dependent β -thalassemia (TDT) patients and 30 controls were included after signing the informed consent. TDT patients were divided into 3 groups: 1) non-splenectomized, 2) splenectomized (SP) and 3) hypersplenic (HS). EV characteristics (cellular origin, cytokine and microRNA content, thrombogenicity) were assessed by FACS, protein array and RT-PCR. Apoptotic effects of patients' EVs on endothelial and liver cells were evaluated (TUNNEL assay). **Results:** EV count / μ L was lower in HS (178 \pm 96) compared to C (410 \pm 167, $p=0.02$) and SP (1812 \pm 1937, $p=0.001$). The % of RBC-EVs was lower in HS vs. controls ($p=0.03$) and SP ($p=0.06$) and it correlated with the hematocrit in HS and SP groups. The % of annexin-V labelled EVs were lower in patients vs. controls ($p=0.01$). Substantial differences in cytokines and angiogenic proteins and in microRNA profile were found between the controls and patients EVs. The amount of 11 proteins in SP-EVs was twice as high compared to that of controls. Patient EVs induced higher in vitro apoptotic rate on endothelial and liver cells (16.5% \pm 10; 20.9% \pm 6.6) compared to control EVs (7.6% \pm 5, $p=0.007$; 14.8% \pm 5, $p=0.09$), respectively. **Conclusions:** Important differences in EV properties (EV number, exposure of negatively charged phospholipids, cell origin and cytokine, protein and miRNA content) were found between patient and control EVs. Patient EVs increased apoptosis in cultured cells, suggesting a novel mechanism of organ damage in TM.

PT3.10

Pro-coagulant activity of platelet-derived extracellular vesicles

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Please see OPW1.8

PT3.11

Impact of leukocyte-derived procoagulant extracellular vesicles on endothelial cells: significance in blood-mediated pancreatic islets graft reaction

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Please see OPW2.4

PT3.12

Microvesicles and Microvesicles associated miRNA as biomarker of acute kidney injury in a paediatric population following cardiac surgery

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AKI is very common in paediatric cardiac surgery patients. The diagnosis relies on changes in serum creatinine and urine output, which lack specificity and sensitivity especially in new-borns and premature infants. Microvesicles have been shown to be altered in animal models of AKI where they regulate injury responses. MV and associated miRNA could provide specific and early-detection markers for AKI and a tool to investigate AKI pathogenesis. Study subjects were 24 children (=17 yrs), undergoing cardiac surgery with cardiopulmonary bypass (CPB) without pre-existing inflammatory state, AKI or extracorporeal life support. AKI was diagnosed following the KDIGO definition. Blood and urine samples were collected pre-operatively and at 6 and 24 hours. Cytokines were measured on a MAGPIX platform. Leukocyte and platelets activation, and MV derivation was assessed by flow cytometry and NanoSight device. miRNAs were isolated from MV preps and analyzed by RTPCR using Applied Biosystems kits. AKI was diagnosed in 14 patients and was confirmed by urinary NGAL increase at 6 hours after the operation (25 \pm 3.45 ng/ml vs 10.44 \pm 2.9 ng/ml; $p=0.006$). Patients with AKI were younger, underwent longer CPB, received higher volumes of red blood cells, and required higher inotrope support. AKI subjects had increased levels of monocyte and β 1-integrin MV. That correlated well with IL-8 and MCP-3 levels and monocyte activation. These changes were also reflected in miRNA profile. In fact we observed a different expression of several miRNA in the two groups only at 6–12 hrs and associated with an inflammatory response and renal ischemia/reperfusion injury. Our data indicate that miRNA could potentially serve as early AKI markers in children and their analysis reveals mechanistic parallels with adults.

PT3.13

Decrease of concentration of extracellular vesicles in blood isolates of 27 athletes one day after long distance running

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Objectives: In order to study the effect of physical effort on mechanisms of blood cell membrane vesiculation we measured concentration of extracellular vesicles (EVs) in blood isolates, inflammation markers TNF alpha and IL6 in plasma, and activity of glutathione S-transferases (GST) and cholinesterases (ChE) in blood and in plasma, two days before and one day after long distance running (10 km, 21 km, 42 km). We also measured blood cell counts and biochemical parameters of blood the day after running. **Methods:** The study was approved by the National Medical Ethics Committee (82/07/14). After 12 h fasting, blood was sampled and erythrocytes were separated from plasma by centrifugation. EVs were isolated from plasma by repetitive centrifugation (at 17570 g) and washing, and counted by flow cytometry. Inflammation markers were measured by ELISA. GST and ChE activities were measured by elution and colorimetry. Standard blood parameters were assessed in a clinical laboratory. **Results:** We found on the average a considerable (59%) and statistically significant decrease of the concentration of EVs in

isolates from blood (probability $p < 0.01$), of IL6 (92%, $p = 0.04$), of GST activity in erythrocytes (17%, $P < 0.01$), of ChE activity in erythrocytes (75%, $p < 0.01$), in blood plasma (41%, $p < 0.01$) and in whole blood (62%, $p < 0.01$). We found a positive and statistically significant correlation between the concentration of EVs after the effort and the mean platelet volume after the effort ($p = 0.04$). Concentration of C reactive protein in serum was on the average slightly elevated after running (7.3 mg/l; ref. value 5 mg/ml) and statistically significantly correlated with the running distance ($p < 0.01$). **Conclusions:** Vesiculability of blood cells a day after physical effort on the average decreased concomitantly with a decrease of some markers of inflammation and oxidative stress in blood and plasma, presumably due to adaptation mechanisms and prandial state of the participants.

PT3.14

Altered distribution of HDL- and extracellular vesicle associated circulating microRNAs in diabetic nephropathy and systemic microvascular damage

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Introduction: We previously demonstrated circulating miRNAs can serve as biomarkers for microvascular injury in diabetic nephropathy (DN) patients. Recent studies revealed that circulating microRNAs also play a causal role in the pathogenesis of microvascular injury which depends on their association with a specific plasma carrier. To assess the plasma carrier distribution of microvascular injury-associated microRNAs in DN compared to diabetic control subjects we developed a method to isolate HDL and extracellular vesicles (EVs) from plasma of these patients and profiled the miRNA content of plasma and isolated fractions. **Methods:** EVs were isolated from EDTA-plasma (125 μ L) of DB ($n = 20$) and DN control patients ($n = 20$) using Sepharose CL-2B size-exclusion chromatography (SEC). EVs in fractions were validated by western blot and electron microscopy and quantified by tunable resistive pulse sensing. HDL was isolated from plasma using KBr density gradient ultracentrifugation and further purified with Sepharose CL-2B to deprive HDL from EVs with the same density. EV- and HDL-miRNA expression profiles were determined using a custom TaqMan(r) MicroRNA Array Card containing 48 selected miRNAs and controls. **Results:** Our method allowed isolation of non-contaminated HDL (45% recovery) and EVs from 900 μ L plasma samples. Differential circulating levels of microvascular injury associated miRNAs were confirmed in total plasma samples in the DN group compared to DB controls. Interestingly, preliminary data suggests for some miRNAs the carrier distribution was selectively altered in HDL or EVs (e.g. miR-331 in EVs, 4.7-fold difference), suggesting a different pathophysiological origin. **Conclusion:** Sub-fractionation of miRNA-carrier fractions improves the selectivity and sensitivity of biomarkers for microvascular injury in DN and enables in vitro studies to assess the impact of transfer of HDL-

and EV-derived post-transcriptional regulators on microvascular integrity.

PT3.15

Quick screening for microparticle content in platelet-rich plasma of cardiovascular patients

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Please see OPW1.5

PT3.16

Levels of circulating microvesicles are associated with plasma lipids and ectopic fat accumulation in type 2 diabetic men with low testosterone and are unchanged after testosterone replacement therapy

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Low testosterone in men has been associated with the metabolic syndrome (MetSy) and type 2 diabetes (T2D), and testosterone replacement therapy (TRT) shown to improve several components associated with higher T2D risk. The multifunctional receptor CD36 may be involved in dyslipidaemia, insulin resistance and ectopic fat storage in MetSy. We aimed to investigate the hypothesis that effects elicited by TRT are reflected in levels of circulating microvesicles (MVs), and investigated how MV phenotypes are associated with insulin sensitivity, body composition, plasma lipids and ectopic fat accumulation in the liver. Thirty-nine Caucasian males with T2D and low testosterone levels were assigned to either TRT or placebo (CTRL) groups, subjected to a 24-week treatment regime, and evaluated at baseline and after 24 weeks. MVs were analysed by flow cytometry and defined as lactadherin binding particles within the 0.1–1.0 μ m gate. MVs of platelet (PMV), monocyte (MMV) and endothelial cell (EMV) origin were identified by cell-specific markers and their expression of CD36 was investigated. Data were analysed by Wilcoxon's signed rank test and Spearman's ranked correlation analysis. No differences in any MV levels were identified between TRT and CTRL at the end of the trial period. Triglycerides correlated with PMVs, CD36+PMVs, EMVs, CD36+EMVs, MMVs and CD36+MMVs ($r_s = 0.37-0.58$, $p < 0.05$). Furthermore, indicators of ectopic liver fat, alanine aminotransferase (ALT) and gamma glutamyltransferase (GGT) correlated with PMVs, EMVs, CD36+PMVs and CD36+MMVs ($r_s = 0.33-0.49$, $p < 0.05$). Body composition measures were associated with CD36+MMV ($r_s = 0.33-0.35$, $p < 0.05$), while insulin sensitivity was not correlated with any of the studied MV phenotypes. Although TRT improved body composition measures, levels of MV phenotypes were unaffected, thus refuting our hypothesis. MetSy components were associated with MV phenotypes, in particular CD36+MVs, which may support the involvement of CD36 in MetSy pathogenesis.

Poster Session 4 - EVs in stem cells

Chairs: Ewa Stepien and Steven Jay

5:00-8:00 p.m.

PT4.01

Extracellular vesicles produced by the microenvironment of low-grade gliomas at different risk of anaplastic progression differ in terms of miRNA content

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Introduction: Although considered benign, 70% of low-grade gliomas (LGG) undergo anaplastic transformation. Up to now, adjuvant chemo and/or radiotherapy are not indicated, since they can cause chronic neurocognitive impairment not justifiable in patients that may be at low risk of progression. Therefore, new drugs specifically designed to act on LGG are requested. Researchers have identified in the tumour microenvironment (TME) a new therapeutic target. In this regard, we isolated from human gliomas a population of glioma-associated stem cells (GASC), representing an in vitro model of the glioma microenvironment. GASC, through the release of extracellular vesicles (EV), support the glioma growth. Aim of the present study was to study, by means of gene sequencing analysis and bioinformatics, the miRNA content of GASC isolated from LGG at high- (LGG-BAD) and low- (LGG-GOOD) risk of progression and their respective EV. **Methods:** We selected 3 LGG-GOOD and 3 LGG-BAD. EVs were collected through precipitation and microRNA extracted. GASC- and exosomes- miRNAs were sequenced by Illumina platform (HiSeq2000). Data were verified and count of reads for individual miRNAs in the individual samples were normalized to obtain a matrix of expression for the subsequent analysis of comparison between categories (edgeR package). Functional analysis was performed using the IPA program. **Results:** The content in miRNAs of GASC and their respective EV differs significantly, stressing that the EV production is a finely tuned process. In addition, the miRNA profile of GASC and EV differs for LGG-GOOD and LGG-BAD. For example, in LGG-GOOD GASC, miR-1-3p and miR-1304-3p were, respectively, up and down regulated. While in LGG-GOOD exosomes, miR-6087 and miR-374a were, respectively, up- and down-regulated. Functional analyses will be presented. **Conclusions:** The analysis of exosomal miRNA can open the way to possible new strategies aimed at blunting the tumour-supporting function of the TME.

PT4.02

Extracellular vesicles released by Glioma Stem Cells activate astrocytes and induce the acquisition of neural stem-like potential by astrocytes

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Introduction: Cellular heterogeneity and plasticity is a hallmark of glioblastoma multiforme (GBM) and the role played by its micro-environment (TME) is crucial for tumour progression. Findings suggest that GBM TME is populated by glial cells that undergo a process of activation resembling reactive gliosis, along with the acquisition of stem cell potential. We evaluated if exosomes produced by glioblastoma stem-like cells (GSC) could induce in normal astrocytes an activated phenotype and stem-like features. **Methods:** GSC and glioblastoma primary cells (GC) were isolated from human GBM

(n=3). EV were obtained as in Bourkoulou et al., 2014. Human astrocytes (CellScience) were cultured in a medium conditioned or not by EV derived from GSC, GC, U87 and Wi38 fibroblasts and subsequently evaluated in terms of growth kinetics, acquisition of a reactive phenotype (anchorage-independent growth, specific protein expression) and stem-like features (neurosphere assay). The miRNA content of GSC-derived EV was evaluated by next-generation sequencing and validated by qRT-PCR. The role played by miR-181a and miR-92b in activating in astrocytes a stem-cell program was evaluated taking advantage of miRNA mimic and antagomir. **Results:** EV, both from U87, GC and GSC, but not Wi38, were able to induce in astrocytes morphology and function of "reactive" cells. However, only exosomes derived from GSC promoted the de-differentiation of human astrocytes, increasing their ability to form neurospheres. It was then identified in the exosomal miRNA-181a and miRNA-92b the responsible for this activation. The two miRNAs acted reducing the levels of the protein Nemo-like kinase, activating the β -catenin pathway thus inducing the expression of a set of genes responsible for de-differentiation of astrocytes. **Conclusions:** Glioma cells utilize EV in order to modify the biological behaviour of normal glia inducing an activated phenotype and increasing its plasticity.

PT4.03

Extracellular vesicles released by stem cells isolated from the glioma microenvironment enhance the in vivo aggressiveness of glioblastoma cells

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Introduction: Studies highlight the importance of tumour-supporting cells in cancer progression and the role played by extracellular vesicles (EV) in tumour-stroma interactions. Recently, we optimized a method to grow in culture a population of human Glioma-Associated Stem Cells (GASC) that, although devoid of genetic alterations, are characterized by stem cell properties and are able, through the release of exosomes, to modify the biological properties of glioma stem cells. We wanted to verify whether GASC and GASC-derived exosomes exert a pro-tumourigenic effect in an orthotopic murine model of human glioma. **Methods:** EVs were isolated from GASC culture supernatants by ExoQuick-TC and their presence verified by FACS and NanoSight. 1.5x10⁵ U87 were xenotransplanted into the striatum of SCID/Beige mice alone (5), in combination with GASC (5) or with GASC-derived EVs (5). After 3 weeks, animals were sacrificed and tumour size as well as histology was evaluated. Human origin, proliferation and expression of genes related to angiogenesis, invasiveness and epithelial to mesenchymal transition (EMT) were evaluated. GASC-derived miRNAs were sequenced. **Results:** Tumours developed in the animals injected with U87 + GASC or U87 + GASC-derived EVs presented, with respect to those receiving U87 only, a twofold increase in the diameter. Immunofluorescence showed that a tumour derived by the co-injection of U87 and GASC and U87 and GASC-derived EVs are characterized by an increased number of vessels, nestin and CD44 expression, as well as an increased expression of gene related to EMT. EV-derived miRNAs possible responsible of the tumour supporting function were identified. **Conclusion:** GASC enhance the aggressiveness of glioma cells through an exosome-mediated mechanism. Studying the molecular cargo of exosomes could be important to understand the tumour biology, opening the

way to new therapeutic approaches aimed at interfering with the tumour-stroma interplay.

PT4.04

The contribution of the cancer initiating cell markers EpCAM and claudin7 to metastatic progression

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Functional activity of EpCAM (EpC) in colorectal and pancreatic cancer progression depends on its association with palmitoylated claudin7 (cld7) which is excluded from tight junctions and is recruited to glycolipid-enriched membrane microdomains (GEM) cooperating with EpC, additional transmembrane and cytosolic molecules. We now explored how palmitoylated cld7 contributes establishing a metastatic phenotype. The loss of metastatic capacity of a rat pancreatic adenocarcinoma by a knockdown (kd) of EpC or cld7 is waved by the rescue of wt EpC, but not EpC with a mutation in the cld7 binding site (EpCmAG) or cld7 with a mutation in the palmitoylation site (cld7mPalm). Cld7mPalm also does not rescue anchorage-independent growth, motility, invasiveness and apoptosis resistance. This is due to cld7, but not cld7mPalm associating with a3, b4, ezrin, uPAR and MMP14, which support motility and invasion. Palmitoylated cld7 additionally is engaged in Pten repression, which allows Akt phosphorylation accounting for apoptosis resistance. Cld7 supports EMT gene expression and nuclear translocation of Snail, Sox2 and Notch. These activities of GEM-located palmitoylated cld7 are transferred into TEX, cld7kd TEX failing to modulated the host surrounding or to induce EMT in non cancer-initiating cells. Finally, several components of the vesicle transport machinery required for exosome biogenesis and exosome release preferentially commu-noprecipitate with palmitoylated cld7 such that TEX release by cld7kd cells is strongly reduced and uptake by host cells and non-cancer-initiating cells is severely impaired. GEM-located palmitoylated cld7 acts as a cancer-initiating cell marker by associating with signalling molecules and EMT genes, which are transferred together with cld7 into TEX and account for the crosstalk with host and tumour cells. Furthermore, by the association with the vesicle transporter machinery GEM-located, palmitoylated cld7 actively contributes to TEX biogenesis.

PT4.05

Paediatric brain cancer stem cells and their exosomal miRNA content

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Introduction: Brain tumours are the leading cause of cancer-related mortality and morbidity in children. A major problem is the recurrence of cancer, which is thought to be dependent on cancer stem cells. In order to increase survival of patients with brain tumours a proper understanding of the cancer stem cell mechanisms are required. The generation and maintenance of cancer stem cells seems to be orchestrated by different epigenetic changes. One important epigenetic change that can contribute to the cancer stem cell features in tumour progression is driven by miRNAs. These short non-coding RNAs are enriched in exosomes and based on the current knowledge it is reasonable to consider a role of exosomes in cancer stem cells mechanisms. **Methods:** Extracellular vesicles released from paediatric brain cancer stem cells were isolated with differential centrifugation. Exosomes were characterised by size determination with Nanoparticle Tracking Analysis (NTA) and Transmission Electron Microscopy (TEM), characteristic exosomal protein markers were detected with western blot. Exosomal miRNA content was studied with 3D-Gene(tm) Human miRNA array chips from Toray Industries. **Results:** We identified several deregulated miRNAs in paediatric cancer stem cells and exosomes. Pathway analysis revealed the role of these miRNAs in Glioma, ErbB signalling pathway, p53 signalling pathway and several other cancer related pathways. **Conclusions:** Our investigation on the altered expression patterns

of miRNA expression in paediatric brain cancer stem cells and their exosomes revealed a possible role of exosomal miRNAs in brain tumour biology.

PT4.06

The regenerative potential of the amniotic fluid stem cell microvesicles

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Introduction: Human amniotic fluid stem cells (hAFS) have been described as endowed with a distinct secretory profile and significant regenerative potential. Here we aim at characterising the hAFS-derived EV (hAFS-EV) as biological mediators of therapeutic paracrine effect within the hAFS secretome. **Method:** hAFS were isolated upon expression of c-kit from left over samples of amniotic fluid collected for prenatal diagnosis, following informed consent. hAFS were cultured under normoxia (20% O₂) versus hypoxia (1% O₂) preconditioning for 24 h to improve the secretion of EV. EV were obtained by ultracentrifugation of the hAFS-conditioned medium and characterised by transmission electron microscope, NanoSight analysis, western blot and flow cytometry. Their microRNA content was evaluated by Agilent technology and qRT-PCR. The hAFS-EV proliferative and pro-survival capacity was assessed on human dermal fibroblast and myoblast C2C12 cells. The regenerative potential of the hAFS-EV was also tested in a mouse preclinical model of spinal muscular atrophy, (HSA-Cre, SmnF7/F7 mice) with clinical features of human muscular atrophies, in which transplantation of hAFS previously enhanced muscle strength and survival. **Results:** hAFS showed to secrete 100–1000 nm sized EV, with hypoxic preconditioning leading to CD81 exosomal marker enrichment. hAFS-EV isolated under hypoxic conditions showed up-regulation of specific microRNA involved in regeneration and repair. In vitro analysis suggested a role for the hAFS-EV as biologically mediators of cell proliferation and survival following oxidative stress. Preliminary results on atrophic mice advised a modulatory role of the hAFS-EV in decreasing skeletal muscle inflammation. **Conclusion:** This is the first study showing that hAFS can actively secrete EV with hypoxic preconditioning improving their isolation efficiency. These promising findings suggest a novel translational approach based on exploiting hAFS-EV for future paracrine therapy.

PT4.07

Hedgehog associated to microparticles inhibits adipocyte differentiation via a non-canonical pathway

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Hedgehog (Hh) is a critical regulator of adipogenesis. Extracellular vesicles are natural Hh carriers, as illustrated by activated/apoptotic

lymphocytes specifically shedding microparticles (MP) bearing the morphogen (MPHh+). We show that MPHh+ inhibit adipocyte differentiation and orientate mesenchymal stem cells towards a pro-osteogenic program. Despite a Smoothed (Smo)-dependency, MPHh+ anti-adipogenic effects do not activate a canonical Hh signalling pathway in contrast to those elicited either by the Smo agonist SAG or recombinant Sonic Hedgehog. The effects of MPHh+ on adipogenesis are abrogated by pharmacological inhibition of Smo with LDE225. We further underscore the activation of a Smo/Lkb1/ Ampk axis as the non-canonical Hh pathway elicited by MPHh+ to inhibit adipocyte differentiation. Our results highlight for the first time the ability of Hh-enriched MP to signal via a non-canonical pathway opening new perspectives to modulate fat development.

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PT4.08

Augmented production of mesenchymal stem cell-derived therapeutic extracellular vesicles via 3D-bioprocess

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Introduction: Recent studies have drawn attention to extracellular vesicles (EVs) secreted from mesenchymal stem cells (MSCs), as an ideal therapeutic candidate. However, the intrinsic ability of MSCs is significantly deteriorated by conventional 2D culture, so that the clinical scale production of EVs is problematic requiring countless batches with significant impact on costs. In this study, we employed effectual 3D-bioprocessing techniques to enhance MSCs' biological properties for a large-scale production of clinically applicable MSC-derived EVs. **Methods:** MSCs were seeded onto the array composed of 1,225 poly-ethylene glycol microwells with 200 µm of diameter at a density of 5 × 10⁵ cells/array. After spontaneously formed in the microwells, MSC-aggregates were subsequently moved to suspension culture under 35 rpm of an orbital-shaking condition. On 3, 5, 7 days of culture, microvesicles (MVs) secreted from MSCs were quantitatively measured in terms of production rates and inclusions of cytokines and micro-RNAs. Therapeutic efficacy of the MVs was also evaluated in neurogenesis and angiogenesis models *in vitro*. **Results:** MSC-aggregates were spontaneously formed with a homogeneously regulated size in 12 h after cell inoculation in the microwells. Live and Dead assay displayed that most cells were highly viable in the 3D cellular constructs during culture. The highly augmented rate of MV production from MSC-aggregates was quantitatively shown about 100-fold greater than those from the 2D control. Those MVs were enriched with various angiogenic/immunomodulatory and neurotrophic factors. **Summary and conclusion:** A set of 3D-bioprocessing techniques employed in this study enabled the large-scale production of MSC-derived EVs and will possibly possess custom-engineered therapeutic properties in our future study. Consequently, our research could contribute to extended benefits of powerful therapeutic capacity of stem cells with an economic consideration.

PT4.09

Exosomes from placental MSCs as vehicles for the Na/I symporter (hNIS): a new antitumoural therapy

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The Na/I symporter gene (hNIS) is expressed in the thyroid and allows the accumulation of iodine from the diet, to form T3 and T4 hormones. Moreover, it is used (i) as a reporter gene for molecular imaging (when the positron emitter isotope is I124 for PET or Tc99 for SPECT) or (ii) as a therapeutic gene for cancer therapy, mediated by the accumulation of I131. An unresolved challenge is how to direct this gene specifically to the tumoural area. Previously, our group demonstrated the migratory capacity of placental mesenchymal stem cells (MSCs) to tumours, carrying adenovirus expressing hNIS. However as hNIS is expressed at the placental tissue (because it transfers iodine to the foetus from the maternal blood) we also observed that placental MSCs expressed hNIS endogenously. Here, we studied whether exosomes derived from those MSCs (1) expressed hNIS endogenously (2) reached the tumoural areas when they were intratumourally injected. Here we present the therapeutic capacity of the exosomes isolated from those of placenta for cancer therapy and imaging.

Funding: This work has been funded by Universidad Francisco de Vitoria.

PT4.10

Effect of ageing on pro-inflammatory micro-RNAs contained in MSC-derived extracellular vesicles

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Please see OPW1.6

PT4.11

Cancer stem cell markers and exosomes

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It is unknown whether there is link between cancer stem cell (CSC) markers and metastasis/EMT promoting activities of CSC TEX. The question was explored by a knockdown (kd) of the CSC markers CD44v6, Tspan8, EpCAM and cld7 in gastrointestinal cancer. Each kd was accompanied by loss of EMT and metastases formation, wt TEX repairing the defects. As a kd of one CSC marker suffices for a CSC breakdown we performed proteome and miRNA analyses of wt and kd TEX searching for joint features. The CSC makers are located in internalization prone microdomains (GEM). GEM-located Tspan8 recruits a6b4, the cMET-CD44v6 complex is recruited upon ligand binding. Internalized cld7 becomes palmitoylated, which fosters GEM location and EpCAM association. Tspan8 and cld7 are engaged in TEX biogenesis. Tspan8 associates with molecular complexes promoting GEM internalization and scission. Tspan8 and palmitoylated cld7 associate with intracellular vesicle transporters. Proteome analysis and immunoprecipitation also confirmed GEM-located complex internalization including cytoskeletal linker and signalling molecules at the inner membrane. By regulating cMET and Tspan8 transcription, CD44v6 favours a second level of connectivity and due to CSC marker recruitment via Tspan8, TEX biogenesis. There is a strong overlap in missing miRNA recruitment in CSC marker kd TEX arguing for a contribution in miRNA recruitment. CD44 also is involved in miRNA processing. In TEX targeting Tspan8 and CD44v6 play a major role, joint features relying on Tspan8 and CD44v6 associating with

integrins and proteases, cytoskeletal components and signalling molecules. The intracellular domain of EpCAM and CD44v6 promote EMT gene transcription. CSC protein markers are recovered in TEX and are engaged in biogenesis including loading. Joint target modulation is promoted by associations between CSC markers and additional TEX components. Thus, TEX-based diagnosis and therapy should appreciate the central role of CSC markers.

PT4.12

Human iPSC-derived extracellular vesicles: unique properties and biological activities in vitro and in vivo

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Human induced pluripotent stem cells (iPSCs) derived from somatic cells of patients provide an unprecedented source of cells for research into future cell therapies. Unlike many adult human stem cells, human iPSCs can be expanded in culture indefinitely and efficiently (10–12-fold every 3 days), while maintaining their developmental potential to generate nearly any cell type. We and others recently developed a highly defined culture system to expand human iPSCs under either adherent or suspension condition. This system includes the E8 culture medium that is free of any serum or animal proteins, and human recombinant vitronectin as a culture substrate. We analyzed extracellular vesicles (EVs) made by iPSCs by using transmission electron microscopy and NanoSight, following standard methods recommended by ISEV. We observed a concentration of 2x10⁹ particles per millilitre of conditioned medium, with diameters of 100 and 200 nm as dominant peaks. The purified EVs contain RNAs, but not genomic DNA. Proteomic and flow cytometric analyses confirm that they express CD9, CD63 and CD81, as well as many membrane and cytoplasmic proteins of human iPSCs. EVs from human iPSCs stimulated endothelial cell growth in culture. We also investigated iPSC-derived EVs that stably express the firefly luciferase (fluc) gene as a marker. We confirmed that EVs express fluc, indicating the EVs are membrane-enclosed and metabolic active. We further investigated the EV uptake and activities in vitro (cell cultures) and in vivo (in NSG immune-deficient mice), by monitoring horizontal transfer of the fluc activity in live cells once a cell-permeable substrate, D-luciferin, is provided. One day after delivering fluc-labelled EVs into the retro-orbital vein of NSG mouse eyes, we detected luciferase signal in the injected eye. We envision that human iPSC-derived EVs, especially those with genetic enhancement, may provide a novel and safer cell-free modality of “cellular” therapies.

PT4.13

Extracellular vesicles released by renal cancer stem cells inhibit immune response

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Tumour immune-escape has been related to the ability of cancer cells to inhibit T cell activation and dendritic cell (DC) differentiation. A CD105+ population of renal clear cell cancer that fulfil the characteristic of tumour stem cells (CSCs) was shown to release extracellular vesicles (EVs) that favour angiogenesis and metastasis. In the present study we compare the ability of EVs derived from CSCs and from non-tumour initiating population CD105- (TCs) to inhibit dendritic cell differentiation and T cell activation. The results obtained demonstrate that EVs from both CSCs and TCs impaired the differentiation process of DCs from monocytes and the activation of T cells. After monocyte incubation with cancer cell-derived EVs an

impaired expression of HLA-DR, and of CD83 and CD40 differentiation and activation markers was observed by FACS analysis. Moreover, EVs maintained CD14 expression and reduced the acquisition of the specific dendritic marker CD1a and down regulated the expression of costimulatory molecules (CD80 and CD86) and of adhesion molecules (α4 and α5 integrin and CD54). The ability of DCs to induce T lymphocytes proliferation detected by BrdU incorporation was also inhibited. However, these immunomodulatory effects were significantly higher in the presence of EVs derived from CSCs in respect to those derived from TCs. IL10 release was significantly increased in the supernatant of DCs stimulated with cancer cell-derived EVs. In addition, EVs carried HLA-G which has been previously involved in cancer immune escape. HLA-G blockade on EVs significantly decreased the ability of EVs to suppress T cell activation and DC differentiation. In conclusion, these results suggest that EVs released by renal cancer cells and in particular those derived from CSCs inhibited activation of T cells by preventing DC differentiation. This immunomodulatory effect of EVs was at least in part ascribed to the presence of HLA-G in the EV cargo.

PT4.14

Role of argonaute 2-Alix interaction in miRNA enrichment in extracellular vesicle released by human liver stem cells

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Several evidence reports that the biogenesis of extracellular vesicles (EV) involves the endosomal sorting complex required for transport (ESCRT). Alix is an accessory protein of ESCRT with several domains exhibiting multiple protein-binding sites and activities. In addition, EV contain argonaute 2 (Ago 2) ribonucleoprotein which is involved in binding and processing of miRNA. The aim of this study was to better understand the mechanism of miRNA enrichment in EV. We evaluated whether an interaction between Alix and Ago2 occurs during packaging of miRNA within EV released by human liver stem cells (HLSC). We observed that EV isolated from HLSC supernatants expressed different ESCRT proteins, such as Hrs, Tsg101, CHMP4a,b,c, and Alix, which was one of the most abundant. In addition EV contained several RNA-binding proteins, such as TIA-1, TIAR, HuR, Stau-1, 2 and Ago 2, and were enriched in several miRNA (miR24, miR31, miR125b, miR99b, miR221, miR16 and miR21) involved in tissue regeneration and anti-tumoural activities. Experiments of co-immunoprecipitation (co-IP) showed the interaction between Ago2 and Alix. By qRT-PCR we showed the association of miRNA with the Ago2-Alix co-IP. Moreover, EV derived from Alix knock down-HLSC showed a significant decreased expression of Ago2 and of miRNA, even if the number of released EV was not affected. In conclusion, these results suggest that Alix by binding to Ago2 is instrumental in the accumulation of miRNA within EV during their biogenesis.

PT4.15

Regulation of macrophage polarization by mesenchymal stem cell derived-exosomes

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Background: Mesenchymal stem cells (MSCs) have been used to successfully treat a variety of inflammatory diseases in preclinical models. Recent evidence has implicated MSC exosome (MEX) secretion as the predominant mechanism of MSC therapeutic activity. Macrophage polarization plays an important role in regulating the immune response and inflammation. However, it remains

unclear as to whether MEX harnesses the MSCs therapeutic immunomodulatory capacity. We aim to investigate the ability of MEX to modulate macrophage polarization states in vitro. *Methods:* MEX were isolated by gradient density and size exclusion chromatography (SEC). MEX were characterized by electron microscopy, nanoparticle tracking analysis, fluorescence microscopy and western blot. The immunomodulatory capacity of MEX was assessed in vitro where alveolar macrophages (MH-S cell line) were polarized to M1 (classical - LPS, IFN γ), M2 (alternative - IL4, IL13) and M2r (anti-inflammatory - IL4, IL10, TGF- β) phenotypes in the presence (0.05–0.5 $\times 10^6$ MSC cell equivalents) or absence of MEX. Macrophage mRNA levels were assessed by RT-qPCR. *Results:* MEX immunoblots were positive for CD9, TSG101, and Alix. Macrophage-MEX interaction was confirmed by fluorescence microscopy. The addition of MEX to

alveolar macrophages significantly reduced the mRNA induction of classically-activated macrophage markers such as TNF α and CCL5, $p < 0.05$. MEX treatment super-induced ARG-1 mRNA in the M2 state ($p < 0.05$) but did not affect ARG-1 levels in the M2r phenotype $p > 0.05$. Separating vesicle populations via SEC demonstrated that the immunomodulatory capacity of MSC-vesicles is attributed solely to MEX, microvesicles had little effect on macrophage polarity. *Conclusion:* We demonstrate a novel mechanism where MEX may modulate the lung macrophage polarity, suppressing the pro-inflammatory M1 and shifting the alternatively-activated M2 phenotype, to favor the anti-inflammatory (M2r-like) phenotype.

Poster Session 5 - EVs as cancer biomarkers

Chairs: Casey Maguire and Carolina Soekmadji

5:00-8:00 p.m.

PT5.01

Extensive phenotyping of plasma EVs from small cell lung cancer patients undergoing treatment

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Introduction: With increasing incidence, lung cancer is a leading cause of cancer death worldwide. Small cell lung cancer (SCLC) accounts for app. 13% of the total Lung Cancer diagnoses, and it is related with early metastasis as well as poor prognosis with 5-year survival rates of app. 10% and 5% in limited and extensive disease (ED), respectively. Thus, it is crucial to identify new biomarkers of diagnostic and prognostic value to target SCLC. Here, we use an internationally unique study-based SCLC cohort (RASTEN: a randomized phase III study of standard treatment ± enoxaparin) featuring baseline samples, samples taken after two cycles of treatment, and follow-up samples, to clarify the potential role of EVs as prognostic predictors. **Methods:** Plasma from 245 SCLC patients (108 with limited and 137 with ED) were subjected to analysis by the extracellular vesicle array (EV Array). The array was composed of 29 capturing antibodies targeting well known EV-associated as well as hypoxia and lung cancer-related proteins. The binding of EVs was visualized and recorded using a cocktail of biotin-conjugated CD9, CD63 and CD81 antibodies combined with fluorescently-labelled streptavidin. **Results:** We were able to detect and phenotype EVs in all samples from only 10 µL of plasma. The array was composed of general EV-related markers (e.g. CD9, CD63, CD81, TSG101, and CD82), selected relevant cancer biomarkers (e.g. CD151, c-MET, and VEGFR2), and markers of hypoxia (CAIX and CAXII). On the basis of this analysis the potential predictive role of EVs in prognostics has been clarified. **Summary/conclusion:** Compared to healthy controls, SCLC patients have a specific protein signature regarding EVs in plasma. The EV Array technique states itself as a relatively simple, non-invasive tool with potential to provide prognostic information on SCLC patients.

PT5.02

Potentials of plasma EVs in relation to diagnostics and prognostics in lung cancers

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Introduction: The majority of lung cancer patients present advanced stage disease at the time of diagnosis and the outcome is often fatal. Hence, new clinical tools are needed to secure a better and earlier diagnosis. Studies have identified plasma EVs as useful markers in several diseases including late stages of non-small cell lung cancer (NSCLC, Jakobsen et al., 2015, JEV). In this study, we focused on identifying the potential of plasma EVs for detection of early stages of NSCLC. Small-cell lung cancer (SCLC) is associated with a poor prognosis and survival. A cohort of SCLC patients treated with enoxaparin or standard treatment was included in the current study to follow the changes in plasma EVs as a response to treatment.

Methods: Plasma samples from cohorts of patients with non-cancerous lung diseases, patients with SCLC, and NSCLC were analyzed for their content of EVs. The cohort of NSCLC patients included all stages of adenocarcinoma (stage I – IV). The cohort of SCLC patients are included in the international study “RASTEN” (a randomized phase III study of standard treatment ± enoxaparin). The EVs were extensively phenotyped with the use of the EV Array technology (Joergensen et al., 2013, JEV). **Results:** Analysis of more than 30 different EV markers in the plasma samples made it possible to generate EV profile signatures for the various cohorts. Multivariate data analysis was performed and revealed that smoking tended to have great influence on the EV protein profiles of non-cancerous lung disease patients and NSCLC patients implying that smoking influences the protein profiles of plasma EVs. The predictive use of plasma EVs was investigated with the cohort of enoxaparin treated SCLC patients. Each of the 245 patients revealed a personal plasma EV profile and a time line data analysis was performed. **Summary/conclusions:** Using the EV Array technology, we demonstrate how plasma EVs serves as a potential diagnostic and predictive tool in different types of lung cancer.

PT5.03

miR126 is mainly delivered through exosomes in early-stage NSCLC patients

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Introduction: Lung cancer is one of the leading causes of cancer-related death. The major issue of this disease is that diagnosis occurs mostly at locally advanced or metastatic stage. Therefore, new diagnostic approaches based on early detection of cancer biomarkers are needed. Recently, increasing attention has been paid to the role of exosomal microRNAs (exo-miRNAs) in tumour metastasis and angiogenesis. Such discoveries open the possibility of using exo-miRNAs as cancer biomarkers and treatment–response predictors. In this study, we evaluated expression levels of miRNA-126 (miR126) in serum and exosomes of non-small cell lung cancer (NSCLC) patients. **Methods:** Stem-loop RT-PCR was performed for expression analysis of miR126. Exosomes were purified by ultracentrifugation over a sucrose cushion and quantified using Nanoparticle tracking analysis (NTA) technology. **Results:** The miR126 expression in serum showed no significant difference among NSCLC patients at early stage and healthy controls, but much higher in the exosome fraction of patients with NSCLC. In cancer patients there is a shift in miR126 distribution in exosomes of about 30% of all miR126 present in the serum. In the healthy control group, the exosomal miR126 was of 10%. **Summary/conclusion:** Our results showed that in lung cancer patients, miR126 is preferably transported by exosomes, which might suggest that this mechanism plays an important role in the lung cancer development.

PT5.04

Tissue-compatible EGFR genotyping in tumour-derived extracellular vesicles from malignant pleural effusions

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Background: Tumour cells shed extracellular vesicles (TEV) into body fluids. These TEV are a potential source for biomarkers that could be used for non-invasive molecular diagnosis. We investigated whether TEV contain cell-specific mutant EGFR DNA using PC9 cells harbouring an exon 19 deletion (E19 del) and PC9/GR cells harbouring

a T790M mutation. In addition, we investigated whether the TEV from malignant pleural effusions can be used for EGFR mutation testing. **Materials and methods:** TEV were isolated from the culture medium of PC9 and PC9/GR cells or pleural effusions by serial centrifugations. Isolated TEV were inspected by DLS to determine their size. EGFR mutation testing was performed using PNA-mediated clamping PCR. **Results:** EGFR genotyping of TEV DNA revealed exactly the same EGFR mutations with cellular DNA in the PC9 and PC9/GR cells. These findings suggest that TEV contain cell-specific mutant DNA that may contain an oncogenic molecular signature such as mutations in EGFR. Testing of the TEV from malignant effusions in 23 NSCLC patients yielded the same genotyping results as from tissue samples. We observed T790M mutation in addition to the initial E19 del and L858R mutations from seven patients who developed acquired gefitinib-resistance. **Conclusions:** In this study, we demonstrated that TEV contain cell-specific oncogenic DNA, including EGFR mutants in the cell lines and malignant pleural effusions.

PT5.05

Circulating RNAs as biomarkers in Epstein Barr virus-associated nasopharyngeal carcinoma

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Please see OPW3.1

PT5.06

Molecular profiling of metastatic versus non-metastatic breast cancer cell-derived exosomes

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Introduction: Breast cancer (BrCa) is the most frequent cancer type in women and a leading cause of cancer related deaths in the world. Despite the decrease in mortality due to better diagnostics and palliative care, there is a lack of prognostic biomarkers for metastasis. Early detection of metastasis is crucial for the survival of the BrCa patients. Recently, the examination of liquid biopsies and in particular of the enriched extracellular vesicles has shown promise in the identification of such predictive biomarkers. **Methods:** In this study we compared the proteomic content of exosomes derived from the syngeneic, non-metastatic 67NR and highly metastatic 4T1 mouse BrCa model. We demonstrate that concentration and protein content of the 67NR and 4T1 exosomes differ significantly. We validated our findings in human cell lines (MCF7 and MDA-MB-231) and in a small cohort of patient samples with early disease or lymph node (LN) metastasis. **Results:** We found that the exosomes isolated from the mouse cell lines, human cell lines and plasma of BrCa patients with LN metastasis were enriched in N-cadherin and periostin. Moreover exosomes from mouse cell lines and plasma of BrCa patients with LN metastasis had higher number of larger exosomes. Furthermore, we identified differences in the uptake of exosomes, with the ones derived from 4T1 cells being taken up more readily and having a bigger effect in the migration of the non-metastatic 67NR recipient cells. **Summary/conclusion:** Exosomes from non-metastatic versus metastatic breast cancer cell lines have both molecular and functional differences. Moreover, these differences are present in samples from patients with non-metastatic disease versus LN metastatic patients.

PT5.07

Development of blood exosome-based immunoassays and PCR tests for active surveillance of neurofibromatosis patients and early detection of malignant peripheral nerve sheath tumours (MPNST)

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Background: Our project was directed towards the identification of MPNST specific exosome associated markers, with the ultimate aim of developing a non-invasive, sensitive and specific multimarker assay for identification of neurofibromatosis (NF1) patients with the risk of MPNST development and early detection. NF1 is a hereditary syndrome characterized by growth of multiple benign tumours that impose high risk of malignant transformation, representing thus a paradigm for surveillance of high risk individuals, likely to benefit from employing circulating exosomes as early sentinels of cancer onset and progression. **Methods:** Exosome associated MPNST proteins and RNAs were revealed via screening of exosomes purified from patient-derived MPNST and plexiform NF lines. Selected markers related to tumour metabolism, adhesion and growth factor signalling were used as targets for exosome-based sandwich immunoassays validated in spiked-in plasma and serum samples, and subsequently for monitoring of tumour growth in a bioluminescent MPNST xenograft murine model, and comparison of MPNST and NF patient serum samples. miRNA (8) and mRNA molecules (10–24) were analysed in a semi-quantitative RT-PCR and in Taqman miRNA array. NTA analysis was performed for sample normalization. **Results:** ELISA assays employing some proprietary and commercial antibodies (i.e. Flotillin1, NEK2, HIF1) and a restricted RNA panel (5 mRNAs + 5 miRNAs) displayed high sensitivity for monitoring of MPNST in a mouse model in terms of early post-graft signals and overall correlation with tumour size. These data partially matched the observations in human serum samples, with behaviour of several candidate markers (two proteins, four mRNAs and three miRNAs) that warrant further validation complemented with identification of additional markers. **Conclusions:** Exosome MPNST markers identified in ex vivo blood samples pave the way to fine assay validation in longitudinal samples from NF patients as well as in other solid tumours.

PT5.08

Characterization of microRNAs and proteomic cargo in exosomes secreted by neuroblastoma

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Introduction: Neuroblastoma (NB) is the most common extracranial paediatric solid tumour. At diagnosis, about 70% of patients with metastatic disease present bone-marrow (BM) infiltration. However, the mechanism underlying the BM tropism remains incompletely elucidated. It has been demonstrated that tumour-derived exosomes can support metastatic progression. Here, we characterize NB exosomes cargo setting the stage for future functional investigations. **Methods:** We characterized exosomes from five NB cell lines: one derived from primary tumour (IMR32) and four from BM metastasis (SKNSH, SHSY5Y, SKNBe2c and LAN1). We isolated exosomes using standard ultracentrifugation and confirmed their number/size by NanoSight and TEM. We analyzed protein content by BCA and proteomic approach. We further characterized exosomal microRNAs

(miRs) of SHSY5Y and IMR32 by microarray profiling. *Results:* TEM on SHSY5Y confirmed a population of exosome-like vesicles (30–100 nm). The protein content of SKNSH and SHSY5Y exosomes was significantly increased compared to IMR32. Exosomal miR profiling showed 153 miRs expressed both in SHSY5Y and IMR32, whereas nine and 12 miRs were found exclusively in SHSY5Y and IMR32, respectively. We found 30 miRs more than two-fold up-regulated in IMR32 exosomes and 28 down-regulated compared to SHSY5Y. The proteomic analysis of exosomes showed 666 common proteins among all NB lines and 18 proteins exclusively present in exosomes from BM metastasis. Classification of exosomal proteins by biological process showed proteins mainly involved in cell growth, proliferation, death and protein synthesis. Furthermore, we found proteins involved in RNA post-transcriptional modification and invasion/metastasis. *Summary/conclusion:* our data suggest that NB-derived exosomes contain a specific signature. Further studies are needed to determine the functional role of NB-derived exosomes in tumour progression and their potential use as prognostic biomarkers of metastasis and BM infiltration.

PT5.09

Circulating cancer-associated extracellular vesicles as early detection biomarkers for pancreatic ductal adenocarcinoma

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Introduction: Pancreatic ductal adenocarcinoma (PDAC) is usually found at an advanced stage, although diagnosis at an early stage is unequivocally associated with better long-term survival. Therefore, there is an urgent need to develop early detection methods to improve these outcomes. Extracellular vesicles (EVs) attract much attention as potential biomarker because tumour cells have been shown to release EVs into circulation, which mirror their cellular origin. Detection of cancer-associated EVs in body fluids from the patients could serve as a non-invasive liquid biopsy for diagnosis and monitoring of cancer. The main objective of this study is identification and detection of PDAC-specific EVs in serum from the patients. *Methods:* To identify the protein profile of EVs in serum from PDAC patients and healthy donors, major serum proteins in blood were removed using ultracentrifugation and affinity spin column for 14 highly abundant serum proteins, and then total EV proteins were purified from 3 different stages of PDAC (stage II, III and IV, each group: 6 patient sera pooled) and healthy donor. Mass spectrometry was performed with purified EV proteins, and the biomarker candidates were validated by immunoblotting in PDAC patient blood samples. *Results:* Proteomics analysis individually identified over 500 proteins in each pooled samples, and we identified 9 membrane proteins, which were detected in only PDAC patients, not in healthy donors. We focused on 2 proteins and performed immunoblotting for the validation of potential biomarkers in 3 stages of PDAC blood samples. As a result, these proteins were detected exclusively in EVs of PDAC patient sera including stage II. *Summary/conclusion:* We identified 2 potential EV-associated proteins as PDAC biomarkers. Further validation of the candidate markers is still needed on large patient cohorts including early stage of pancreatic cancer patients.

PT5.10

Plasma-derived exosomes as a reservoir of biomarkers for leukaemia patients stratification

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We have analysed the transcriptome content of blood circulating exosomes isolated from lymphoid and myeloid malignancies. We

evaluated for each disease (ALL, CLL, MBL, AML, MDS, MPN) and for the healthy donors (HD) the overall composition of the various RNA species that can be detected using the sequencing methodology designed to detect mature miRNA, that is, small RNA sequencing (miRNA-seq), and whole transcriptome, that is, non-polyA selected stranded total RNA sequencing (WT-seq). Specifically, we detected both coding and non-coding RNAs (mRNAs, miRNAs, snRNAs, snoRNAs, lincRNAs, circRNAs) transported by the exosomes. We have analysed by miRNAs-seq 256 samples and 123 samples by WT-seq. From the comparison between AML, ALL, CLL and HD specimens, we identified subsets of coding and non-coding RNAs that were disease specific. Specifically, we detected a subset of AML-specific miRNAs and subsets of CLL-specific miRNAs/mRNAs/lincRNAs. Using the miRNA AML-specific signature, we managed to stratify MPN and MDS, diseases that in some cases can progress in AML, AML-like, HD-like and MPN/MDS-only subgroups. Using the mRNA and lincRNAs CLL-specific signatures, we managed to discriminate efficiently MBL from CLL. Interestingly, our data suggest that exosomes transcriptome provides extra features for the discrimination of MBL from CLL, which cannot be grasped in case miRNAs from the intracellular compartment are analysed. Preliminary observations suggest that the signature allows the prediction of the evolution of MBL in CLL. Taken together, our results indicate that exosome transcriptome represents a powerful new instrument for the discovery of new biomarkers for disease stratification.

PT5.11

Exosomes secreted by apoptosis-resistant AML blasts harbour regulatory network proteins potentially involved in antagonism of apoptosis

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Introduction: Expression levels of proteins promoting apoptosis-resistance in acute myeloid leukaemia (AML) bone marrow (BM) blasts at diagnosis are associated with shorter disease-free survival. We have previously measured, by flow cytometry, expression of anti-apoptotic and pro-apoptotic proteins in leukaemic combining them to define the anti-apoptosis index (AAI). The AAI of normal lymphocytes in the AML patients paralleled that of AML blasts of the same patient. This points to a role of microenvironment in regulation of apoptosis in AML BM cells. The aim of this study was to characterize the microenvironment generated in the BM with apoptosis-resistant AML cells, in terms of its protein composition. *Methods:* To characterize the AML microenvironment, conditioned media (18 h) from patient samples displaying either high AAI (n=5) or low AAI (n=6) were analysed using mass spectrometry-based proteomics. We further investigated protein profiles of purified extracellular vesicles (EVs) of 2 high AAI patients and 1 low AAI patient combined with confocal imaging of the EV transfer between cells. *Results:* The total secretome data set consisted of 1492 proteins. Our comparative proteomics analysis showed that the major functional protein clusters upregulated in secretomes of the high-AAI AML were involved in mRNA splicing, protein translation and chromatin remodelling. EV proteomics indicated that these functional protein networks are cargo of EVs that are enriched for exosome markers. Finally, confocal microscopy-based co-localization studies suggest that EVs, containing splicing factors and secreted by high AAI cells, are taken up by low AAI cells. *Conclusion:* Transfer of functional proteins between cells by EVs is a well-documented phenomenon. Therefore, it is conceivable that the regulatory proteins detected in EVs excreted by AML blasts are involved in regulation of apoptosis in recipient bone marrow cells, whereby apoptosis resistance in AML blasts contributes to therapy resistance.

PT5.12

Plasma extracellular vesicles as a liquid biopsy source for RNAseq-based detection of cancer-associated microRNAs

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Current treatment strategies for cancer patients aim towards personalized therapy by early identification of the effectiveness of treatment and detection of relapse during follow-up. Non-invasive, sensitive diagnostic tools are highly desirable. Extracellular vesicles (EVs) that encapsulate miRNAs with biomarker potential are actively secreted into circulation. We used SEC to separate plasma EVs and protein/HDL from Hodgkin lymphoma (cHL) patients and healthy donors and prepared libraries for RNAseq analysis. The distribution of different small RNA species is similar in cHL compared to healthy plasma EVs. Libraries from cHL EVs yielded a larger amount of miRNAs than EVs from healthy donors and the proportion of miRNAs in EVs outnumber miRNAs identified in protein/HDL. Importantly, the number of different miRNAs does not correlate with the total amount of reads and is therefore not merely caused by sequence depth but seems related to pathological conditions. We examined the potential role of non-templated nucleotide additions (NTAs) in miRNA secretion. Circulating miRNAs in the EVs from cHL patients but not healthy individuals have a high percentage of NTA(U) compared to NTA(A), suggesting disease-associated alterations in EV-mediated isomiR secretion. In a direct comparison of individual miRNAs, several potential biomarkers were identified in plasma EVs. MiR21-5p, let7a-5p, miR24-3p, miR155-5p and miR127-3p were significantly increased in plasma EVs of cHL patients. qRT-PCR analysis confirmed these RNAseq results and allowed us to monitor patient response during and after treatment. We show that several candidate miRNA biomarkers in plasma EVs decrease significantly in clinical responders, as determined by FDG/PET scan. Moreover, a perfect correlation was found with serum TARC, a chemokine secreted by Hodgkin tumour cells. Altogether these data show that plasma EVs can be used for biomarker discovery by RNAseq and that these biomarkers directly reflect the presence of vital tumour cells.

PT5.13

Depletion of normal blood cell-derived exosomes from plasma to enhance a tumour-associated exosomal mRNA signature

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Introduction: Exosomes are released from cells, including tumour cells, into biofluids and carry nucleic acids and proteins from the cell of origin. Exosome-based liquid biopsies enable non-invasive

monitoring of a patient's tumour status in real time, an advantage over invasive tissue-based tests. However, the tumour signature must be identified against a background of exosomes originating from non-malignant cells. To reduce this background, we developed a platform to deplete exosomes from normal blood cells. We can remove (a) reticulocyte-derived exosomes, (b) platelet cell exosomes and (c) leukocyte-derived exosomes. We hypothesize that removal of normal blood cell-derived exosomes will enrich tumour-derived exosomes. **Methods:** Plasma exosomal mRNA (exoRNA) was extracted from 40 plasma samples (36 metastatic melanoma, 4 normal) using two extraction methods, total exosome isolation using exoRNeasy and reticulocyte-depleted exosomes using ExosomeDx Depletion/Enrichment (EDDE) Platform. Analysis of exoRNA utilized the Open-Array Human Inflammation Panel. The impact of removing reticulocyte exosomes was examined by comparing genes from EDDE Platform versus total exosomes. **Results:** For each sample, 607 genes were ranked based on their Ct value. A systematic assessment of the delta rank was performed to determine the utility of the EDDE Platform. This assessment revealed that the EDDE Platform enriched 13 genes and reduced 4 genes on average, significantly more compared to total exosomes with 0 genes under the same selection criteria. When comparing plasma exoRNA signatures of metastatic melanoma immunotherapy patients who responded to treatment compared to non-responders, only the EDDE platform revealed gene changes consistent with response. The removal of targeted exosomes was validated using western blot and ELISA. **Conclusions:** We have developed a customizable platform to deplete normal exosomes from plasma which increases the power to discriminate tumour-associated exoRNA.

PT5.14

Exosomes from hypoxic colorectal cancer cells – miRNA cargo as indicator of adverse CRC biology?

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Introduction: Tumour hypoxia (oxygenation deficiency) contributes significantly to treatment resistance and metastatic progression in colorectal cancer (CRC), and evidence supports a central role of exosomes in the aggravated biology caused by tumour hypoxia. We therefore aimed to characterize exosomes released by normoxic and hypoxic CRC cells and study their miRNA cargo. **Methods:** The CRC cell line HCT 116 was cultured in RPMI-1640 supplemented with 1% bovine serum albumin under normoxia (21% O₂) or hypoxia (0.2% O₂) for 24 h. Vesicles were isolated from conditioned media by differential ultracentrifugation and characterized by western blot and flow cytometry. Particle size was measured by nanoparticle tracking analysis (NTA) using NanoSight (Malvern), and vesicle integrity and size were determined by cryo-electron microscopy (cryoEM). Expression profiling of exosomal miRNA was conducted using the miRCURY LNA Universal RT microRNA PCR Human panel I (Exiqon). **Results:** Normoxic and hypoxic HCT 116 cells released vesicles that expressed proteins known to be present in exosomes (CD9, CD63, CD81, Alix) and with the absence of contamination markers (the endoplasmic reticulum markers GRP78 and calnexin and the Golgi marker GM130). Vesicles isolated by using CD81-antibody-coated beads counterstained with anti-CD9, anti-CD63 and anti-CD81 in flow cytometry analysis. Vesicle size ranged from 30 to 130 nm, confirmed by cryoEM and NTA, and supported the presence of exosomes. Preliminary data confirmed the presence of miRNA in exosomes and an altered miRNA profile in response to hypoxia. **Summary/conclusion:** Using differential centrifugation, vesicles released from both normoxic and hypoxic CRC cells were isolated and further characterized and defined as exosomes. We are currently investigating the miRNA cargo in exosomes from CRC cells and will further analyse plasma samples from patients with locally advanced rectal cancer, and whether exosomal miRNA could serve as a novel biomarker of tumour hypoxia.

PT5.15

Targeting tumour-associated exosomes with novel integrin-binding peptides

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Introduction: The lag in exploiting the potential of extracellular vesicles (EVs) as tumour biomarkers is largely due to the inability of easily distinguishing tumour-associated vesicles from healthy ones. Here, we report our recent development of a set of novel peptide ligands capable of binding exosomes derived from specific tumour cells. **Methods:** Via a combinatorial-based screening method of tumour cells against libraries containing millions of solid-phase bound peptides, some were found to specifically bind either ovarian (SKOV-3) or leukaemia (Jurkat) tumour cells through their integrin surface proteins. By mass spectrometry proteomics measurement, we found that exosomes produced from these cells (isolated both by differential and density gradient ultracentrifugation methodologies) similarly overexpress these integrins. Those exosomes were then pre-mixed with a dye-labelled analogue of the respective integrin-binding peptide ligand and subsequently bound to latex beads for flow cytometry analysis. Additionally, the ligands were functionalized with a Raman tag and mixed with the target exosomes before Raman laser trapping measurement for the presence of the ligand on a single exosome. **Results:** Both flow cytometry and Raman laser trapping experiments confirmed the specificity of binding for the peptide ligands to their respective tumour cell-isolated exosomes and furthermore showed little affinity to other types of normal cell-derived exosomes. Finally, scrambled analogues of the peptides did not exhibit binding to the tumour-associated exosomes. **Summary:** Given the overexpression of certain integrins present at the surface of EVs released from a variety of tumour types, we believe this technology will be useful in separating circulating EVs according to their tissue of origin or state of disease. Therefore, we are further investigating these ligands for use as exosome markers for a variety of clinical applications.

PT5.16

High-throughput, purification-free, multiplexed profiling of circulating miRNA for discovery, validation and diagnostics

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We have developed the multiplexed circulating microRNA assay that allows the detection of up to 68 microRNA targets per sample. The assay combines particle-based multiplexing, using patented firefly hydrogel particles, with single-step RT-PCR signal. Thus, the circulating microRNA assay leverages PCR sensitivity while eliminating the need for separate reverse transcription reactions and mitigating amplification biases introduced by target-specific qPCR. Furthermore, the ability to multiplex targets in each well eliminates the need

to split valuable samples into multiple reactions. Results from the circulating microRNA assay are interpreted using Firefly Analysis Workbench, which allows visualization, normalization and export of experimental data. To aid discovery and validation of biomarkers, we have generated fixed panels for oncology, cardiology, neurology, immunology and liver toxicology. Here we present the data from several studies investigating circulating and tumour microRNA, showcasing the ability of the technology to sensitively and specifically detect microRNA biomarker signatures from fluid specimens.

PT5.17

Metabolomics of urinary EVs: main pathways, normalization and potential for biomarker studies

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Introduction: Extracellular vesicles (EVs) are promising sources of biomarkers. Metabolomics of EVs is an unexplored field, although it holds the potential to reveal dynamic changes in the metabolism downstream of genetic and proteomic regulation. Here we sought to clarify, what metabolites are found within urinary EVs, could EV metabolites act as biomarkers in prostate cancer and how to normalize the data. **Methods:** We purified EVs from urine samples of three consented prostate cancer patients before and after radical prostatectomy and 3 controls, and subjected them together with their source urine samples to UPLC-MS-MS analysis of 102 metabolites. For normalization, EV-derived (CD9, particle number and volume) or urine-derived parameters (volume and creatinine) and calculation of metabolite proportions were tested. **Results:** We were able to quantify 32–55% of the screened metabolites in EV samples containing as little as 6×10^9 EVs or EVs from 10 mL of urine. The EV metabolome differed clearly from the urine. While most of the metabolites were more abundant in urine, some were better detected in the EVs. All EV samples contained nucleotide, amino acid, vitamin B, carnitine, amine or related metabolites representing mainly urea cycle, purine nucleotide, glutathione and carnitine shuttle pathways. Normalization of the data with EV-derived factors or metabolite proportions together revealed reduction of glucuronate, ribose-5-phosphate and isobutyrylcarnitine in cancer samples compared to control or post-prostatectomy samples. No changes were observed by normalization to urine-derived parameters. **Conclusion:** Relatively small number of EVs is sufficient for detection of EV metabolites. Normalization to EV-derived parameters or metabolite proportions reveals differences between samples better than urine-derived parameters. The observed changes in the cancer samples relate to cancer metabolism and suggest that EV metabolites could act as prostate cancer biomarkers.

Poster Session 6 – EVs in the CNS

Chairs: Julie Saugstad and Roosmarijn Vandenbroucke

5:00–8:00 p.m.

PT6.01

Autophagy activation prevents alpha-synuclein secretion in exosomesRita Perfeito¹, Vanessa Anjos¹, Rui Nobre¹, Manuel Garrido^{1,2} and Luís Pereira De Almeida¹¹Center for Neuroscience and Cell Biology, Coimbra, Portugal; ²Genibet Biopharmaceuticals, Lisbon, Portugal

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the accumulation of misfolded protein aggregates of alpha-synuclein (a-syn). a-syn has been described to be transmitted from neuron to neuron through exosomes, propagating aggregate pathology to different brain areas and contributing to PD progression. a-Syn accumulation in neurons has also been linked to an ineffective clearance of this protein by autophagy. The main goal of this work was to investigate whether the transport of a-syn occurred via exosomes using an in vitro model of mouse neuroblastoma cells (N2a) expressing human WT a-syn. We aimed to clarify a possible interaction between a-syn exosome secretion and autophagy by investigating whether pharmacological activation of autophagy would prevent secretion of a-syn in exosomes. Results show that upon incubation of exosomes isolated from N2a cells expressing human a-syn with non-transfected N2a cells, a-syn was detected in the latter, suggesting that it was transferred from cell-to-cell via exosomes. Pharmacological activation of autophagy in N2a cells overexpressing human WT a-syn effectively reduced the levels of this protein. A decrease in the number of aggregates positive for a-syn was also observed. Importantly, we found that in exosomes isolated from transfected N2a cells treated with an autophagy activator during 12 h, a-syn levels were significantly decreased, suggesting that this strategy of autophagy activation prevented the secretion of a-syn via exosomes. Our data strengthen previous evidence that the autophagy pathway plays an important role in the clearance of a-syn levels and provides new information on a possible interaction between the autophagic process and the spreading of a-syn via exosomes. We propose that inhibiting a-syn release in exosomes and inducing degradation of a-syn and/or aggregates by autophagy may constitute a novel pharmacological approach for treatment of synucleinopathies such as PD.

PT6.02

Investigation of the role exosomes play in the propagation of tau pathologyAaron Bradshaw¹, Danning Li¹, Rohan De Silva², J. Mark Cooper³ and Lydia Alvarez-Erviti⁴¹University College London, England; ²University College London, Institute of Neurology, London, England; ³Department of Clinical Neurosciences, University College London, England; ⁴Centro De Investigacion Biomedica De La Rioja, Spain

Introduction: Microtubule-associated protein tau exists in neurons as 6 alternatively spliced isoforms. The misfolding and intracellular aggregation of tau is a pathological hallmark of Alzheimer's disease (AD) brains and correlates with neuronal loss. In AD tau, pathology follows a typical spatial progression through the brain suggesting active propagation of the pathological protein, a common feature in several neurodegenerative diseases. One potential mechanism underlying this spread is via release in exosomes. Tau protein has been observed in exosomes although the factors underlying its exosomal targeting are unknown. Previous work with alpha-synuclein suggests the presence of a chaperone-mediated autophagy pentapeptide motif (of which tau has two) may play a key role in targeting specific proteins to exosomes. Defects in protein degrada-

tion also increase the release of misfolded proteins via this pathway. We aim to understand the factors influencing exosomal tau release and if this plays a key role in the spread of tau pathology. **Methods:** We have used SH-SY5Y neuroblastoma clones stably expressing different tau isoforms. Exosomes were purified from conditioned medium using ultracentrifugation. Genetic and pharmacological techniques were used to study the degradation pathways of tau and the effects upon exosomal tau release and transmission to naïve cells. **Results:** We have confirmed the full length tau isoform (2N4R) and alternatively spliced isoforms (2N3R, 0N3R, 0N4R) are present in the exosomal fraction isolated from conditioned medium. We will present data regarding the intracellular degradation pathways of tau protein and how genetic and pharmacological manipulation affects exosomal tau release and transmission. **Summary:** We have confirmed different tau isoforms are released from neuronal cultures in exosomes and are beginning to understand the factors that influence the propagation of tau aggregation, which will be important targets for therapeutic intervention.

PT6.03

Microvesicles in amyotrophic lateral sclerosis: potential biomarkers for disease propagation and therapeutic targetsDaisy Sproviero¹, Sabrina La Salvia^{1,2}, Federico Colombo³, Marta Giannini², Luca Diamanti⁴, Paola Bini⁴, Orietta Pansarasa¹, Laura Porretti³ and Cristina Cereda¹¹Laboratory of Experimental Neurobiology, "C. Mondino" National Neurological Institute, Pavia, Italy; ²Department of Brain and Behavioral Sciences, University of Pavia Pavia, Italy; ³Clinical Chemistry and Microbiology Laboratory, Flow Cytometry and Experimental Hepatology Service, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy; ⁴Neurology Department, IRCCS National Neurological Institute C. Mondino, University of Pavia, Pavia, Italy

Introduction: The lack of biomarkers in neurodegenerative diseases makes impossible to determine the stage of illness in patients delaying therapeutic trials. Blood contains microvesicles (MVs), pro-inflammatory vesicles that transfer mRNA, non-coding RNA or transcription factors among different cell types. This facilitates the spreading of the disease through the delivery of genetic material and pathogenic proteins. A new hypothesis about disease transmission merges several neurological diseases associated with protein misfolding and aggregation, in the statement of "prion-like diseases." MVs can initiate prion propagation from prion-infected neuronal cells to uninfected cells. The aim of our study is to investigate MVs in plasma of amyotrophic lateral sclerosis (ALS) patients, in order to support the "prion-like theory" and discover a new mechanism in disease progression. **Methods:** Peripheral blood (5 mL in sodium citrate tubes) from 20 ALS, 20 Alzheimer's disease (AD) patients and 20 healthy volunteers was centrifuged in two steps: 1000xg for 10 min and 1600xg for 20 min. The plasma obtained was then centrifuged at 20,000xg for 1 h and the MVs pellet was immunostained with anti-annexin V and anti-CD45 (pan-leucocyte antigen) antibodies and then analysed by flow cytometry. Misfolded protein level was detected by western blot. **Results:** The number of annexin-V+CD45+ MVs and annexin-V-CD45+ were increased in the plasma of ALS patients, respectively, of 2- and 4-fold compared to healthy controls and AD patients (ANOVA test, $p < 0.05$). Different misfolded protein levels (SOD1, FUS, TDP43 and A β) were found in plasma-derived MVs of ALS patients compared to healthy controls and AD patients. **Summary and conclusion:** Leukocyte-derived MVs are overexpressed in ALS patients, and this could have a relevant role in the disease propagation of ALS. Moreover, they might be the "carriers" of misfolded proteins, main cause of disease progression.

PT6.04

Exosomes as a source of new biomarkers and candidate therapeutic targets in Parkinson's disease

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Parkinson's disease is characterized by multi-focal, progressive neurodegeneration that often begins in olfactory and vagal neurons, clinically presenting as a movement disorder only when substantia nigra neurons are severely compromised. Efforts to restore or replace neuronal populations post-presentation are likely to suffer from the existing and progressive load of misfolded and aggregated alpha-synuclein. We suggest that the development of disease altering medications must focus upon identification of early markers of disease spread between neurons and characterization of these molecular mechanisms as candidate therapeutic targets. We are investigating the potential of exosomes to mediate transfer of different alpha-synuclein variants between multiple cell-types and attempting to identify molecular markers of toxic vesicles as candidate therapeutic targets. We have isolated exosomes from various cellular models and biological fluids and characterized their physical properties by using electron and atomic force microscopy, analysed their chemical compositions by LC-MS proteomics and lipidomics, as well as performed cell-based assays for functional studies. Our results show that pathological conditions such as mitochondrial stress can affect both physical properties and chemical composition of exosomes. In particular, stress-induced exosomes appeared to be smaller and more homogeneous in size than those produced by the cells growing in normal conditions. Using targeted and untargeted LC-MS methods, we have identified a number of proteins and bioactive lipids that are significantly altered in stress-induced exosomes. Together these results indicate that altered surface and cargo molecules could change exosomal targeting, signalling or enzymatic properties and suggest that these models have a strong potential to identify candidate therapeutic targets to reduce the progressive spread of protein aggregation diseases.

PT6.05

Salivary vesicles expression in traumatic brain injury

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Background: Studies have been conducted to determine if biomarkers in body fluids such as cerebral spinal fluid and blood are predictive of degenerative brain disease in patients post-traumatic brain injury (TBI). Aberrant levels of neurofilaments, cleaved tau, microtubule-associated protein 2, myelin basic protein, neuron-specific enolase, S100B, glial fibrillary acidic protein, ubiquitin carboxyl terminal hydrolase L1 and alpha-II spectrin breakdown products can be detected post-TBI (Dash, 2010; Yokobori, 2013; Zetterberg, 2013). Increased levels of beta amyloid, tau and TDP-43 have been shown to be associated with Alzheimer's disease (AD) in patients post-TBI (Blennhow, 2010). In addition, increased levels of caspase-3, S100Bm, Gfap and TrkB have been found in the brains of patients that died due to TBI and other cerebral traumas (Staffa, 2012). We wished to determine if aberrant levels of similar genes could be found in salivary extracellular vesicles (EVs) of subjects after TBI. **Methods:** Saliva was collected from current athletes who either had a confirmed head impact or no recorded impact, and emergency room (ER) patients to isolate EVs. Athletes had samples collected prior to and after their game. ER patients that suffered head trauma had saliva samples collected post event. EVs were isolated via

differential centrifugation and analysed for mRNA content. **Results:** Salivary EVs derived from athletes were different in number and size and had different gene expression profiles (pre- vs. post-game). ER patients admitted due to head trauma had elevated salivary EV expression of genes associated with TBI and AD (vs. subjects with no known head trauma). One ER patient with a confirmed concussion had the highest expression of AD genes. **Conclusion:** Salivary EV profiling could be developed as a non-invasive test that might predict the development of degenerative brain disease post-TBI.

PT6.06

MicroRNAs in human cerebrospinal fluid as biomarkers for Alzheimer's disease

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Alzheimer's disease (AD) is the most common form of dementia; nearly 44 million people worldwide are affected today. There are currently no biomarkers to confirm the onset of AD, but a preclinical tool that can diagnose AD earlier in stage, allowing treatments to be initiated earlier in the disease, would be a great asset to patients, family and caregivers. We examined miRNA expression in 49 controls and 50 moderate AD patients. Cerebrospinal fluid samples from living donors were obtained from the Oregon Alzheimer's Disease Center. Total RNA was isolated using the mirVana PARIS kit, and RNA expression was quantified using TaqMan Human MicroRNA Arrays. Cycle threshold (Ct) values for each miRNA were determined using QuantStudio(tm) software (v1.2.2), using automated baseline and threshold values. Starting with a set of 151 miRNAs meeting quality and attestation criteria, we performed 5 phases of inferential analysis on the Ct values, normalized to U6 snRNA. The first 2 phases assessed per-target associations with AD using log-rank testing of Kaplan-Meier curves and predictions from logistic regression models while accounting for censoring of the Ct values. The next 2 phases used different random forest classifiers to gauge the predictive importance of miRNA targets while accounting for cross-miRNA interactions. Thirty-six miRNAs were flagged in more than one phase and carried forward for the final phase, where we used best-subsets logistic regression to rank multimarker classification performance. Top-performing 3- and 4-marker models have AUC of 0.79–0.83, increasing to 0.84–0.86 when combined with ApoE4 gene status, a current biomarker. We identified a panel of miRNAs that can discriminate AD patients from control subjects. Multiple miRNAs show better biomarker performance than ApoE4 alone, and combining ApoE4 and miRNA together provides a powerful AD biomarker tool. Funded by NIH NCATS TR000903 (JAS & JFQ), and NIA AG08017 (JFQ).

PT6.07

Serum exosomal RNA as a biomarker in multiple sclerosis

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Multiple sclerosis (MS) is an idiopathic chronic inflammatory demyelinating disease of the central nervous system, which leads to progressive motor and cognitive disability. There is currently no blood-based assay to detect the disease, to monitor its progression or to judge therapeutic response. We are studying small non-coding

RNAs derived from exosomes in patient serum to assess their potential as biomarkers in MS using unbiased deep sequencing assays and quantitative RT-PCR. We have collected serum from over 160 MS patients at different stages of disease (first demyelinating event, relapsing-remitting MS, secondary- and primary-progressive MS) with extensive clinical annotations. Our initial results based on a small discovery set have identified several microRNAs (miRNAs) dysregulated in disease compared to healthy age and gender-matched controls. Some of these miRNAs have been previously reported as dysregulated in whole blood or plasma analysis of MS patients; however, we have also identified novel dysregulated miRNAs that have predicted effects on endothelial cell maturation and/or TGF-beta signalling. We are currently completing additional discovery set investigations as well as exploring our initial findings in larger validation sets.

PT6.08

ApoE-containing particles excreted by astrocytes and neurons: Exosomes or lipoproteins?

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Introduction: The e4 allele of the APOE gene is the strongest genetic risk factor for the development of late-onset Alzheimer's disease. Apolipoprotein (Apo) E synthesized by the liver is best known for its role in cholesterol trafficking in circulatory lipoproteins. Notably, ApoE is also highly synthesized in the brain, predominantly by astrocytes and likely to be involved in the lipid trafficking. The brain produces most of its cholesterol endogenously and does not rely on cholesterol from the circulation. Astrocytes secrete ApoE-containing lipoprotein-like particles, which are thought to best resemble high-density lipoproteins (HDL). However, these ApoE-carrying particles also share features with exosomes, and ApoE has recently been detected on exosomes excreted in vitro by osteoblasts, platelets and pigment cells. Our aim is to better characterize the kind of ApoE-carrying particles that are secreted by astrocytes. **Materials and methods:** Particles derived from the human astrocytoma cell line CCF-STTG1 are isolated from conditioned media by ultracentrifugation and immunoprecipitation using magnetic beads. The isolated particles are characterized using cryo-electron microscopy, mass spectrometry, western blotting, size distribution, mRNA and proteomics. **Results:** Western blot analysis showed the presence of both CD9 and ApoE in particle preparations isolated from CCF-STTG1 conditioned media. Furthermore, we found no expression of ApoAI, an essential protein of HDL. Results of other analyses are pending. **Conclusions:** Our preliminary results suggest that the ApoE-containing particles secreted by astrocytes may represent exosomes rather than HDL-like lipoproteins. This could potentially lead to the identification of novel mechanisms by which ApoE4 contributes to the development of Alzheimer's disease.

PT6.09

Proteomics characterization and functional assays of leech microglia extracellular vesicles

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The medicinal leech (*Hirudo medicinalis*) is a well-known model in neurobiology due to its ability to naturally repair the central nervous

system (CNS) after injury. The present study is focused on the involvement of resident microglial cells in this repair mechanism. In particular, we investigated the crosstalk between microglial cells and damaged neurons mediated by extracellular vesicles (EVs) inducing neuroprotection. In mammals, microglial cells are considered as the resident immune cells in CNS. Many studies demonstrated that, after injury, these cells were activated and recruited at the site of lesion. Leech microglia present a similar pattern of activation and migration upon experimental lesion of CNS. In addition, we observed that this activation is linked to the release of a large amount of EVs. In order to identify in the leech the functional properties of microglial cells leading to a nerve repair, EVs released by naïve or ATP-stimulated microglia were collected. Then, their protein content was analysed using mass spectrometry approaches. Results showed the presence of specific proteins in differentially stimulated samples. Interestingly, an important amount of proteins found in leech microglia EVs were previously described in mammals. On the other hand, EVs issued from ATP-stimulated microglial cells were assessed for their neuritic outgrowth potential on leech neurons and mammal cell lines. The measurement showed an increase in neurite size compared to the controls. These results suggest a conservation of pathways involved in neuroprotection. The present study will permit to determine the molecular signature of microglial EVs exerting a neurotrophic effects and would lead to target-specific proteins promoting CNS repair.

PT6.11

Microvesicles derived from specific cells of the neurovascular unit associate with white matter hyperintensities in brains of middle aged women with and without history of preeclampsia

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Background: A history of preeclampsia (PE) has been associated with cerebral white matter lesions, which may be associated with small vessel ischemic disease. Our objective was to quantify circulating microvesicles (MVs) derived from cells of neurovascular unit and to determine their association with small vessel ischemic disease markers, white matter hyperintensities (WMH) in middle-age women with and without a history of PE. **Methods:** This study was approved by the Institutional Review Board at Mayo Clinic. Age-matched women (median, 60 years) free of cerebrovascular events: n = 40 with and n = 40 without a history of PE participated in this study. The MV positive for low-density lipoprotein receptor-related receptor (blood-brain barrier endothelium), glial fibrillar acidic protein (astrocytes), Iba1 (ionized calcium adaptor molecule 1, microglia), Tuj-1 (neuron specific class III beta-tubulin) and Tau or amyloid β 1-42 (Alzheimer's disease) were quantified by digital flow cytometer. Volume of WMH was quantified by FLAIR MRI of the brain. MV variables were analysed for an association with WMH volume on the combined group of women (n = 80), after adjustment for history of PE, BMI and HOMA-IR in multivariable linear regression. **Results:** Numbers of MVs derived from the cells of neurovascular unit were not statistically different between women with and without a PE history. Multivariable linear regression analysis showed a negative correlation between astrocyte-derived MVs and WMH volume (p = 0.025). **Conclusions:** MVs derived from cells of neurovascular unit can be detected in peripheral blood of asymptomatic women. Astrocyte-derived MVs were found to be associated with WMH suggesting that reactive astrocytes may contribute in the pathophysiology of WMH. Further validation of these results in different cohorts may identify novel cellular processes that are associated with WMH, cerebrovascular disease and cognitive impairment.

PT6.13

Extracellular vesicles as mediators of periphery to brain communication: relevance for stress-induced behavioural disorders
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Introduction: Understanding the aetio-pathophysiology of depression is the route to identification of efficacious therapies. A major theory is that stress-induced inflammation is aetiological in depression and that one of its effects is to alter dopamine signalling leading to depressive symptoms. Extracellular vesicles (EVs) comprise a group of secreted vesicles that are able to transfer biomolecules between cells and organs. EVs derived from hematopoietic cells deliver miRNAs to CNS cells during inflammatory conditions. The objective of this study is to (a) investigate the effect of psychosocial stress on the peripheral immune system and on dopamine (DA) neurons and (b) assess the role of EVs in the communication between activated

peripheral immune cells and DA neurons. *Methods:* Mice are exposed to chronic social defeat (CSD) stress and the effects of CSD on immune cells are studied using flow cytometry. To investigate the effect of CSD on EVs, plasma EVs are isolated and miRNA content is analysed. Using Vav1-iCre x Rosa26-GFP mice, neurons receiving EVs cargo from (Vav1+) hematopoietic cells are identified by Cre-mediated GFP expression, and their gene expression pattern is examined. *Results:* Mice exposed to CSD exhibit peripheral inflammation: increased splenic granulocytes, inflammatory monocytes and T helper 17 cells. The immune response co-occurs with attenuation of dopamine signalling. Future experiments will investigate (1) CSD effects on miRNA content of EVs secreted by peripheral immune cells, (2) CSD effects on EVs shuttling to the brain and cargo delivery to brain cells and (3) influence of EVs cargo on target dopamine neurons function. *Conclusion:* Analysis of EV-associated miRNA that are transferred to neurons and modulate their function will be important for the identification of pathophysiological pathways for stress-induced behavioural disorders and will serve as the basis for the discovery of peripheral biomarkers and the development of targeted therapies.

Poster Session 7 - EV characterization from different sources or different subtypes

Chairs: *Masood Kamali-Moghaddam and Takahori Ichiki* 5:00-8:00 p.m.

PT7.01

Extracellular vesicles from oral squamous cell carcinoma cells in culture: isolation and characterization

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Oral squamous cell carcinoma (OSCC) incidence is increasing and is the most common oral malignant tumour. Tumour-derived extracellular vesicles (EVs) have been described to be involved in tumour development and progression but their role in the processes is unclear. Traditionally, EVs isolation has been achieved by ultracentrifugation. This requires specialized equipment, is time consuming and may cause vesicle damage. Here we aimed to establish a fast and effective protocol for isolation of EVs from OSCC cells by ultrafiltration followed by size exclusion chromatography (SEC). PE/CA-PJ49 (OSCC) cells were cultured in advanced DMEM (Gibco) with L-glutamine, PSA and exosome-depleted FBS (1% V/V, Thermo Fisher Scientific) until 80 and 100% confluence. Conditioned culture media were concentrated by ultrafiltration (Amicon Ultra Centrifugal Filters, Ultracel 50K and 100K – 50 and 100 KDa cut-off, respectively) followed by SEC (Sephacel CL-2B, GE Healthcare). Fractions were quantified for protein amount by spectrophotometry (A280 nm). Particle number determined by nanoparticle tracking analysis (NTA) using NanoSight NS500, followed by detection of magnetic bead-bound CD9 positive EVs by flow cytometry (median fluorescence intensity (MFI), BD Accuri C6). Preliminary results from NTA showed no variation from cell culture at 80% confluence between both ultrafiltration devices. Still, a slight decrease (approx. 15%) was observed in 100% confluence culture run in Ultracel 100K compared to 50K. Flow detection of CD9 positive EVs revealed > 10% reduction in MFI for both culture conditions when concentrated with Ultracel 100K. Our findings entail a successful isolation of EVs from cultured OSCC cells using both Ultracel (100K and 50K) devices. However, our data imply that loss of EVs may occur when supernatants are concentrated with Ultracel 100K. More details on the impact of the different ultrafiltration devices on EVs are to be elucidated by western blot and electron microscopy.

PT7.02

Role of extracellular vesicles in epithelial–endothelial communication during thyroid development

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Intercellular communications are important for organ development and homeostasis, as well as for pathological and repair processes. Our lab is interested in epithelial–endothelial communications during thyroid gland development. We have shown that epithelial production of VEGFa first allows endothelial cell recruitment and invasion around and in the developing thyroid lobes. In turn, endothelial cells instruct epithelial cells to organize in polarized spheres, thereby forming angio-follicular unit. Indeed, Vegfa cKO mice display reduced endothelial cell density and impaired follicle formation. Using an ex vivo culture system of thyroid lobes, we demonstrated that medium conditioned (CM) by endothelial progenitor cells (EPC) promote follicle formation and that partial activity was present in the high-speed pellet (H-SP) isolated from this CM. We postulated that the H-SP contains extracellular vesicles

(EVs). Using classical differential centrifugation protocol, we characterized the H-SP by western blotting, floatation density gradient and electron microscopy. The H-SP was positive for CD63 and Flot-1, but negative for cellular marker calnexin, had a density of 1.09 g/mL and an average particle size of 100 nm. Altogether, these data indicate that H-SP contains EVs. We then confirmed that EVs from EPCs can stimulate follicle formation in thyroid lobes. To identify potential folliculogenic factors carried by EVs, we resorted to mass spectrometry. Candidate molecules potentially involved in this process are currently under study. In parallel, we developed a protocol to isolate EVs from embryonic thyroid lobes and demonstrated that an H-SP isolated from embryonic thyroid tissue was positive for CD63 and Flot-1, while it was negative for calnexin. Our future aims will be to test candidate folliculogenic factors, identify which cell type of the explants is targeted by the EVs produced by EPCs and continue the characterization of the H-SP isolated from embryonic thyroid.

PT7.03

Concomitant evaluation of a panel of exosome proteins and miRs for qualification of cultured human corneal endothelial cells

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Purpose: Cultured human corneal endothelial cells (hCECs) serve as an alternative to donor corneas for the medication of corneal endothelial dysfunction. However, predisposition of hCECs into a senescence phenotype, epithelial-mesenchymal transition has been hampering the practice in clinical settings. The aim of this study was to find the way to utilize secreted miRNA (miR) profiles to qualify hCECs adaptable for cell injection therapy. *Methods:* The variations of hCECs in their composites of heterogeneous subpopulations (SPs) were certified in the context of their surface CD markers. The integrated analysis of miRNA profiles in culture supernatants were investigated by 3D-gene (Toray). To validate 3D gene results, qRT-PCR was carried out for a vast number of cultures, distinct in their morphology and the composites of SPs. The exosomes and miRNAs in culture supernatants (CS) were also analysed. *Results:* Secreted miRNA profiles among morphologically diverse hCEC SPs proved to be useful for their distinction each other. The candidate miRs to discriminate CD44-SPs from those with CD44 + + + phenotypes were miRs 221-3p, 1246, 1915-3p and 4732-5p. The levels of the latter 3 miRs decreased dramatically in CS of hCECs without CST, whereas those from hCECs with senescence-like CST showed the increase. Of note, miR184 only decreased inversely in parallel with the upregulation of CD44 on hCECs. CD9+ exosomes were far elevated in CS of hCECs with senescence-like CST than those without CST, indicating the possible import of these extracellular vesicles (EVs) into hCECs without CST. *Conclusions:* The hCECs sharing a CD44-phenotype may be discriminated by measuring the amount of miRs or exosome in CS. Thus, miRs in CS may serve as the tool to qualify cultured hCECs. The future extended analysis of cell-to-cell communication through these EVs might open the new aspect for the better understanding of cell state transition of cultured cells.

PT7.04

Visualization and characterization of extracellular vesicles derived from human bone marrow mesenchymal stem cells using different staining analysis

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Introduction: Mesenchymal stem/stromal cells (MSCs) are of great interest in regenerative therapies. The predominant mechanism by which MSCs participate in tissue repair is mainly related to their paracrine activity. In parallel to soluble factors, MSCs release microvesicles that contribute to the reparative processes. The aim of the study was to optimize the method of staining for EVs derived from MSCs in term of their visualization after transplantation. **Methods:** The experiments were performed on human bone marrow mesenchymal stem cells (hBM-MSCs) (Lonza). hBM-MSCs were labelled with two different dyes: lipophilic stain PKH26 and iron nanoparticles conjugated with rhodamine (Molday) and co-stained with anti-CD9, anti-CD63 and anti-CD81 (tetraspanins) antibodies. Then, EVs were isolated from the culture media of previously labelled hBM-MSCs by centrifugation at 1800 x g for 30 min at 4°C followed by two times ultracentrifugation at 100,000 x g for 60 min at 4°C. To observe the transfer of EVs to hBM-MSCs in vitro, isolated EVs were co-cultured with hBM-MSC during 24 or 48 h. The results were analysed by confocal microscopy and transmission electron microscopy. **Results:** Our results depicted the presence of intracellular structures positively stained with PKH26 or iron nanoparticles visible inside hBM-MSCs, which co-expressed CD9, CD63 and CD81 markers specific for EVs. The isolated EVs represent heterogeneous population differed with size and content as confirmed by electron microscopy analysis. Co-culture of EVs with hBM-MSCs revealed their uptake by cells during short time of observation. **Conclusions:** PKH26 and Molday proved to be efficient stains, biocompatible with EVs and the labelling did not interfere with the capability of EVs to re-enter hBM-MSCs during in vitro co-culture studies.

This work was supported by KNOW 06 project: "The role of bone marrow mesenchymal stem cells and microvesicles derived from these cells in CNS repair of brain ischemia disorders."

PT7.05

Analysis of extracellular vesicles obtained from clear cell renal cell carcinoma tissue

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Introduction: In the last decade, extracellular vesicles (EVs) have emerged as a novel mediator of tumorigenesis. Many reports suggest that EVs are inseparable part of cancer microenvironment from the experiments using cell lines and body fluids. However, these observations have not yet been experimentally proven in vivo. **Methods:** We attempted to obtain EVs directly from fresh clear cell renal cell carcinoma (ccRCC) clinical specimens by an ultracentrifuge method. Obtained tissue-derived EVs (TEVs) were analysed by western blot, transmission electron microscopy (TEM) and LC/MS/MS analysis. Furthermore, obtained TEVs were used for the functional analysis on HUVEC cells. **Results:** EVs obtained from ccRCC clinical specimens expressed CD9, CD63 and CD81. To identify the ccRCC-TEVs-specific cargo, we focused on protein signatures by LC/MS/MS analysis. The expression signature revealed that several proteins were enriched in ccRCC-TEVs but not in EVs obtained from adjacent normal kidney tissue (Normal-TEVs). Moreover, ccRCC-TEVs-specific protein "protein X" was only detectable in the serum of ccRCC patients and not in the serum of healthy control. Functional analysis revealed that angiogenesis and endothelial permeability were upregulated by ccRCC-TEVs treatment but not by the Normal-TEVs. **Summary/conclusion:** In this study, we successfully obtained EVs directly from the clinical specimens and found that those TEVs have biological activities against endothelial cells. Moreover, those biological functions were different

between Normal- and ccRCC-TEVs. Taken together, TEVs might be a powerful tool for researching the in vivo EVs.

PT7.06

Characterization of retinal progenitor cell microvesicle content

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Microvesicles (MVs) are membrane-covered cell fragments released from the plasma membrane of a range of cell types including central nervous system microglia, astrocytes and neurons. MVs engulf cytoplasm through membrane blebbing leading to encapsulation of unique combinations of microRNA, mRNA and proteins similar to those present in the cells from which they originate. MVs carry functional molecules, transfer genetic information between cells and mediate physiologic and pathologic processes. Here we describe MV release, content and transfer from mouse retinal progenitor cells (mRPCs). MV release rate and ultrastructure were described using SEM and NanoSight analysis. Under standard culture conditions, mRPCs were shown to release ~4800 MVs per cell/48 h, with an average size of ~100 nm. Using immunogold TEM and immunohistochemical techniques, we established that mRPC-derived MV structure incorporated the tetraspanin protein, CD63. Following genetic content analysis, mRPC-derived MVs were shown to contain mRNA, miRNA and proteins that reflect the cell's expression state. MVs transferred GFP mRNA to non-GFP recipient cells in a transwell assay. These findings demonstrate that mRPC-derived MVs contain genetic material with reflective of the expression states of the cells of origin. Our established release rates and the MV-mediated transfer of GFP to recipient cells suggest that MV-mediated genetic transfer may play a role in communication between mRPCs in vitro.

PT7.07

Differential characterization of microvesicles and exosomes from activated and apoptotic human lymphocytes

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Introduction: Extracellular vesicles (EVs), microvesicles (MVs) and exosomes (Exos) are released from activated and apoptotic lymphocytes and may have an influence in the pathogenesis of autoimmune diseases. MVs are shed from the plasma membrane, while Exos are released from an intracellular compartment, the multivesicular bodies. Since MVs and Exos are released either after cellular activation or apoptosis induction, we wanted to systematically characterize EVs released after different stimuli. Further, we have been interested in the interactions between EVs and dendritic cells (DC). **Methods:** MVs and Exos from activated and apoptotic human lymphocytes were isolated by filtration and ultracentrifugation (MV: 10,000g; Exos: 100,000g). Isolated vesicles were analysed by transmission electron microscopy (TEM) and NanoSight and were used to stimulate monocyte-derived DC. EV protein-profile was analysed by western blot analysis and 2D gel electrophoresis. **Results:** Western blot analysis presented a distinct protein expression pattern in activated and apoptotic EV. We found proteins of the T-cell receptor signalling complex (LAT, LCK) exclusively in MVs. Actin, a cytoskeletal protein, and ERK1 were concentrated especially in MVs released from apoptotic cells. From around 8000 2D gel spots, we picked 60 and analysed them by mass spectrometry. Some proteins (n = 30) had a distinct regulation between activation and apoptosis (e.g. LAP3; DDB1), whereas others were differentially regulated in MVs when compared to Exos (e.g. YWHAQ;

HSPD1). Finally, DC-stimulated MV/Exos had higher expression of CD83, CD80, CD86 and CD274, compared to untreated DC, while MHCII was significantly less expressed on MV-treated DC. **Conclusions:** We observed a distinct protein expression in MVs and Exos with a differential regulation, depending on distinct stimuli, implicating possibly new markers for vesicle identification. Moreover, we show that MVs and Exos cause a distinct regulation of DC maturation.

PT7.08

Characterization of mycobacterial membrane vesicles

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Please see OPW2.8

PT7.09

Diversity of extracellular vesicles addressed by immunoaffinity beads

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Introduction: Extracellular vesicles (EVs) are loaded with specific sets of proteins, lipids and nucleic acids, depending on the originating cell. EVs can be distinguished by surface marker profiling and markers can be used to separate EV populations. **Methods:** Defined blood cell populations were separated and cultivated. EVs were isolated by serial centrifugation, filtration and ultracentrifugation. A multiplex bead assay was used to analyse EV markers: EVs were incubated with a mix of 39 distinct labelled antibody beads. Beads were discriminated by flow cytometry and bound EVs were detected by labelled antibodies. For immunomagnetic isolation, EVs were incubated with microbeads, separated on a magnetic column, washed and eluted. Eluted EVs were analysed by western blot or the multiplex assay. **Results:** We investigated surface proteins of EVs from NK cells, platelets and activated B cells. The multiplex analysis showed that CD9 was low on NK cell EVs and platelet EVs hardly carry CD81. Using a mixture of NK cell/platelet EVs, we proved that EV populations can be separated by immunomagnetic microbeads. EVs of activated B cells comprised also EVs due to contaminating platelets in the B cell culture. To purify the EVs, we depleted platelet EVs using anti-CD61 microbeads. Low signals for platelet markers (CD41b, CD42a, CD62P) in the flow through fraction confirmed the depletion of platelet EVs. As the CD9 signal also disappeared and was recovered in the eluted CD61+ fraction, we conclude that B-cell EVs are CD9-. Positive signals on CD80 and CD86 beads indicate the transfer of activation markers from B cells to EVs. A subpopulation of CD80 and CD86 EVs likely derive from cells before activation. In addition, we found a subpopulation of B-cell EVs that carry high levels of CD20. **Summary:** We demonstrate that EVs differ in surface protein composition. Marker profiles can reveal subpopulations. Cells secreting diverse EVs might address different target cells or instruct distinct EV tasks.

PT7.10

Fractionation of discrete MDA-MB-231-derived extracellular vesicle sub-populations reveals distinct protein and RNA profiles

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Cells release an array of extracellular vesicles (EVs) consisting of a lipid bilayer and transmembrane proteins enclosing soluble cellular content and encompassing a broad size range (~30 to 1000 nm). One of the most common methods to isolate EVs is by differential velocity

ultracentrifugation culminating with a 100,000 × g spin. Unfortunately, downstream analyses from vesicles isolated by this method suffer from the co-isolation of multiple EV subpopulations and protein aggregates. To resolve this issue, we developed a fractionation method combining differential velocity centrifugation and buoyant density flotation into a 5–25% linear iodixanol gradient. Using this method, we separated at least 2 different EV subpopulations in HEK 293T cells and resolved vesicles from protein aggregates. The linear gradient showed differential distribution of soluble (HSC70, Alix, TSG101) and membrane protein (CD63, CD9, MFG8 and flotillin-2) EV markers. Immun isolation experiments showed different co-purification patterns for EV markers, confirming our previous observations. We next probed the RNA content of the distinct vesicle populations. The RNA amount across the gradient varied by an order of ten. The highest RNA levels were found in the very dense fractions, likely associated with ribonucleoprotein complexes rather than vesicles. The EV fractions were enriched in small RNA species, while the higher density fractions displayed longer RNAs. Interestingly, a miRNA (miR-223) that is highly enriched in exosomes from HEK 293T cells was clearly associated with the EV fraction and depleted from the other fractions. We used the same approach to isolate EV-subpopulations from MDA-MB-231 cells and are evaluating differences in their RNA content through RNA seq. In conclusion, purification of EVs from debris and other vesicle subpopulations can dramatically influence the downstream protein and RNA analysis and is essential for a detailed examination of EV cargo and function.

PT7.11

MSC secretes at least three EV types each with a unique permutation of membrane lipid, protein and RNA

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Mesenchymal stem cell (MSC), a widely used adult stem cell candidate for regenerative medicine, has been shown to exert some of its therapeutic effects through the secretion of extracellular vesicles (EVs). These homogeneously sized EVs of 100–150 nm exhibited many exosome-like biophysical and biochemical properties and carry both proteins and RNAs. Recently, exosome-associated proteins in this MSC EV preparation were found to segregate primarily to those EVs that bind cholera toxin B chain (CTB), a GM1 ganglioside-specific ligand, and pulse-chase experiments demonstrated that these EVs have endosomal origin and carried many of the exosome-associated markers. Here we report that only a fraction of the MSC EV proteome was found in CTB-bound EVs. Using annexin V (AV) and Shiga toxin B subunit (ST) with affinities for phosphatidylserine and globotriaosylceramide, respectively, an AV- and an ST-binding EV were identified. CTB-, AV- and ST-binding EVs all carried actin. However, the AV-binding EVs carry low or no detectable levels of the exosome-associated proteins. Only the ST-binding EVs carry RNA and EDA-containing fibronectin. Proteins in AV-binding EVs are also different from those released by apoptotic MSCs. CTB- and AV-binding activities are localized to the plasma membrane and cytoplasm of MSCs, while ST-binding activity is localized to the nucleus. Together, this study demonstrates that cells secrete many types of EVs, specifically MSCs secrete at least 3 types. They can be differentially isolated based on their affinities for membrane lipid-binding ligands. As the subcellular sites of the binding activities of these ligands and cargo load were different for each EV type, they are likely to have a different biogenesis pathway and possibly different functions.

PT7.12

Presence of DNA in subsets of exosome-like extracellular vesicles

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Introduction: The RNA cargo of extracellular vesicles (EVs) is relatively well investigated, but less is known about the presence of DNA in EVs. Currently, it has been confirmed that EVs carry mitochondrial DNA, single-stranded and double-stranded DNA, as well as oncogene amplifications within their cargo. The DNA content associated with EVs has a great potential to be used as a biomarker for several diseases, but needs to be better characterized among vesicle subsets. The aim of this study was to determine the DNA content of different exosome-like EV subsets to achieve a better understanding of their nucleic acid content. **Methods:** EVs were isolated from human HMC1 mast cells and erythroleukaemic TF1 cells by differential centrifugation at 120,000 x g for 2.5 h, after removal of debris and larger vesicles. The vesicles were further separated by iodixanol density gradient, resulting in two main subsets: low-density EVs and high-density EVs. Both subpopulations were characterized with western blot and particle concentration with ZetaView analyser. Isolation of DNA and RNA was performed using QIAamp DNA and miRCURY RNA kits, respectively, and the nucleic acid profiles were analysed with Bioanalyzer. Furthermore, the association of the DNA in or outside the EVs was evaluated using DNase I treatment comparing DNA concentrations and profiles of treated/untreated vesicles. **Results:** Low-density EVs contained low quantities of DNA and exhibited RNA profiles with 18S and 28S ribosomal RNA peaks. In comparison, high-density EVs contained greater quantities of DNA, and the RNA profiles consisted mainly in small RNAs without ribosomal RNA peaks. Most of the DNA protected from DNase degradation, suggesting it was intravesicular. **Summary/conclusion:** The DNA content varies in different subsets of exosome-like EVs. Further characterization of the EV-DNA cargo can help to clarify its physiological significance, as well as its possible utilization in biomarker discovery studies.

PT7.13

Transcriptome of different types of human blood circulating complexes

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RNAs of extracellular circulating complexes – exosomes, microvesicles and ribonucleoproteins – are an important component, which mediates functions of circulating complexes after their internalization by recipient cells. Each type of circulating complexes is seemed to be enriched with the specific array of RNA species different from the RNA content of donor cells. In order to characterize transcriptome of human blood plasma microvesicles, exosomes and extracellular ribonucleoproteins, we processed blood of healthy donors, patients with squamous cell lung cancer or lung adenocarcinoma by sequential centrifugation steps at 1200g, 16,000g and 160,000g and used each pellet and final supernatant for RNA extraction and sequencing with high-throughput SOLiD 5500xl platform. On average, 14 millions of reads per cDNA library were obtained. High-throughput RNA sequencing data were analysed using Bowtie/Cufflinks pipeline via step-by-step mapping to mitochondrial transcripts, transcribed human repeats, human RefSeq RNAs and human genome reference sequences. Differential expression analysis showed that main distinctive feature of 160,000g pellet transcriptome in comparison with other examined blood plasma subfractions and blood cells is an enrichment with fragments of mRNAs (such as KRTAP10-5, TSHB, IK, PMP22, HOXB8, OR9Q2, UCHL3) and non-coding RNAs (RNU11, set of microRNAs). The RNA content of blood plasma 16,000g pellets was enriched with microRNAs, snoRNAs and vault RNA. We suppose that explored differences related to biogenesis of human blood extracellular circulating complexes, and thus, this study will promote to a better understanding of their underlying biological functions.

The work was supported by the RFBR 16-04-01457.

PT7.14

Comparative transcriptomic analysis of human and *Drosophila* extracellular vesicles reveals extensive conservation

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Extracellular vesicles (EVs) are membrane-enclosed nanoparticles containing specific repertoires of genetic material. In mammals, EVs can mediate the horizontal transfer of various cargos and signalling molecules, notably miRNA and mRNA species. Whether this form of intercellular communication prevails in other metazoans remains unclear. Here, we report the first parallel comparative morphologic and transcriptomic characterization of EVs from *Drosophila* and human cellular models. Electronic microscopy revealed that human and *Drosophila* cells release similar exosome-like EVs with diameters ranging from 30 to 200 nm, which contain complex populations of transcripts. RNA-seq identified abundant ribosomal RNA pseudogenes and retrotransposons in human and *Drosophila* EVs. Vault RNAs and Y RNAs abounded in human samples, whereas small nucleolar RNAs involved in pseudouridylation were most prevalent in *Drosophila* EVs. Numerous mRNAs were identified, largely consisting of exonic sequences displaying full-length read coverage and enriched for translation and electronic transport chain functions. By analogy with human systems, these extensive similarities suggest that EVs could potentially enable RNA-mediated intercellular communication in *Drosophila*.

PT7.15

Membrane proteomics of extracellular vesicles

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Introduction: Plasma membrane proteins play diverse roles in intercellular communication by accepting or releasing signalling. Extracellular vesicles (EVs) are intercellular communicators, which contain multiple plasma membrane proteins and bioactive surface molecules. Identification of those highly bioactive surface molecules is important to understand the role of EVs in intercellular communication. However, it is difficult to identify these molecules by standard LC-MS/MS, because of their low abundance. Here, we present a method for identifying low abundant membrane proteins on EVs. **Methods:** EVs were isolated from the human mast cell line HMC-1 by differential centrifugation and density gradient. Then, EVs were treated with high pH solution to open the membrane and to eliminate cytosolic proteins. Non-treated and high pH treated EVs were subjected to LC-MS/MS. Relative protein abundance was obtained by label free quantification. **Results:** In total, 1658 and 1492 proteins were identified from non-treated and high pH treated EVs, respectively. The relative abundance of cytosolic proteins was greatly reduced after high pH treatment. By contrast, low abundant receptors (e.g. VEGFR1 and IL9R) and growth factors were identified after high pH treatment. **Conclusions:** Our results show that high pH treatment increases the identification of bioactive, but low abundant, surface molecules by LC-MS/MS. This study can contribute to EV research fields by providing a new method for identifying surface molecules of EVs.

Poster Session 8 – Late Breaking Poster Session 1

Chairs: Margot Zöller and Malene M. Jorgensen

5:00–8:00 p.m.

PT8.01**Surface markers profiling of prostate cancer exosomes**

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Introduction: Prostate cancer is one of the most common types of cancer in men. The markers capable to better distinguish cancerous from benign conditions and slow-growing cancers from aggressive ones are required. This study is aimed to analysis of expression of CD9, CD81, CD147, CD117 CD29 and EGF markers on the exosomes isolated from the urine of patients with histologically confirmed prostate cancer and age matched patients without cancer. **Materials & Methods:** Urine samples from men with or without PC were collected after digital rectal exam (DRE). Urine exosomes were isolated either by differential centrifugation or using Exosome Precipitation solution (Machery-Nagel). The purity of exosome population was analysed using TEM. Pre-purified exosomes (5 ml of urine per sample) were incubated with CD9 exosomes dynabeads for 36 hrs at 4 C followed by antibody staining and flow analysis. **Results & Discussion:** In the test experiments exosomes from four urine samples were purified by differential centrifugation and with Exosome Precipitation solution. In our hands the highest CD81 fluorescence signal was observed for exosomes purified by differential centrifugation. This method was further used for exosomes purification. Our Initial data showed essential difference between the patients in expression of CD9/CD81 markers and markers associated with PC progression. A further larger-scale study is required to confirm our observations. This work was supported by Ministry of Education and Science of the Russian Federation (grant No. 14.607.21.0068, Sep 23, 2014).

PT8.02**Detection and characterization of extracellular vesicles in pancreatic juice and blood of patients with pancreatic cancer**

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Introduction: In recent past, the prognosis of patients with pancreatic adenocarcinoma has remained virtually unchanged with a 95% mortality rate over 5 years. An earlier detection would provide a time window of opportunity for treatment and prevention of deaths. In recent past, many reports have been published on the potential of extracellular vesicles (EVs) to carry biomarkers for different diseases including pancreatic cancer. In the present study we attempted to extend the list of EV biomarkers for pancreatic cancer by directly assessing EVs secreted into the pancreatic juice of patients. **Methods:** We obtained pancreatic juice by intraoperative puncture and blood from 36 patients, 13 with pancreatic head tumor, 12 with Vater ampulla tumor, and 11 with chronic pancreatitis. These EVs were

characterized by flow cytometry using fluorochrome conjugated anti-CD9, -CD63, as well as Annexin V followed by lysis with Triton X-100. The EV concentration and size distribution was measured using tunable resistive pulse sensing (qNano, IZON Science). Morphology of EVs was assessed by transmission electron microscopy. Protein composition of EVs was analyzed by mass spectrometry (MS). **Results:** Here we show for the first time the presence of EVs directly in human pancreatic juice samples. These EVs could be isolated for MS characterization of their cargo and were surprisingly stable despite their presence within a lipase and protease rich body fluid. The mean EV size in pancreatic juice was 149.93 ± 35.37 nm (n=18) and did not differ between disease groups. Transmission electron microscopy showed typical EV morphology. Importantly, we found that certain pancreatic juice and blood EV proteins distinguished patients with pancreatic carcinoma from those with chronic pancreas inflammation. **Conclusions:** These data suggest that detection of EVs directly in pancreatic juice may provide new hints regarding pancreatic cancer biomarkers.

PT8.03**Breast cancer cell-derived exosome secretion is regulated by intracellular calcium concentration**

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Introduction: In exosome biogenesis, many factors are related to exosome secretion pathway such as ESCRT, ceramide, Rab-GTPase proteins, calcium and so on. It has been reported that intracellular calcium concentration is the one of the important factors to regulate exosome secretion in K562 cell. Here, we show that breast cancer cell-derived exosomes are regulated by intracellular calcium concentration. **Methods:** To understand the effect of intracellular calcium on exosome secretion in breast cancer cell lines, we used chemical A that regulates intracellular calcium concentration. After treatment of chemical A to breast cancer cell lines, secreted exosomes were collected and concentration of secreted exosomes were measured by NTA system. To confirm the effect of chemical A on exosome secretion, we measured the intracellular calcium concentration and performed RT-PCR and western blot to check the Rab11 RNA and protein expression. **Results:** Our results indicate that breast cancer cell-derived exosome secretion is inhibited by chemical A. The effect of chemical A on exosome release is caused via a decrease of intracellular calcium concentrations. Rab11 protein and RNA expression levels are decreased by treatment of chemical A to breast cancer cell lines in calcium-dependent manner. **Summary/Conclusion:** Taken together, exosome secretion is regulated by intracellular calcium concentration in breast cancer cell lines. Decrease of intracellular calcium concentrations influence exosome secretion. Chemical A might act as exosome secretion inhibitor by regulating intracellular calcium concentration and Rab11 expression.

PT8.04**Tumor-education of platelets by pre-mRNA splicing and alternative RNA processing enables blood-based detection of cancer**

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Introduction: Tumor-educated platelets (TEPs) are a new blood-based biosource for the detection of cancer. TEPs respond to local and systemic stimuli during tumor progression by altering their RNA profile. Platelets are anucleated, but contain an RNA processing and translation machinery and a functional spliceosome. **Methods:** Blood platelet samples of 283 patients with cancer covering six tumor types (60 non-small cell lung cancer, 41 colorectal cancer, 39 glioblastoma, 35 pancreatic cancer, 14 hepatobiliary cancer, and 39 breast cancer) and of 55 healthy donors were isolated from whole blood by differential centrifugation. Total platelet RNA was isolated, subjected to SMARTer mRNA amplification and subjected to mRNA sequencing on the HiSeq 2500 platform. RNA-seq data was mapped to the human reference genome, and subjected to MISO, Samtools- and R-based bioinformatic algorithms. **Results:** The 283 platelet samples were successfully sequenced (Best et al. Cancer Cell 2015), and the RNA-seq read coverage analysis demonstrated an increased abundance of spliced RNAs in patients with cancer (n=228) compared to healthy controls (n=55). Deduction of RNA isoforms enabled generation of isoform-based TEP signatures for cancer classification. Finally, several alternative RNA processing events, such as exon skipping and intron retention, were identified in TEP RNA. **Conclusion:** Molecular interrogation of TEP-based liquid biopsies allows for cancer detection. The TEP RNA signatures are characterized by alternative RNA splicing events, indicating complex regulation of the RNAs in TEPs. Additional biological insight into the altered RNA signatures is necessary for understanding the mechanism of platelet-education and further optimization of TEP-based diagnostics.

PT8.05

Metabolomic profiling of breast cancer-derived extracellular vesicles

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Introduction: Cancer cells secrete a lot of extracellular vesicles (EVs), and these cancer cell-derived EVs are known to mediate cell-cell communication in order to modulate cancer microenvironment. EVs contain various molecular constituents such as proteins, mRNAs, and micro RNA (miRNAs) to act on around cell. On the one hand, the metabolisms of cancer cells are different from normal cells, and these the cancer metabolites were reported of suppress immune response. However, the existence of metabolites in EVs and their functions remain largely unknown. Therefore, this study aimed to perform a metabolomic analysis of breast cancer cells and breast cancer cell-derived EVs. **Methods:** Metabolomic analysis of cell and EVs were performed on two breast cancer cell lines, MDA-MB-231-D3H1 (D3H1) and MDA-MB-231-D3H2LN (D3H2LN). D3H1 is low-metastatic cell line and D3H2LN is high-metastatic cell line. EVs were purified from cell supernatant by ultracentrifugation. EV samples were then washed with 5% mannitol for metabolomics analysis. Next, ethanol solution or methanol containing internal standard was added to the sample. The metabolomic analysis was performed by LC-TOFMS and CE-TOFMS. **Results:** Based on the analysis by LC-TOFMS, we found that cells contain the 149 metabolites (Positive ionization mode (Pos): 117, Negative ionization mode (Neg): 32) and EVs contain the 99 metabolites (Pos: 76, Neg: 23). The 46 metabolites (Pos: 39, Neg: 7) were detected to be a higher amount in D3H2LN cell-derived EVs than D3H1 cell-derived EVs, and the 15 metabolites (Pos: 12, Neg: 3) were significantly a higher amount in D3H2LN cells. **Summary/Conclusion:** The breast cancer cell-derived EVs contain a lot of metabolites, and some metabolites are characteristic in D3H2LN cell-derived EVs which compared with D3H1-derived EVs.

Disclosure of interest: None Declared.

PT8.06

EXO-DNA applications: a novel EV-based method for detection of actionable mutations from the blood of cancer patients

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Introduction: Circulating DNA is emerging as a novel non-invasive tool for patient's stratification and disease monitoring. While most of the research has focused on circulating cell-free (cfDNA) or circulating-tumor-cell-(CTC)-derived DNA, extracellular vesicle-(EVs)-associated DNA (EV-DNA) is emerging as a third valuable "liquid biopsy" platform. We developed a novel protocol, using EXO-DNA kit, for the isolation of cfDNA and EV-DNA from blood and subsequent detection of actionable mutations by PCR and next-generation-sequencing (NGS). We assessed BRAF WT and BRAFV600E levels in patients with metastatic melanoma and compared our protocol with the current gold-standard kit for circulating nucleic acids extraction. **Methods:** Immuno-affinity and chemical precipitation were evaluated for enrichment of tumor-derived EVs from healthy donor samples spiked-in with melanoma-cell-derived EVs and "real" melanoma samples. DNA was extracted with EXO-DNA kit (HansaBioMed) and amplified by allele-specific-locked-nucleic-acid-(LNA)-qPCR. DNase protection assay and electropherogram analysis were performed to assess the source and integrity of the isolated DNA. Advantages over benchmark methods (Qiagen kit for circulating nucleic acids) were also assessed by digital PCR and NGS. **Results:** Affinity isolation method yielded the highest BRAFV600E copy number/ml and BRAFV600E/BRAF WT ratio, suggesting a significant enrichment of tumor-derived DNA versus background circulating DNA. LNA-PCR detected BRAFV600E in the serum of 6 out of 7 patients previously stratified as BRAFV600E-positive from tissue biopsy. Only 10–20% of DNA was found to be protected from DNase digestion but its quality was sufficient for amplification. The enrichment of actionable mutations over a generic kit was confirmed by digital PCR and NGS analyses. **Conclusions:** The novel EXO-DNA protocol enriches for cfDNA and a significant fraction of EV-DNA conferring a competitive advantage to "liquid biopsy" applications.

PT8.07

Energy-requiring uptake of prostasomes and PC3 cell-derived exosomes into non-malignant and malignant cells

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Epithelial cells lining the prostate acini release, in a regulated manner (exocytosis), nanosized vesicles called prostasomes that belong to the exosome family. Prostate cancer cells have preserved this ability to generate and export exosomes to the extracellular space. We previously demonstrated that human prostasomes have an ATP-forming capacity. In this study, we compared the capacity of extracellular vesicles (EVs) to generate ATP between normal seminal prostasomes and exosomes secreted by PC3 cells (PC3 exosomes), a prostate cancer cell line. Proteomic analyses identified enzymes of the glycolytic chain in both prostasomes and PC3 exosomes, and we found that both of them were capable of generating ATP when supplied with substrates. Notably, the net production of extracellular ATP was low for prostasomes due to a high ATPase activity contrary to an elevated net ATP level for PC3 exosomes because of their low ATPase activity. The uptake of the 2 types of EVs by normal prostate epithelial cells (CRL2221) and prostate cancer cells (PC3) was

visualized and measured, demonstrating differential kinetics. Interestingly, this uptake was dependent upon an ongoing glycolytic flux involving extracellular ATP formation by EVs and/or intracellular ATP produced from the recipient cells. We conclude that the internalization of EVs into recipient cells is an energy-requiring process also demanding an active V-ATPase and the capacity of EVs to generate extracellular ATP may play a role in this process.

PT8.08

Exosomes and microparticles: extracellular vesicles with functionally different roles in colorectal cancer progression

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Introduction: Activated stroma promotes tumour progression. Fibroblasts are the most populous stromal cell. Paracrine signalling between fibroblasts and cancer cells occurs in part through shuttling of extracellular vesicles (EVs). We aimed to investigate the effect of fibroblast EVs on colorectal cancer cells and vice versa. We consider exosomes as sub-100 nm vesicles with a specific structural signature and microparticles (MPs) as all EVs larger than this. **Methods:** MPs and exosomes were isolated from DLD-1 colorectal cancer cells and MRC5 fibroblasts by selective centrifugation, labelled with a lipophilic dye and validated by transmission electron microscopy, flow cytometry and western blotting. 4 mg of MPs or exosomes (by protein) were reciprocally co-cultured with 1×10^6 DLD-1 or MRC5 cells for 24 h. Uptake of EVs was confirmed by fluorescence microscopy. Protein expression was analysed by western blotting, proliferation by cell counting and chemoresistance by mitochondrial depolarisation (after 24 h 300 mM oxaliplatin treatment). **Results:** Cancer exosomes induced phosphorylation of AKT and its downstream target Bad in fibroblasts, corresponding with increased resistance to oxaliplatin; there was no effect on ERK activity. Cancer MPs had no effect on ERK or AKT signalling in fibroblasts. In contrast, fibroblast exosomes strongly induced ERK phosphorylation in cancer cells, corresponding with increased proliferation on days 1–4; AKT/Bad signalling was concomitantly induced with associated oxaliplatin resistance. Interestingly, fibroblast MPs did induce AKT signalling in cancer cells without Bad activation or chemoresistance and had no effect on ERK activity. **Conclusion:** Exosomes are reciprocally shuttled between fibroblasts and cancer cells in the tumour microenvironment (TME), making both more resistant to chemotherapy. Fibroblast exosomes increase proliferation of cancer cells, another hallmark of cancer. Exosomes and MPs have distinct cellular and functional effects.

PT8.09

"Artificial neoepitope"- bearing exosomes derived from tumor cells being genetically modified to express Mycobacterium tuberculosis antigen: a novel vaccine for cancer therapy

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Introduction: Tumor-derived exosomes can be regarded as miniatures of the originating tumor cells containing same antigens, and were expected as cancer vaccines. However, treatment with those exosomes often failed to elicit efficient antitumour T-cell responses in vivo experiments or clinical studies. Moreover, recent evidences suggest that tumor-derived exosomes possess rather immunosuppressive properties, and facilitate immune evasion of the tumor cells. To overcome these problems, we prepared the exosomes bearing highly immunogenic bacterial epitopes as "artificial neoepitopes" by

gene transfection of the parent tumor cells. Ability of these exosomes to evoke the cellular immunity against tumor cells, and their potential as a cancer vaccine was examined. **Methods:** B16 melanoma cells were transfected with a plasmid encoding the Mycobacterium tuberculosis antigen, early secretory antigenic target-6 (ESAT-6), and the secreted exosomes were isolated. They were injected into mice to examine their ability to evoke the cellular immunity against ESAT-6, and to the tumor cells. Antitumor therapeutic efficacy of the "artificial neoepitope"-bearing exosomes was also explored in mice with B16 tumors. **Results:** Exosomes bearing ESAT-6 epitopes effectively evoked high cellular immunity against both ESAT-6, and B16 tumor cells. Intratumoral injection of the exosomes demonstrated significant tumor growth suppression in mice with syngeneic tumor transplants, while those derived from the non-transfected B16 cells showed no effect on the tumor growth. **Conclusions:** The tumor-derived exosomes bearing Mycobacterium tuberculosis antigen epitopes as "artificial neoepitopes" were shown to have a high potential as a candidate for cancer vaccine to overcome the low immunogenicity of the tumor cells. **Acknowledgments:** This work was supported by JSPS KAKENHI Grant Number 25350555, and The Cosmetology Research Foundation.

PT8.10

Extracellular vesicles are involved in MAPC[®] promotion of oligodendrocyte differentiation in vitro

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Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system. While the exact cause of MS is unknown, considerable research has targeted a hyperactive immune system as a mediator of disease progression. Infiltration by a heterogeneous population of immune cells including T cells, B cells and macrophages, along with increased levels of pro inflammatory cytokines and chemokines contribute to disease progression. Current standard of care focuses on diminishing the inflammatory response as opposed to promoting remyelination to improve quality of life. Multipotent adult progenitor cells (MAPC) are a well characterized population of bone marrow-derived cells that exhibit immunomodulatory effects in central nervous system injury models such as traumatic brain injury, stroke and spinal cord injury. Further, MAPC cell therapy has been previously shown to reduce deficits in mouse models of EAE. However, whether MAPC cells promote recovery by decreasing inflammation, promoting repair, or both is unknown. Here, we show that MAPC cells and MAPC cell-secreted factors promote the differentiation of oligodendrocytes from C57BL/6 mouse postnatal neural stem cells in vitro. We observed a significant increase in the number of MBP and O1 positive oligodendrocytes in the presence of MAPC cells or MAPC cell-conditioned media when compared to control. Depletion of extracellular vesicles from MAPC cell-conditioned media diminished the increase of MBP and O1 positive cells. Our results suggest that MAPC cell-secreted soluble factor(s) and/or extracellular vesicles are capable of driving oligodendrocyte differentiation. We are currently investigating the role of MAPC cell therapy in the regulation of human oligodendrocyte maturation.

PT8.11

Dendritic cell exosomes are rich in chemotactic mediators and recruit mesenchymal stem/stromal cells

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Introduction: Orchestration of cell-cell communication is crucial for tissue repair, and reported to be influenced by Extracellular Vesicles (EV), namely exosomes. Immune cells, including dendritic cells (DC), are present at initial stages of tissue repair, secrete many mediators and high amounts of EV. Mesenchymal stem/stromal cells (MSC) migrate to injury locations and contribute to repair. Thus, herein we investigate the content and functional impact of EV secreted by human DC on human MSC. **Methods:** Primary human bone marrow MSC and monocyte-derived DC were used. EV were isolated from DC cultures (DC-EV) by differential centrifugation (final ultracentrifugation 100000 × g), and characterized by standard techniques. Content of DC-EV was evaluated using an antibody array, combined with Western Blot, imaging flow cytometry and gelatin zymography. EV effect on MSC was tested by transwell cell migration assay, Ki67 immunostaining and gene expression. **Results:** DC-EV were highly enriched in exosomes, and contained several chemotactic factors, such as MCP-1 and osteopontin. MMP9 was also enriched inside EV. DC-EV were internalized by MSC, and were able to recruit MSC in a dose-dependent manner. On the other hand, DC-EV decreased the number of proliferating cells and did not impact significantly MSC differentiation. **Conclusion:** Taken together our results show that DC-EV are packed with chemoattractants and able to recruit MSC, suggesting a functional role for these vesicles in linking inflammation and tissue repair. **Acknowledgments:** We thank Centro Hospitalar S.João (Porto, Portugal) for sample donation. Work funded by FEDER, through COMPETE 2020-Operational Programme for Competitiveness and Internationalisation (POCI), Portugal 2020; and Portuguese funds via FCT/Ministério da Ciência, Tecnologia e Inovação, Project "Institute for Research and Innovation in Health Sciences"-POCI-01-0145-FEDER-007274, and fellowships SFRH/BD/85968/2012; SFRH/BPD/91011/2012; SFRH/BD/112832/2015.

PT8.12

Female patients with suspected adverse effects of vaccination against human papilloma virus, have a different composition of extracellular vesicles in plasma compared with a healthy female control group
Kim Varming¹, Rikke Baek¹, Malene Moeller Joergensen², Louise Brinth³ and Jesper Mehlsen³

¹Aalborg University Hospital, Aalborg, Denmark; ²Department of Immunology, Aalborg University Hospital, Aalborg, Denmark; ³Frederiksberg Hospital, Frederiksberg, Denmark

Introduction: In Denmark around 2000 patients with suspected adverse effects of vaccination against human papilloma virus have been referred to 5 hospital centers. In one center 177 patients have been investigated for possible biomarkers. Most of these patients were diagnosed with the chronic fatigue syndrome, a syndrome which most clinicians perceive as a functional disease. The aim of this study is to explore the potential of using surface proteins from extracellular vesicles as biomarkers for this illness. **Methods:** Plasma from 177 female patients and 96 healthy female controls were subjected to analysis by the extracellular vesicle array (EV Array) (Joergensen et al., 2013, JEV). The array was composed of 29 capturing antibodies targeting well-known EV-associated proteins. The binding of EVs was visualized and recorded using a cocktail of biotin conjugated CD9, CD63 and CD81 antibodies combined with fluorescently-labeled streptavidin. **Results:** The protein profiles of the plasma EVs were analyzed in a multivariate manner with a random forests prediction model. The best obtained model using all 29 markers, resulted in a sensitivity of 0.72 and a specificity of 0.65 (AUC: 0.70). **Conclusion:** Female patients with suspected adverse effects of vaccination against human papilloma virus, have a different composition of extracellular vesicles in plasma compared with a healthy

female control group, which indicate that we are facing a biological state instead of functional disease.

PT8.13

Canine Wharton's Jelly mesenchymal stem cell suppression of T helper cell division is mediated by exosomes

Sarah Crain, Kristen E. Thane, Airlie M. Davis and Andrew M. Hoffman
Veterinary Clinical Sciences, Cummings School of Veterinary Medicine at Tufts University, North Grafton, Massachusetts, USA

Introduction: Mesenchymal stem cells (MSC) are known to suppress activation and proliferation of T helper (TH) cells, but the specific contribution of extracellular vesicles (EV) remains unclear. Knowledge of signaling roles for EV may lead to novel EV-based therapies for chronic inflammatory diseases. We hypothesized that MSC EV suppress TH cell division. **Methods:** EV blockade was tested by incubating canine Wharton's Jelly MSC (WJ-MSC; CD44+ CD90+ CD34- CD45- MHCII-) with GW4869 (2.5–10 µM) for 48 hr and EV output assessed using nanoparticle tracking analysis (NTA; NS300, NanoSight NTA3.0 software). For lymphocyte suppression assays, WJ-MSC cell lines (n=5) were exposed to 6 µM GW4869 or vehicle. Peripheral blood mononuclear cells from healthy beagle donors (n=2) were stained with CFSE and co-cultured (10:1) with WJ-MSC across transwell membranes (0.4 µm pore size) for 72 hours. Analysis of CFSE fluorescence using FlowJo yielded percent CD4+ divided and mean number of cell divisions in dividing CD4+ cells. **Results:** EV from WJ-MSC conditioned medium were mainly (87.2%) in the exosome size range (30–200 nm) based on NTA. Concentrations of 2.5, 5, and 10 µM GW4869 reduced exosome counts by 36%, 55%, and 73%, respectively. The percentage of CD4+ T cells that divided in response to ConA alone, ConA+WJ-MSC, or ConA+WJ-MSC+GW4869 (6 µM) was 74.4+10.6%, 52.5+12.4% (P < 0.01 vs ConA), and 64.8+10.7% (P < 0.01 vs. ConA+WJ-MSC), respectively. Therefore exosomes contributed to 56% of the effect of WJ-MSC to reduce the percentage of dividing CD4+ cells. No significant effect was noted on the number of cell divisions in dividing CD4+ cells (P=0.14). **Summary/Conclusions:** These data support a significant role for exosomes to alter CD4+ cell kinetics in response to ConA, specifically to suppress entry into the cell cycle. Further exploration is needed to identify the specific contents of exosomes (e.g. miRNA, proteins) and exosome-CD4+ T cell interactions responsible for this effect.

PT8.14

Extracellular vesicle miRNA 371-5p is a biomarker candidate in isolated hepatic perfusion from patients with metastatic uveal melanoma

Rossella Crescitelli¹, Taral Lunavat¹, Valerio Belgrano², Roger Olofsson Bagge², Hideo Akiyama³, Cecilia Lässer¹ and Jan Lötvall¹

¹Krefting Research Centre, The Sahlgrenska Academy, University of Göteborg, Göteborg, Sweden; ²Department of Surgery, Institute of Clinical Sciences, Sahlgrenska Academy at University of Gothenburg, Sahlgrenska University Hospital, Gothenburg, Göteborg, Sweden; ³New Projects Development Division, Toray Industries, Inc., Kamakura, Japan

Introduction: Uveal melanoma arises from the eye and 30% of patients develop liver metastases that can be treated by isolation of the liver circulation for local chemotherapy. We have previously shown that liver perfusates contains miRNA-containing extracellular vesicles (EVs), and the aim was now to compare the extracellular miRNAs in liver circulation EVs with that of tumor tissue and liver tissue. **Method:** One superficial liver melanoma metastasis and a small piece of normal liver tissue from each patient were extracted during surgery. EVs were isolated from perfusates by multiple centrifugations. Presence of miRNAs was analyzed with Toray 3D-Gene platform. **Results:** miRNAs expressed in melanoma and liver tissues were com-

pared with miRNAs from perfusate EVs. Melanoma and liver tissues expressed similar miRNAs (80% overlap). Comparison between miRNAs in perfusate plasma and melanoma tissue showed the presence of 11 and 16 miRNAs overlapping uniquely between melanoma tissue and perfusate EVs for each patient respectively. The melanoma specific miR-371b-5p was commonly expressed in EVs between the two patients, and was found in the peripheral

circulation of one of these patients. The miR-371b-5p found primarily in larger EVs (classically called microvesicles). *Conclusions:* miRNAs from melanoma tissue can be detected in larger vesicles in the liver circulation and in peripheral plasma of uveal melanoma patients. It is possible that microRNAs in larger EVs can be a source of uveal melanoma biomarkers and miR-371b-5p is one possible candidate.

Room: Jurriaanse

Satellite Event - ISEV-ISAC-ISTH Workshop

Chairs: *Joanne Lannigan and Marca Wauben*

6:30-8:30 p.m.

Satellite meeting ISEV-ISAC-ISTH EV flow cytometry work group

This satellite event will provide insight into the scientific interaction of three big international societies, and will also give opportunity for young researchers to present the first results of this collaboration

6:30-6:35: Marca Wauben - Introduction of the ISEV-ISAC-ISTH EV flow cytometry work group

6:35-6:45: Leonie de Rond - Size and refractive index determination to standardize vesicle flow cytometry

6:45-6:55: Sten Libregts - Interference of non-fluorescent particles in fluorescence EV measurements

6:55-7:10: Rienk Nieuwland - Update on ISTH EV flow cytometry workgroup studies (scatter-based measurements)

7:10-7:35: John Nolan - Standards and calibration in flow cytometry & workgroup Spring 2016 activities

7:35-7:45: Joanne Lannigan - Update on the ISAC MV IDG workshop

7:45-8:30: Discussion about next steps

Scientific Program ISEV2016 meeting Friday May 6, 2016

Room: Willem Burger

Meet the Experts Session 4

EVs as Modulators of Drug Resistance and Tumor Metastasis

Speakers: *Janusz Rak and Alissa Weaver*

Moderator: *Lorraine O'Driscoll*

8:00-8:45 a.m.

Room: Jurriaanse

Meet the Experts Session 5

EVs-Associated Versus Soluble Functional Molecules

Speakers: *Aled Clayton and Guillaume van Niel*

Moderator: *Maria Yanez-Mo*

8:00-8:45 a.m.

Room: van Weelde

Meet the Experts Session 6

Extracellular RNAs: EVs or not EVs

Speakers: *Andrey Turchinovich and D. Michiel Pegtel*

Moderator: *Jan Lötvall*

8:00-8:45 a.m.

Oral Presentations

Room: Jurriaanse

Symposium Session 19 - EVs in the immune system

Chairs: Jan Lötvall and Esther Nolte-*t* Hoen

9:00-10:15 a.m.

OF19.1

Macrophages preferentially package TNF within microvesicles in response to ATP

Soni Sanoj, Ying Ying Tan, Kahori Cho, Michael Wilson, Kieran O'Dea, Masao Takata
Imperial College London, England

Introduction: Tumour necrosis factor (TNF) is a potent pro-inflammatory cytokine, playing a crucial role in various inflammatory diseases. Microvesicles (MVs), carrying a variety of mediators including cytokines, provide a vital alternative pathway for inflammatory signalling, yet there is a paucity of literature examining MV-associated TNF transfer. Here we investigate the effect of ATP, a typical danger signal that also stimulates MV production, on the manner of TNF release by macrophages. **Methods:** RAW 264.7 cells were stimulated with 1 µg/mL LPS for 1 h and then treated with an additional "second hit" of ATP 6 mM/ecto-ATPase inhibitor 1 mM for 1 h to produce MVs. Cells were analysed by flow cytometry for TNF expression. Cell-free supernatant underwent high speed centrifugation to separate MV pellet and MV-free supernatant, and TNF/IL-6 content of both fractions were quantified by ELISA/western blotting. Controls included cells stimulated with LPS alone for 2 h. **Results:** Compared to LPS alone, surface TNF expression on RAW cells was lower with the second hit of ATP (20 ± 3 vs. 2 ± 2 , mean fluorescent intensity, $p < 0.01$, $N = 3$). ATP treatment almost abolished soluble TNF release into the media (471 ± 127 vs. 32 ± 14 pg, $p < 0.05$, $N = 3$) but substantively upregulated TNF content within MVs (1.1 ± 0.2 vs. 23 ± 7 pg, $p < 0.05$, $N = 3$), as measured by ELISA. Western blotting confirmed the presence of TNF within MVs. The profile of IL-6 release between the soluble vs. MV-associated fractions were not altered by ATP, suggesting this "switch" phenomenon may be specific to TNF. **Conclusion:** These data show, for the first time, that ATP preferentially packages TNF within MVs, while concurrently inhibiting soluble TNF release. Our results indicate that danger signals superimposed onto pro-inflammatory signals can switch macrophage TNF release from the ordinary soluble form to the MV-associated form, suggesting a novel mechanism of long-range MV-associated TNF transfer in case of significant tissue injury.

OF19.2

Extracellular vesicles rapidly and massively released from degranulating mast cells are enriched in distinct phospholipid species and contain active carboxypeptidase

Tom Groot Kormelink¹, Willie J. C. Geerts², Ger J. A. Arkesteijn^{1,3}, Maarten Altelaar⁴, Frank A. Redegeld⁵, Esther N. M. Nolte-*t* Hoen¹, Marca H. M. Wauben¹

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Degranulating mast cells, found in tissues during allergic reactions and chronic inflammation, release a plethora of soluble mediators.

Previously, we showed that degranulation also resulted in the instant release of large numbers of extracellular vesicles (EVs). We here analysed the morphology, phospholipid and protein composition of EVs to define their putative biogenesis and functional properties. Unstimulated murine serosal mast cells were cultured for 24 h, or degranulation was induced for 1.5 h. EVs were isolated by differential centrifugation and floatation into iodixanol density gradients. EV sizes were determined by Cryo-EM. Phospholipids were isolated by Bligh and Dyer extraction followed by LC-MS analysis, and proteins were identified using LC-MS/MS proteomics and western blotting. Carboxypeptidase (CPA) activity was determined by measuring hydrolysis of N-(4-methoxyphenylazofornyl)-Phe-OH. EVs released after degranulation were between 40 and 80 nm, whereas those released from unstimulated cells were more heterogeneous in size (70–300 nm). These EV types differed in phospholipid composition, with a particular enrichment of phosphatidylinositol in EVs released after degranulation. These latter EVs contained mast cell-specific proteases as identified by proteomics and confirmed by western blotting for CPA. EV-associated CPA showed significant functional activity. C-Kit and Fc receptor gamma chain, both associated with mast cells, and proteins with immune-modulating capacities were also identified. Although exclusively considered as soluble mediators, mast cell-specific proteases also associate with a subset of relatively small EVs. Whether the distinct phospholipids present in these EVs imply differences in function or biogenesis is subject of current research. The EV-associated transfer may enhance relocation of locally high concentrations of active proteases, ensuring a functionally significant activity at sites distant from the degranulating mast cells.

OF19.3

Apoptotic cell-derived extracellular vesicles (ACdEV) – a matter of life and death

Andrew Devitt¹, Allan Cameron¹, Roberta Liccardo¹, Khaled Alghareeb¹, Parbata Chauhan¹ and Ivana Milic¹

¹Aston Research Centre for Healthy Ageing and School of Life & Health Sciences, Birmingham, England

Introduction: Damaged, aged or unwanted cells are removed from the body by apoptosis. This highly orchestrated programme results in the exposure of "flags" at the dying cell surface and the release of attractive signals to recruit phagocytes. Together these changes ensure efficient phagocytic removal of dying cells, which is central to the control of inflammation. We have shown that dying lymphocytes release apoptotic cell-derived extracellular vesicles (ACdEV) and these are strongly attractive to phagocytes in vitro. Our work seeks to characterize ACdEV, their release, physical characteristics and their molecular structure-to-function relationships. **Methods:** ACdEV were enumerated using qNano to identify kinetics of EV release. Monocyte migration to ACdEV was assayed using horizontal Dunn chemotaxis chambers, Cell-IQ live cell imaging and an in vivo mouse tumour model. The involvement of specific molecules was assessed using mAbs or reduced antigen expression. Additionally, the molecular profile of ACdEV was assessed using mass spectrometry. **Results:** ACdEV released early in apoptosis promote strong directional migration of phagocytes but the molecular mechanisms mediating this attraction vary between dying cells. Monoclonal antibody blockade of ICAM-3 on ACdEV can reduce phagocyte migration towards apoptotic foam cells, while CX3CL1 mAbs reduce migration

to apoptotic macrophages. Using transwell studies, we further demonstrate that THP-phagocytes are attracted across endothelial cell barriers to dying cells, also in an ICAM-3-dependent manner. Finally, our *in vivo* studies indicate that macrophages migrate towards dying cells in a manner that is ACdEV-ICAM-3 dependent. **Summary:** Our data suggest ICAM-3 may play an important role in monocyte recruitment to sites of cell death, for example, tumours and atherosclerotic plaques. Furthermore, the inhibition of monocyte migration in the presence of anti-ICAM-3 mAbs suggests ICAM-3 may be a useful target for modulation of monocyte recruitment for therapeutic gain.

OF19.4

Vitamin D3-tolerized dendritic cells release extracellular vesicles that suppress pro-inflammatory cytokine production

Susanne van der Grein, Tom Driedonks, Tom Groot Kormelink, Henrike Jekel, Marca Wauben and Esther Nolte-'t Hoen
Department of Biochemistry and Cell Biology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands

Introduction: Antigen presenting dendritic cells (DC) are key regulators of the adaptive immune system that collect and interpret information on the local immune status of peripheral tissues. Vitamin D3 is an immune suppressive environmental signal that induces tolerogenic DC (tol-DC), which have been implicated in repression of Th17-driven immune responses. Th17 cells are pro-inflammatory CD4+ T cells that produce interleukin 17 (IL-17) and play an important role in the clearance of extracellular bacteria, but are also strongly associated with autoimmune disorders. DC-derived extracellular vesicles (EVs) are thought to play an important role in the communication between DC and T cells and the control of immune responses. We therefore aimed to study the immune regulatory function of EVs released by tol-DC. **Methods:** Tol-DC were generated by treating murine bone marrow-derived DC with 1 α ,25-dihydroxyvitamin D3. EVs released by tol-DC or control DC were isolated by density gradient ultracentrifugation and added to *in vitro* DC-CD4+ T co-cultures using various stimulation and differentiation regimes. Modulation of the T-cell response was assessed by flow cytometry and cytokine profile analysis. **Results:** Tol-DC-derived EVs were able to modulate T cells during DC-CD4+ T-cell co-cultures. The modulated T cells displayed largely reduced secretion of Th17-related pro-inflammatory cytokines upon T-cell re-stimulation under Th17-skewing conditions. This repression of Th17 responses by tol-DC-derived EV was apparent under suboptimal T-cell stimulating conditions and could be overcome by strong

T-cell stimulation. Initial data indicate that the modulating effect of tol-DC-derived EVs on T cells is not antigen specific and requires T-cell interaction with a DC. **Conclusion:** This study suggests a role for tol-DC EVs in creating a local immune suppressive milieu in which tolerogenic DC inhibit potentially harmful Th17 responses until a strong danger signal is perceived.

OF19.5

Polyclonal T-cell proliferation is inhibited by umbilical cord MSC-derived EVs but not by MSC conditioned media

Marta Monguió-Tortajada, Santiago Roura, Anna Oliveira-Tercero, Marcel·La Franquesa, Antoni Bayes-Genis and Francesc E. Borràs
Germans Trias i Pujol Health Sciences Research Institute (IGTP), Can Ruti Campus, Badalona, Spain

Introduction: Mesenchymal stem cells (MSC) have been proposed as powerful inhibitors of unwanted immune responses. MSC's conditioned medium (CM) partially mediate MSC's immune suppressive paracrine activity but is often less effective than the MSC themselves. Our aim is to isolate EVs from umbilical cord MSC-CM, compare their suppressive effect to the MSC and derived CM and investigate their mechanism of action. **Methods:** MSC 48 h-CM was concentrated by ultrafiltration. MSC-EVs were isolated by size-exclusion chromatography (SEC) and characterized by NTA and Cryo-TEM and checked for EV markers by bead-based flow cytometry. CM or purified MSC-EV were co-cultured with allogenic T cells stimulated with anti-CD2/CD3/CD28 loaded beads and proliferation was assessed by CFSE. Cytokines produced were analysed by multiplex ELISA. **Results:** SEC-purified EVs were enriched in CD9 and CD63, and bulk proteins were eliminated from EV fractions. Importantly, the MSC markers CD73 and CD90 were found in EV-containing SEC fractions. Also, the immune suppressive molecule CD73 seemed to be specifically sorted to MSC-EV with IFN γ conditioning, while levels in MSCs and EV production remained unaltered. Functionally, purified MSC-EVs suppressed polyclonal proliferation in a dose-dependent manner, to the same extent as the MSC, whereas neither the CM nor the concentrated CM was able to affect T-cell proliferation. While CD73 did have an important role in MSC-mediated reduction of T-cell proliferation, inhibition of CD73 did not modify MSC-EV immune suppressive functions. **Conclusions:** Our results demonstrate the higher potential of purified MSC-EV over CM to suppress T-cell proliferation. While CD73 is an important immune suppressive molecule used by MSC, it seemed to have a marginal role in EV-mediated suppression of T-cell proliferation.

Room: Willem Burger
 Symposium Session 20 - EVs in cancer metastasis I
 Chairs: *Dolores Di Vizio and Suresh Mathivanan*

9:00-10:15 a.m.

OF20.1**Positive effects of vitamin D in bone metastases: role for extracellular vesicles?**

Marjolein Van Driel¹, Resti Rudjito¹, Iris Robbesom¹, Hideki Chiba² and Johannes Van Leeuwen¹

¹Department of Internal Medicine, Erasmus MC, Rotterdam, The Netherlands;

²Fukushima Medical University, Fukushima, Japan

The bone microenvironment provides a fertile soil for cancer cells to metastasize to. These cells de-arrange bone metabolism that stimulates cancer cell growth. Compounds that restore bone integrity are promising therapies, such as vitamin D (1 α ,25-OH₂D₃), a bone stimulatory and anti-cancer agent. Novel routes of metastatic communication have been shown via extracellular vesicles (EVs). Here, we aim to investigate the potential of vitamin D as a novel bone-targeted therapy for bone metastatic cancer and study underlying mechanisms involving EVs. We used a human co-culture model of differentiating osteoblasts (SV-HFO) and bone metastatic prostate cancer cells (PC-3). EVs released by PC-3 cells were isolated via ultracentrifugation (20,000 g and 100,000 g). EVs were quantified on a NanoSight NS300. The effect of 1 α ,25-OH₂D₃ (10⁻⁷ M) was studied on (1) osteoblast differentiation (alkaline phosphatase (ALP) activity) and mineralization (calcium) and PC-3 growth (FACS), (2) EV production by PC-3 cells (NanoSight) and (3) PC-3 EV interaction with osteoblasts (ALP activity). 1 α ,25-OH₂D₃ treatment recovered the negative effects of PC-3 cells on osteoblast differentiation and mineralization and significantly reduced cancer cell growth by 80%. Interestingly, the number of EVs produced by PC-3 cells is increased by 1 α ,25-OH₂D₃ (20,000 g fraction 10-fold, 100,000 g fraction 2-fold). These EVs significantly increased the ALP activity of human osteoblasts with 20%. This is in contrast to EVs from PC-3 cells that were not treated with 1 α ,25-OH₂D₃; these EVs significantly decreased ALP activity of human osteoblasts with 20%. These are the first observations that vitamin D may act as a therapeutic agent in bone metastases, with an involvement of EVs. EVs from bone metastatic prostate cancer cells negatively affect osteoblast differentiation. After vitamin D treatment, EV production by these cancer cells is strongly increased and these EVs positively affect osteoblast differentiation.

OF20.2**Role of bone marrow-mesenchymal stromal cell-derived extracellular vesicles on chronic lymphocytic leukaemia B cells defined by functional studies and microarray analysis**

Emerence Crompton¹, Dominique Bron², Philippe Mineur³, Laurence Lagneaux¹, Basile Stamatopoulos¹, Nathalie Meuleman², Michael Van Damme¹ and Karlien Pieters¹

¹LTCC, Jules Bordet Institute - ULB, Brussels, Belgium; ²Hematology, Jules Bordet Institute, Brussels, Belgium; ³Hematology, Grand Hôpital de Charleroi, Charleroi, Belgium

Introduction: The interactions between chronic lymphocytic leukaemia B cells (CLL-B-cells) and the bone marrow microenvironment (BM-ME) (notably composed by mesenchymal stromal cells-MSC) play an important role in promoting the increased survival of CLL-B-cells. Extracellular vesicles (EVs) produced by CLL-B-cells and the ME may be implicated in this crosstalk. **Methods:** Ultracentrifugation (150,000g) was applied to isolate EVs from supernatant of BM-MSC. Different concentrations of EVs were added to CLL-B-cells to evaluate their

impact on cell survival, migration and chemoresistance. The gene expression change induced by EVs in CLL cells was also defined by microarray analysis (Affymetrix) after their incubation (24 h) with BM-MSC-EVs. Some genes identified as differentially expressed were validated by real-time PCR. **Results:** We demonstrated that BM-MSC EVs are able to enter in CLL-B-cells (PKH67 labelling): after 24 h, >95% of CLL cells had integrated EVs. Apoptosis was assessed by flow cytometry: addition of increasing concentrations of EVs showed a protective effect on CLL-B-cells from spontaneous cell death (n = 21/ p-value <0.0001). CLL-B-cells pre-incubated with EVs (4 h) displayed an increased spontaneous migration index and also in response to SDF1 α (n = 10/ p-value = 0.002). We also observed that EVs rescue CLL-B-cells from drug-induced apoptosis. Microarray analysis reveals a significant effect on the expression of 987 genes after the integration of EVs in CLL-B-cells, notably implicated in apoptosis, epigenetic, adhesion, migration, cell activation, actin cytoskeleton regulation and immune pathways. **Conclusion:** Here we show for the first time that BM-MSC-EVs protect CLL cells from spontaneous apoptosis and influence their migration and chemoresistance capacities. The implication of EVs in several cell functions was observed by microarray analyses. This study provides evidence of the critical role of EVs in the interactions between leukaemic cells and their ME.

OF20.3**Pancreatic cancer initiating cell and exosome CD44v6 affects additional stem cell marker expression and cancer stem cell activities**

Zhe Wang, Anja Von Au and Margot Zoeller

Tumor Cell Biology, Heidelberg University Hospital, Heidelberg, Germany

CD44 variant isoform v6 (CD44v6) is a metastasis-promoting molecule, upregulated in pancreatic cancer initiating cells (CIC). We explored by a CD44v6 knockdown (v6kd) and by CIC enrichment of human pancreatic adenocarcinoma (PaCa) the impact of exosomal CD44v6 on stem cell features and metastases. A CD44v6kd was accompanied by a striking loss in anchorage independent growth, sphere/holoclone-forming capacity and apoptosis resistance. CD44v6kd cells poorly migrated and lost invasive capacity. Exosomes (TEX) from wild-type cells and CIC rescued migratory and invasive capacity. Loss of migratory capacity was accompanied by reduced integrin recovery in cells and TEX and mitigated activation of joint CD44v6-integrin signalling. Loss of invasion was promoted by reduced recovery of CD44v6-associated proteases in cells and TEX. The impact of the TEX CD44v6 - protease liaison on metastasis was verified in vivo, Matrigel embedded CD44v6kd cells not invading the surrounding, but progressing, when supported by CD44v6-competent CIC-TEX. Importantly, beyond affecting integrin and protease expression, CD44v6 exerted a striking effect on CIC markers, including c-Met, the α 6 β 4 integrin, CXCR4 and strongly Tspan8. Tetraspanins playing a central role in TEX generation and delivery, we hypothesized that reduced Tspan8 transcription in CD44v6kd cells might account for the overall collapse of CIC activities. The assumption was supported by the finding that Tspan8-competent TEX promoted upregulation of mesenchymal genes in CD44v6kd cells and in vivo metastatic settlement. On the other hand, an excess of CD44v6kd TEX sufficed to outsmart CIC TEX such that CIC largely lost the capacity to metastasize. CD44v6 affects metastasizing tumour cell motility and invasion by cooperating/associating with integrins and proteases and by regulating expression of additional TEX-enriched CIC markers. Thereby, the deficit of CD44v6 suffices for a collapse of the exosome CIC crosstalk with non-CIC and host cells.

OF20.4

Exosomes mediate intra-tumour communication in pancreatic cancer

Carolina F. Ruvio^{1,2}, Agustin F. Fernandez³, Mario Fraga³,
Jose Luis Costa^{1,2,4}, Jose Carlos Machado^{1,2,4} and Sonia A. Melo^{1,2,4}
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Oncology of Asturias (IUOPA), HUCA, Universidad de Oviedo, Spain;
⁴Department of Pathology Medical Faculty of Porto, Portugal

Tumours are characterized by a great diversity of cancer cells, and this substantial intra-tumour heterogeneity reflects different cell phenotypes and tumorigenic capacity. Pancreatic cancers are known for their heterogeneity and for the presence of hierarchical cancer cell subpopulations. Subpopulations of cancer cells cooperate to drive and maintain tumour progression. Exosomes contribute to horizontal reprogramming and phenotypic re-education of recipient cells. The role that exosomes play in intra-tumour heterogeneity is still unclear. We have identified 5 pancreatic cancer cell subpopulations. Exosomes from each subpopulation were characterized by nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM) and by western blot for common exosomes markers. In order to study the exosomes flow between these 5 different subpopulations of cancer cells, we developed stable clones combining known exosomes markers with a fluorescent reporter protein. Subpopulations of cancer cells that secrete color-coded exosomes were co-cultured and the flow of exosomes analysed by confocal microscopy and flow cytometry. Exosomes from different subpopulations of cancer cells present a different proteome. Additionally, co-cultures demonstrated the presence of multicolour positive cells, which indicates that exosomes are exchanged between different tumour subpopulations. The communication network mediated by exosomes among phenotypically different cancer cells might be involved in tumour progression and metastatic disease.

OF20.5

Hypoxic cancer-derived EVs contain specific miRNA cargo and mediate intercellular communication among hypoxic cancer cells

Aija Line¹, Artūrs Ābols¹, Elina Zandberga¹, Rasita Toleikiene¹, Cristina Bajo¹, Dārta Pūpola¹, Lilite Sadovska¹, Pavel Zayakin¹, Una Riekstiņa²

¹Latvian Biomedical Research and Study Centre, Riga, Latvia; ²Faculty of Medicine, University of Latvia, Riga, Latvia

Introduction: Hypoxia is a common feature of the vast majority of solid cancers, which has been shown to promote cancer cell survival, chemoresistance, motility and self-renewal capacity of cancer stem cells. We hypothesized that hypoxic cancer-derived EVs may transfer these features among cancer cells and the presence of hypoxic EVs in biofluids of cancer patients may serve as a biomarker for aggressive phenotype and reveal hypoxia status in the tumour. **Methods:** Exosome-enriched EVs were isolated from colorectal (SW480 and SW620) and breast cancer (MCF7) cell cultures exposed to hypoxic or normoxic conditions by using sequential centrifugation, filtration and size-exclusion chromatography steps. The EVs were visualized by electron microscopy, quantified by NanoSight and Zetasizer Nano and characterized by western blot analysis. EVs were stained using Exo-APC stain and their uptake by SW480, SW620 and MCF7 cells was analysed by FACS. Small RNA libraries were constructed from hypoxic and normoxic SW480 and SW620 cells and EVs and sequenced using Ion Proton Platform. **Results:** The obtained EV subpopulations ranged in size from 30 to 180 nm and were positive for CD9, CD63, CD81 and Alix and negative for GM130. Importantly, EVs expressed hypoxia-inducible CAIX that potentially might allow isolation of hypoxic EVs from biofluids. Under normoxic conditions, the cells preferentially internalized normoxic EVs, while hypoxic conditions enhanced the uptake of hypoxic EVs. Preliminary RNASeq data analysis revealed a set of miRNAs that were induced by hypoxia in the cells. However, only a few of these miRNAs were also found to be upregulated in hypoxic EVs, while a subset of miRNAs that were not significantly upregulated in the cells were found to be upregulated in the EVs. **Conclusions:** These data suggest that EVs mediate intercellular communication between cancer cells under hypoxic conditions and hypoxia may serve as a signal for sorting specific miRNA cargo into EVs.

Room: van Weelde

Symposium Session 21 - EVs in reproduction and pregnancy

Chairs: Chris Gardiner and Paul Harrison

9:00-10:15 a.m.

OF21.1

Extracellular vesicles – the next small thing in epigenetic inheritance
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Introduction: Epigenetics is the study of heritable changes in gene expression that are not caused by changes in DNA sequence. Environmental effects such as diet and stress are able to affect an individual's epigenotype and phenotype. These effects can persist beyond the initial exposed generation, suggesting that a signal is passed through the germline. However, it is not known what the signal is or the mechanism of transmission. We propose that the signal is somatic-derived non-coding RNA that is packaged inside extracellular vesicles (EVs). We have started to test this hypothesis in vitro using Sertoli cells, a germline-associated somatic cell. We aimed to determine if Sertoli EVs interact with germ cells and investigate whether Sertoli EV cargo is altered by environmental stress. **Methods:** Sertoli cells were exposed to bisphenol A (BPA), a known reproductive toxin for 12 days. EVs were isolated from conditioned culture media by filtration and differential centrifugation. Sertoli EVs were labelled with PKH26 and CFSE dyes and co-cultured with spermatogonial stem cells. EV RNA was extracted with Trizol LS. Small RNA libraries were constructed using a NEBNext Small RNA Library Kit and sequenced on an Illumina HiSeq 2500. Differential expression analysis was performed in R using the edgeR package. **Results:** Sertoli EVs are able to bind and fuse with spermatogonial stem cells in vitro. BPA exposure increases the ratio of EVs released per cell. BPA exposure also alters the selective packaging of small RNA inside Sertoli EVs, with altered miRNAs implicated in defective spermatogonial stem cell renewal and maintenance. **Summary/conclusion:** We show that somatic EVs are able to interact with germ cells. We also show that somatic EV cargo is susceptible to the environment. Taken together, this suggests that environmental effects may be transmitted from the soma to the germline via EVs – that EVs are a vector for transgenerational epigenetic inheritance.

OF21.2

Extracellular vesicles contents alter RNA profile after in vitro treatment of bovine cumulus-oocyte complexJuliano Da Silveira¹, Luiz Coutinho², Aline Cesar², Felipe Perecin¹, Flávio Meirelles², Marcelo Nogueira³, Gabriella Andrade², Marcelo César² and Maite Del Collado²¹Faculty of Animal Science and Food Engineering, Department of Veterinary Medicine, University of São Paulo, Pirassununga, São Paulo, Brazil; ²Faculty of Animal Sciences, Department of Animal Science, "Luiz de Queiroz" College of Agriculture, University of São Paulo, Piracicaba, São Paulo, Brazil; ³Faculty of Sciences and Letters, Department of Biological Science, University of São Paulo State, Assis, São Paulo, Brazil

Intercellular communication is crucial to induce cell proliferation and differentiation during cumulus-oocyte complex (COC) maturation. RNA molecules accumulated within the oocyte and are important during early embryo development. Extracellular vesicles (EVs) were identified in follicular fluid and can mediate cell communication. Our hypothesis is that extracellular vesicles from bovine follicular fluid can modulate changes on mRNA levels during early bovine embryo development. To test this hypothesis, extracellular vesicles isolated

from 3 to 6 mm and pre-ovulatory ovarian follicles were supplemented during oocyte maturation. Follicular fluid was submitted to differential centrifugation for removal of cellular components and debris before freezing at -80°C . COCs were in vitro matured in control media or media supplemented with EVs for 24 h and then fertilized. After 7 days in culture, embryos were collected for RNA analysis. EVs RNA-seq analysis demonstrated a variety of coding and non-coding RNA molecules including epigenetic-modifying enzymes (DNMT1 and DNMT3a) and mRNA-binding proteins (EIF4b and EIF4e). Functional annotation analysis of EVs RNA contents demonstrated to be involved in the regulation of chromatin remodelling or transcription activation. We identified 280 EVs miRNAs involved in regulation of important follicle developmental pathways. EVs supplementation increased levels of DNMT3a, an enzyme involved in de novo methylation of DNA, in embryos treated with exosomes from 3 to 6 mm follicles compared to control embryos. Thus, our results demonstrated that EVs carry RNA molecules involved in chromatin regulation capable of modulating transcript levels. Further experiments are necessary to explore if changes in RNA levels are mediated by extracellular vesicles delivery or gene activation.

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OF21.3

Functional impact of delivery of oviductosomal PMCA4 to Pmca4 null sperm in vitroAmal Al-Dossary¹, Pradeepthi Bathala² and Patricia Martin-Deleón²¹Biological Sciences, University of Dammam, Dammam, Saudi Arabia;²Biological Sciences, Newark, Delaware, USA

Introduction: Reproductive extracellular vesicles (EVs) in the male (epididymosomes and prostasomes) and those in the uterine secretion (uterosomes) play a role in the maturation of sperm and in their functional competency. We hypothesize that the recently identified EVs in the oviductal secretion, oviductosomes (OVS), play a pivotal role in capacitation, the final sperm maturation process. OVS carry an essential fertility-modulating protein, plasma membrane $\text{Ca}(2+)$ -ATPase 4 (PMCA4), which is the major $\text{Ca}(2+)$ efflux pump in murine sperm where its absence leads to male infertility due to loss of sperm motility. Following co-incubation of OVS and sperm, oviductosomal PMCA4 can be delivered to sperm by a fusogenic mechanism. To test our hypothesis of a functional role of oviductosomal PMCA4 in capacitation, we evaluated the impact of PMCA4 delivery in vitro to Pmca4 KO sperm. **Methods:** We examined the effect of fusion of WT and Pmca4 KO OVS and sperm after in vitro co-incubation on (a) $\text{Ca}(2+)$ -ATPase activity, (b) $\text{Ca}(2+)$ loading and (c) motility. The basis of fertility of Pmca4 KO females was assessed by investigating the presence of PMCA4 in OVS, via western analysis. **Results:** After co-incubation, $\text{Ca}(2+)$ -ATPase activity significantly increased ($p < 0.01$) for WT sperm and OVS and for Pmca4 KO sperm and WT OVS ($p < 0.003$). Here, $\text{Ca}(2+)$ levels significantly decreased ($p = 0.015$) and motility rates increased ($p = 0.007$). Fertility of Pmca4 KO females is associated with a significant upregulation of PMCA1 in Pmca4 KO OVS, compensating for PMCA4's loss. **Conclusion:** Our results show that oviductal PMCA4 transferred to sperm via OVS is functional and contributes to proper $\text{Ca}(2+)$ handling, impacting sperm phenotype. Knowledge of OVS transfer of fertility-modulating proteins to sperm provides new insights into molecular interactions at the fertilization site. This has important implications for increasing the fertilizing competence of sperm in the in vitro fertilization clinic.

OF21.4

Exosome trafficking from immune cells to the placenta: a novel mode of maternal-placental messaging

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Introduction: The placenta forms the interface between the maternal and foetal circulations and sheds extracellular vesicles, including exosomes, into the maternal circulation. These interact with maternal immune cells and alter their activation status. We have recently published the first demonstration that this trafficking is bi-directional. We propose that exosomes released by maternal immune cells interact with the foeto-placental unit and impact on placental immune response. **Methods:** Exosomes were isolated from macrophage supernatants and from serum, by ultracentrifugation or by size exclusion chromatography. Exosome membranes were labelled with PKH-26, and RNA and protein cargoes labelled with fluorescent SYTO RNASelect and CFSE, respectively. Uptake by the BeWo trophoblast cell line, human placental explants and the mouse placenta in vivo was measured by flow cytometry and confocal microscopy. Inhibitors of endocytosis were used to determine route of exosome uptake. Functional impact of exosomes on the placenta was measured by cytokine ELISA. **Results:** Uptake of exosomes was observed in the human and mouse placentas. Exosome uptake by trophoblast cells was mediated via clathrin-mediated endocytosis. This interaction has functional impact, as macrophage exosomes induced release of cytokines from the human placenta, with altered responses depending on the macrophage activation status. **Summary/conclusion:** We have demonstrated the trafficking of exosomes from immune cells to the placenta. We are also the first to demonstrate uptake of heterologous exosomes by the placenta. Importantly, we also show functional impact on placental cytokine release, presenting a novel mode of communication between the mother's immune system and the placenta during pregnancy. This has important impact for understanding placental responses to maternal infection, inflammation and vaccination, which could impact on the health of both mother and baby during pregnancy, and beyond.

OF21.5

Extracellular vesicles produced by group B streptococcus disrupts foeto-placental barrier and leads to preterm birth

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Introduction: Preterm rupture of the foetal membranes is a direct consequence loss of amniotic barrier integrity leading to preterm birth. Intrauterine infections and lower genital tract infections are major causes of preterm birth. However, 50–80% women with chorioamnionitis do not have bacteria in their amniotic fluid or in the decidual tissue. This implies the presence of unidentified mechanism of amniotic barrier integrity perturbation and subsequent preterm birth induction by distant infections. Colonization of vagina and cervix of pregnant women with Group B Streptococcus (GBS) significantly increases the probability of preterm birth. In this report, we demonstrate for the first time that GBS produces extracellular membrane vesicles, which lead to preterm birth. **Methods:** We have isolated the MVs from GBS culture supernatant and characterized them both physically and biochemically. We also examined how GBS MVs interacted with host cells in vitro as well as mice amniotic membrane ex vivo. Finally, we performed in vivo studies in pregnant mice to analyse the effects of these vesicles on preterm birth. **Results:** Our results demonstrate that MVs produced by GBS is a strain independent trait, and these are loaded with different virulence factors including proteases and pore-forming toxins. GBS MVs were cytotoxic and led to apoptosis in HeLa cells. Mice amniotic membranes challenged with MVs ex vivo resulted in extensive membrane damage because of connective tissue degradation. This led to loss of mechanical strength, making the membrane prone to rupture. Finally, intra-amniotic injections of GBS MVs in pregnant mice led to chorioamnionitis and resulted in premature delivery and foetal demise. **Conclusions:** Our findings provide a novel insight into how GBS while colonizing in the lower genital tract can orchestrate events at the foetal membrane by secreting extracellular vesicles that lead to premature rupture of amniotic membrane and subsequent preterm birth.

Room: Willem Burger

Plenary Session 3 - EVs in cell biology

Chairs: *Clotilde Théry and D. Michiel Pegtel*

10:45-11:25 a.m.

Speaker:

Francisco Sanchez-Madrid (Universidad Autónoma de Madrid and Immunology Department in the La Princesa Hospital, Spain)

Immune Cell-to-Cell Communication: Mechanisms of microRNA and Proteins Sorting into Exosomes

Room: van Weelde

Symposium Session 22 - EVs in acute and chronic inflammatory disease

Chairs: Philip Askenase and Muthuvel Jayachandran

1:30-2:45 p.m.

OF22.1

Circulating EBV-modified exosomes in SLE patients target the renal tubular epithelium delivering inflammatory EBER1 and EBV-microRNAs
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A possible pathogenic role of Epstein–Barr virus (EBV) in autoimmune diseases such as systemic lupus erythematosus (SLE) is long suspected, but its exact role remains unknown. Recently, we have shown that Epstein–Barr virus-encoded small RNA 1 (EBER1) and some EBV-related microRNAs (miRNA) are selectively released from infected B cells via exosomes and are internalized by human plasmacytoid DCs (pDC's) expressing TIM1 phosphatidylserine receptor, which is a known viral and exosomal target molecule. We found evidence that EBV-modified exosomes target the skin in SLE patients that may involve EBER1-activated pDCs. In this study, we first analysed lupus nephritis (LN) and other kidney disease control biopsies by in situ hybridization, which revealed a defined EBER1 presence in tubular epithelial cells (TEC) of LN. High levels of EBER1 and EBV-miRNAs were detected in LN biopsies by RT-PCRs, while EBV DNA was absent. TIM1 is expressed by TEC cells, especially in higher levels after kidney damage. We confirmed in primary TEC cultures that EBV-modified exosomes are internalized in a PtdSer-dependent manner, delivering inflammatory RNAs into endosomes. To verify whether circulating EBV-modified exosomes may target the kidneys in SLE patients, we isolated exosomes from blood and urine of SLE and LN patients and detected EBV-miRNAs and EBER1. We next analysed the possible inflammatory effects of EBV-modified exosomes in TEC cells, by isolation of exosomes from EBV-infected and non-infected B cells. The addition of EBV-modified exosomes, as well as infection of RNA-isolated from the same exosomes into TEC cells caused an increased level of cytokine production such as IL6 and interferon-stimulated genes. This effect was significantly lower upon addition of non-EBV-modified exosomes, supporting the hypothesis that EBV-modified exosomes might play a significant role in promoting inflammatory responses in autoimmune diseases including SLE and LN.

OF22.2

Molecular pathology of circulating extracellular vesicles in systemic lupus erythematosus (luposomes) provides novel insight into disease mechanisms

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Introduction: Systemic lupus erythematosus (SLE) is a chronic, autoimmune disease of unknown aetiology. The sustained autoimmunity

and the systemic type I interferon response in SLE may be due to clearance defects. It is unknown, however, where such defects reside and if the disease is aggravated or triggered by increased subcellular material, such as cell fragments and vesicles that are normally cleared by non-pro-inflammatory mechanisms. We therefore profiled circulating EVs from SLE patients and controls using tandem mass spectrometry. **Methods:** EVs were isolated from platelet-poor plasma from patients (n=45 SLE; n=38 Systemic sclerosis (SSc)) and healthy controls (HC, n=50) by repeated 18,900xg centrifugations. Samples were processed into peptide fragments and analysed by tandem mass spectrometry. Proteins were annotated and quantitated based on their intensity values and compared between the groups using nonparametrical statistics and corrections for multiple comparisons. **Results:** More than 1000 individual proteins were identified in the SLE/HC (1139 proteins) and the SSc/HC (1032 proteins) cohorts. The circulating EV proteomes were distinctly different between SLE/SSc patients and HC and differences between EVs in the SLE and SSc groups allowed a complete differentiation between these two autoimmune diseases. The data suggest a markedly increased production of circulating abnormal EVs in SLE characterized by profound metabolic, cytoskeletal and mitochondrial alterations. **Summary/conclusion:** The primary pathology in SLE is an increased production of abnormal EVs that are released to the circulation and become immunogenic because the amount or nature of these SLE-specific EVs – that we call luposomes – cannot be appropriately handled by normal non-inflammatory clearance mechanisms. Our findings pave way for new candidate biomarkers, support particle clearance disturbances as a central disease mechanism and give insight into new treatment modalities in SLE.

OF22.3

Suppression of autophagy by extracellular vesicles promotes myofibroblast differentiation in COPD pathogenesis

Tsukasa Kadota^{1,2}, Kuwano Kazuyoshi¹, Kenji Kobayashi¹, Hiromichi Hara¹, Jun Araya¹, Saburo Ito³, Yu Fujita⁴, Yusuke Yoshioka⁵ and Takahiro Ochiya⁵

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Introduction: Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory lung disease that causes obstructed airflow from the lungs. Cigarette smoke is the main causes of COPD and cigarette smoke-stimulated bronchial epithelial cells produce various paracrine factors that affect neighbouring lung fibroblasts (LFs), resulting in airway fibrosis in COPD. Here, we investigated an extracellular vesicle (EV)-mediated intercellular communication mechanism between primary human bronchial epithelial cells (HBEC) and LFs. **Methods:** EV from cigarette smoke extract (CSE)-exposed HBEC were isolated by ultracentrifugation and characterized by electron microscopy, western blot and nanoparticle tracking analysis. The profiles of EV microRNA (miRNA) were examined by microarray. The in situ hybridization was used to detect miRNA expression in non-smokers, non-COPD smokers and COPD lungs. **Results:** Evaluations of the modified EV and COPD lung samples indicated that cigarette smoke induced relative upregulation of cellular and

EV miR-210 expression of bronchial epithelial cells. In co-culture assays, we showed that HBEC-derived EV miR-210 promotes myofibroblast differentiation in LFs. We found that miR-210 directly regulates autophagy processes via targeting ATG7, and expression levels of miR-210 are inversely correlated with ATG7 expression in LFs. Importantly, autophagy induction was significantly decreased in LFs from COPD patients, and silencing ATG7 in LFs led to myofibroblast differentiation. These findings demonstrate that CSE triggers the modification of EV components and identify bronchial epithelial cell-derived miR-210 as a paracrine autophagy mediator of myofibroblast differentiation. *Summary/conclusion:* Our findings show that stressor exposure changes EV compositions as emerging factors, potentially controlling pathological disorders such as airway remodelling in COPD.

OF22.4

Extracellular vesicles and their microRNA cargoes are novel biomarkers and intercellular communicators in alcoholic liver disease

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Introduction: Alcohol induces hepatocyte damage and inflammation in the liver. miR-122 is abundant in hepatocytes and circulating miR-122 was found in liver injury. We hypothesized that extracellular vesicle (EV)-associated miRs can serve as biomarkers and provide communication between hepatocytes and immune cells in alcoholic hepatitis. *Methods:* EVs isolated from sera of chronic alcohol-fed (5 weeks Lieber DeCarli diet) or pair-fed mice and patients with alcoholic hepatitis were characterized by western blot, nanoparticle tracking analysis system and miRNA analysis. *Results:* The number of circulating EVs was significantly increased in alcohol-fed mice compared to controls. Exosomes represented most of the EVs (~80%). MicroRNA array of EVs revealed a significant increase of 7 inflammatory miRs (miR-192, 122, 30a, 744, 1246, 30b and miR-130a) in alcohol-fed mice compared to controls and of those miR-192, 122 and 30a excellent diagnostic value by receiver operating characteristic (ROC) curve analyses. In patients with acute alcoholic hepatitis, we found a significant increase in the number of circulating EVs compared to controls with an increase in miR-192 and miR-30a in their cargo. Serum miR-122 was increased after alcohol binge drinking. In vitro, exosomes derived from ethanol-treated human hepatocytes were taken up by monocytes and transferred mature miR-122 into monocytes. This horizontally transferred miR-122 inhibited hemeoxygenase-1 expression, a target of miR-122 and sensitized monocytes to LPS stimulation to increase production of pro-inflammatory cytokines, TNF- α and IL-1 β ; these effects were

inhibited by exosome-mediated delivery of a miR-122 inhibitor in monocytes. *Summary/conclusions:* Elevated levels of EVs and their miR signature could serve as biomarkers of alcoholic hepatitis. We found a novel EV-mediated mechanism of alcohol-induced communication between hepatocytes and monocytes by transferring hepatocyte-derived miR-122 that reprograms monocytes promoting inflammation in alcoholic hepatitis.

OF22.5

Subtypes of extracellular vesicles released by dendritic cells promote differential CD4+ T-cell responses

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Introduction: Cells secrete into their environment different types of extracellular vesicles (EVs) that have distinct structural and biochemical properties depending on their intracellular site of origin. We have recently shown that different subtypes of EVs secreted by human dendritic cells can be recovered during the steps of the differential ultracentrifugation protocol (1). Large EVs were pelleted at 2000 $\times g$ (200–5000 nm diameter), intermediate size EVs at 10,000 $\times g$ (100–800 nm) and small EVs (50–150 nm), as the exosomes, at 100,000 $\times g$. Whether these EVs have similar or different effects on target cells, as CD4+T cells, has never been addressed. *Methods:* EVs released by human monocyte-derived DCs were recovered at all steps of the differential centrifugation purification protocol. Activation of CD4+T lymphocytes by EV-borne MHC class II was measured by culturing EVs with allogeneic T cells and analysing cell proliferation and cytokine secretion. *Results:* All the pellets were able to induce, to a similar extent, primary CD4+T-cell proliferation. The EVs recovered at 2000 $\times g$ induced Th2 cytokines secretion (IL-13, IL-5 and IL-4), while the EVs recovered at medium/high centrifugation speed induced the secretion of both Th1 and Th17 cytokines (IFN- γ and IL-17A). To study the role of different molecules present on EVs involved in this CD4+T-cell differential polarization, we added blocking antibodies for MHC class II, LFA-1, CD40 and DC-SIGN to the EV-CD4+T cells co-cultures. MHC class II and LFA-1 were involved in the responses induced by all the pellets, while DC-SIGN and CD40 blockade affected only the response induced by the 100,000 $\times g$. *Conclusion:* We observed that all the EVs assayed in this work are able to induce CD4+T-cell activation and proliferation. Interestingly, EVs subtypes promote differential CD4+T-cell polarization, suggesting that cells can spread different responses though the secretion of several types of EVs (1).

Reference

1. Kowal J, et al. 2016. PNAS (113: E968-977).

Room: Jurriaanse

Symposium Session 23 - EVs in cancer metastasis II

Chairs: Richard Simpson and Hector Peinado

1:30-2:45 p.m.

OF23.1**Osteosarcoma exosomes establish a prometastatic inflammatory loop by engaging mesenchymal stem cells**

Serena Rubina Baglio¹, Tonny Lagerweij², Maria Pérez Lanzón¹, Roberta Bonafede³, Ekaterina S. Jordanova⁴, Nicolas Léveillé⁵, Koos Rooijers⁶, Monique A. J. Van Eijndhoven¹, Gloria Bonuccelli⁷, Laura Roncuzzi⁸, Sonia A. Melo⁹, Anne-Marie Cleton-Jansen¹⁰, Thomas Wurdinger², Nicola Baldini⁸ and D. Michiel Pegtel¹
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Introduction: Metastasis formation and resistance to therapy, for example via STAT3 activation in tumour cells, could be a consequence of driver-mutations in cancerous "stem" cells or of cancer-stroma interactions, but a role for mesenchymal stem cells (MSCs) is poorly understood. Here we explored in an in vivo osteosarcoma (OS) model of how tumour exosomes may engage MSCs to facilitate tumour progression. **Methods:** To study the effect of OS exosomes on MSC behaviour, we injected OS exosome-educated MSCs in a bioluminescent orthotopic OS xenograft model. The effects of OS exosomes on MSC cytokine expression were assessed by FACS and ELISA. TGFβ1 was identified as the exosomal component responsible for IL-6 induction by using a combination of assays including ELISA, and exosome uptake and ALK5 inhibition. STAT3 activation in OS tissues was assessed by IHC staining of tissue microarrays. Circulating levels of exosomal TGFβ1 were assessed by size exclusion chromatography followed by TGFβ1 ELISA. The R2 Genomics Analysis Platform was used to correlate our findings with clinical data. **Results:** We found that while OS tumours have elevated pSTAT3 levels, local IL-6 expression is low. We confirmed this in primary OS cells and OS cell lines suggesting that STAT3 activation may be a consequence of an exogenous source. We demonstrated that OS tumour exosomes decorated with TGFβ1 educate MSCs to secrete IL-6 leading to elevated STAT3 activation and malignant progression in vivo. TGFβ1 expression in OS tumours correlates with poor outcome, and circulating levels of exosome-associated TGFβ1 is increased in OS patients compared to controls. Breaking this pro-malignant EV-mediated inflammatory loop by administering the MoAb Tocilizumab to OS-bearing mice reduced tumour growth and metastasis formation. **Conclusion:** Our studies indicate exosomes as powerful messengers in OS progression and provide a rationale for the clinical use of IL-6R blocking agents to stop OS progression.

OF23.2**Role of tumour-secreted exosomes in reprogramming the lymph node microenvironment during metastasis**

Hector Peinado¹, Alberto Benito-Martin², Ana Amor-Lopez¹, Susana Garcia-Silva¹, Irina Matei², Raghu Kataru³, Babak Mehrara³,

David Lyden², Laura Nogues-Vera², Marta Hergueta-Redondo¹, Carolina Villarroya-Beltri⁴ and Lucía Robado De Lope¹

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Metastasis is the most devastating phase of tumour progression. Recent research has demonstrated that microvesicle-based information transfer by exosomes plays a key role in tumour progression. Melanoma-secreted exosomes have been demonstrated to home to the lymph nodes (LNs). Exosomes derived from highly metastatic models (B16-F10) have a faster and wider distribution in the lymphatic system as compared to exosomes derived from low (B16-F1) or non-metastatic model (melan-a) whose distribution is restricted to the sentinel lymph node. Our data demonstrate that exosomes secreted by highly metastatic models promote vascular leakiness in the sentinel lymph node in a short time range. Another main consequence of in vivo exposure of B16-F10-derived exosomes is the promotion of new lymphoangiogenesis in sentinel and distal LNs. In order to identify primary cell targets of melanoma-secreted exosomes, we have performed confocal imaging and flow cytometry analysis. Our findings indicate that there exists a primary uptake of exosomes by lymphatic endothelial cells (LECs) from the lymph nodes. RNAseq and RT-PCR analysis of human LECs incubated with melanoma-derived exosomes confirm upregulation of lymphoangiogenesis-related genes together with the positive enrichment of molecular adhesion-, autoimmune- and cytokine-related signatures among others. Our results support a role of tumour-secreted exosomes in promoting cellular and molecular alterations in the lymph node microenvironment fostering metastasis. In addition, analysis of exosome content in human lymphatic fluid suggests that proteomic cargo and particles are increased in melanoma patients opening the possibility of the use of circulating vesicles in lymphatic fluid as biomarkers and as a source of novel markers in pre-metastatic sentinel lymph nodes that predict relapse and metastatic potential.

OF23.3**Metastatic colon cancer extracellular vesicles spread malignant properties in tumour microenvironment affecting the behaviour of both tumour and endothelial cells**

Odessa Schillaci¹, Riccardo Alessandro¹, Francesca Monteleone¹, Simona Taverna¹, Valentina R. Minciacci², Dolores Di Vizio² and Simona Fontana¹

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Introduction: Intratumoural heterogeneity makes the development of new anticancer treatments difficult. Several studies suggest that exosomes may be an important microenvironmental factor affecting tumour heterogeneity. In this context, our goal was to understand if exosomes derived from highly metastatic cell lines may affect the behaviour of less aggressive cells and the properties of the endothelium, thus promoting metastatic dissemination of cancer cells. **Methods:** Isogenic SW480 and SW620 colon carcinoma lines

derived from primary and secondary tumours resected from the same patient were our model system. We used (1) differential centrifugation and iodixanol gradients to purify EVs; (2) transwell migration, dextran permeability and tube formation assays to test the functional role of EVs; and (3) high-throughput, label-free mass spectrometry SWATH to profile exosome proteins. **Results:** We found that EVs released by metastatic SW620 cells are able to induce a mesenchymal-amoeboid transition in SW480 non-metastatic cells. Treatment of SW480 cells with exosomes from SW620 cells induced non-apoptotic membrane blebbing, associated with higher migratory and invasive capabilities. Moreover, SW620 exosomes, in comparison with SW480 exosomes, induced a stronger permeability of endothelial monolayers, which appeared to be due to destabilization of endothelial junctional systems. RacGap1, which is a well-known activator of RhoA, emerged as enriched in SW620 exosomes by quantitative proteomic analysis. Further experiments demonstrated that the effects of SW620 exosome treatment, both on SW480 and endothelial cells, are mediated by RhoA, responsible of cytoskeletal remodelling. **Conclusions:** We demonstrated that, through exosomes, highly metastatic cells spread their malignant behaviour to less aggressive cells and affect tumour microenvironment.

OF23.4

Exosome-mediated delivery of miR-26a suppresses liver cancer growth in vitro and in vivo

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Background: Accumulating evidence indicates that miRNAs in exosomes function as a communicator to modulate tumour biogenesis and development. Delivery of miR-26a by adeno-associated virus (AAV) potently results in dramatic suppression of liver tumour progression in vivo and in vitro. However, safety consideration limited the clinical application of AAV. In this study, we tried to establish a novel miRNA delivery system based on exosomes. **Methods:** The expression of exosomes miR-26a in healthy, cirrhosis and hepatocellular carcinoma (HCC) patients was investigated. Purified exosomes were loaded with synthetic miR-26a by chemical transduction and then cultured with SMMC7721. The expression of miR-26a and its targeted genes was determined by RT-PCR and validated by luciferase reporter assays (LRA). The proliferation was evaluated by CCK8 assay and Edu dye array. MHCCLM3-luciferase and BALB/c nude mice were adopted to establish orthotopic HCC models. Tumour growth and DiR-labelled exosome distribution were monitored dynamically by IVIS Spectrum. **Results:** Exosomes miR-26a was significantly lower in HCC patient versus healthy and cirrhosis. Patients with metastatic HCC showed lower miR-26a compared with that of non-metastatic HCC ($p < 0.01$). LEF1 level in HCC tissue was inversely correlated with exosomes miR-26a ($r = -0.5478$, $p < 0.001$). Kaplan–Meier analysis showed that tissue LEF1 and exosomes miR-26a were correlated with OS and DFS. LRA demonstrated that exosome-mediated delivery of miR-26a into HCC cells inhibits their proliferation by directly targeting CCND1, CCND2 and CCNE2. In vivo imaging systems demonstrated

that xenografts exosomes distributed mostly in liver even 3 days after intravenously injected. miR-26a loaded exosomes showed dramatic suppression of tumour size and lung metastasis in mice model. **Conclusions:** HCC patients with low miR-26a level in exosomes showed shorter DFS and OS. Delivery miR-26a by exosomes inhibits HCC proliferation and metastasis through Wnt pathway in vitro and in vivo.

OF23.5

CD8+ T cells interrupt tumour progression including invasion and metastasis by exosome-mediated depletion of mesenchymal tumour stromal cells

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Introduction: Fibroblastic mesenchymal tumour stromal cells promote strongly tumour progression in part an exosome-mediated manner. On the other hand, there is no report about the role of CD8+ T-cell-released exosomes on the regulation of tumour progression. In this study, we investigated in murine models whether or not exosomes from CD8+ T cells including CTLs affect the tumour progression focusing on the modification of tumour stromal cells. **Methods:** Supernatants obtained by the cultivation of CD8+ T cells or control cells were collected and subjected to exosome purification by the filtration and ultracentrifugation (25,000 g, 2 h). The purified exosomes were injected into d-10 subcutaneous CMS5a, B16 and B16F10 tumours (1.0–1.5 cm diameter) to investigate growth and CD140a expression of tumours, and subsequent tumour invasion and metastasis. CMS5a-specific CD8+ T cells treated with/without GW4869 were transferred i.v. of CMS5a tumour-bearing mice to examine exosome-dependent depletion of fibroblastic tumour stromal cells. **Results:** CD8+ T-cell-released exosomes from the culture supernatant of CD8+ splenocytes of normal or CMS5a-specific TCR gene-transgenic mice, but not tumour-bearing mice, attenuated CMS5a and B16 growth in correlation with the downregulation of CD140a expression. The detailed studies using SYTO RNASelect-stained exosomes, MSC chimeric mice and the cultured bone-derived MSCs demonstrated that the reduced growth and CD140a expression in CD8+ T-cell-released exosome-treated tumours caused by interrupting tumour progression after apoptotic death of exosome-engulfed fibroblastic mesenchymal tumour stromal cells rather than the direct attenuation of tumour cells. Furthermore, subcutaneous B16F10 tumour lost invasive and lung metastatic properties by i.t. treatment of CD8+ T-cell-released exosomes. **Conclusion:** Our findings provide crucial insights that CD8+ T cells play as an anti-stromal effector in addition to the conventional cytotoxicity of tumour cells and have a possibility to develop effective treatment of patients with advanced cancer.

Room: Willem Burger

Symposium Session 24 - EVs in immune modulation in bacterial and parasitic infections

Chairs: *Hernando del Portillo and Cherie Blenkiron*

1:30-2:45 p.m.

OF24.1**Spleen-dependent immune protection elicited by reticulocyte-derived exosomes from malaria infection revealed by T-cell population changes**

Lorena Martin-Jaular^{1*}, Armando De Menezes-Neto¹,
Marta Monguio-Tortajada², Aleix Elizalde-Torrent¹,
Carmen Fernandez-Becerra¹, Francesc E. Borrás², Maria Montoya³
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Introduction: Reticulocyte-derived exosomes (rex) are released during the maturation of reticulocytes. We previously found that the immunization of mice with rex from Balb/c infected with the reticulocyte-prone non-lethal P.yoelii17X strain (rexPy) in combination with CpG-ODN promoted survival and long lasting protection of mice subsequently challenged with a lethal strain. **Methods:** Exosomes were isolated from *P. yoelii*-infected reticulocytes culture and used to immunize Balb/c mice in combination of CpG-ODN 1826. Importance of the spleen in the protection elicited by rexPy was assessed by immunization of splenectomized mice and by transfer of splenocytes. T-cell responses in the spleen after immunization were analysed by flow cytometry. Analysis of the protein content of rexPy was done by mass spectrometry. In order to translate these results to humans, in vitro experiments with splenocytes from transplantation donors were performed to analyse exosomes uptake and stimulation of splenocytes by these vesicles. **Results:** We show that rexPy-mediated protection is completely lost in splenectomized animals and such protection is 100% achieved after passive transfer of splenocytes obtained from animals immunized with rexPy. Notably, rexPy immunization of mice induced non-exhausted memory T cell expansion with effector phenotype. Proteomics analysis of rexPy confirmed their reticulocyte origin and demonstrated the presence of parasite antigens. Moreover, human splenocytes were able to actively capture plasma-derived exosomes from patients infected with *P. vivax* (exPv) and stimulation of spleen cells with exPv for 72 h lead to an increase in the number of T cells. **Summary/conclusions:** Our studies prove, for what we believe is the first time, that rex from reticulocyte-prone malarial infections are able to induce non-exhausted splenic long-lasting memory responses. All together, these data further support the value of rex as a novel vaccine and platform against malaria.

OF24.2**Malaria parasites regulate secretion of exosomes carrying distinct cargo**

Neta Regev-Rudzki
Weizmann Institute of Science, Rehovot, Israel

Cells use extracellular vesicles to communicate, to coordinate social activities and, in the case of pathogens, to export virulence effectors into host target cells. Previously, we showed that the lethal malaria parasites directly communicate within a population using exosome-like vesicles that are capable of delivering episomal plasmids and these are produced at the ring-stage of the asexual life cycle. Here, we identify these nanovesicles as exosomes and determine their molecular content. By establishing advanced nano-resolution techniques for analysing exosome content, we discovered that the

exosomes deliver endogenous nuclear genes, parasite apicoplast and mitochondrial genomic DNA. Moreover, we show that the nucleotide cargo is packaged into released exosomes only at specific stage of parasite development in host (infected red blood cells), highlighting the selectivity of this process. Our work identifies previously unknown molecular players in signalling pathway of malaria parasite and provides a new insight into our understanding of how malaria parasites can manipulate their host environment.

OF24.3**Immunomodulation of anti-parasitic responses via *Heligmosomoides polygyrus* secreted exosomes**

Gillian Coakley¹, Marissa Lear¹, Rick Maizels², Henry Mcorley¹,
Fabio Simbari¹ and Amy Buck¹

¹Institute of Immunology and Infection Research, Ashworth Laboratories, University of Edinburgh, Scotland; ²The Queen's Medical Research Institute, Centre for Inflammation Research, University of Edinburgh, Scotland

Helminth secretomes have been shown to play a central role in both pathogenicity and the regulation of host defense. Through previous studies, we have demonstrated that secreted vesicles from the murine gastrointestinal nematode *Heligmosomoides polygyrus* exhibit a range of regulatory properties in the murine host. To investigate the fundamental interactions between helminth-derived exosomes and host cells, we have performed comparative studies between mammalian and *H. polygyrus*-derived exosomes. Using the PKH67-labelling system, we were able to track exosome uptake in cells (such as intestinal epithelial cells and primary macrophages) over the course of 24 h. This illustrated a number of factors required for optimal uptake and potential exosome function, including; time of incubation, cell type and exosome origin. Microarray of *H. polygyrus* exosome-treated small intestinal epithelial cells reveals significant host gene changes, including those involved in the anti-parasite response, such as IL1RL1 (the receptor for IL-33). We have shown that *H. polygyrus*-derived exosomes can suppress the onset of alternative activation and reduce expression of the IL33R in macrophages. In addition, we have shown that a prophylactic dose of exosomes can suppress IL33R expression in the IL2 population during an allergic asthma response in vivo. Immunization of mice using an exosome/alum conjugate elicits significant immune protection from a subsequent *H. polygyrus* infection, as seen through a reduction in egg counts and worm burden, whereby exosomes can induce specific antibody responses. Complementary studies are now under way to determine whether exosome-induced immunity is intact in IL33R/ST2 knockout mice. Taken together, these studies suggest that exosomes secreted by nematodes can mediate the transfer of parasitic products into host cells, establishing cross-species communication to modulate the host response, and that we can block this process through vaccination.

OF24.4**Worsening of atopic dermatitis by *Staphylococcus aureus*-derived membrane vesicles**

Je Chul Lee, Hyejin Jeon, Hyo Il Kwon, Seok Hyun Na, Yoo Jeong Kim and So Hyun Jun

¹Kyungpook National University School of Medicine, Daegu, Republic of Korea

Skin colonization or infection with *Staphylococcus aureus* is known to trigger aggravation of atopic dermatitis (AD). We investigated whether and how *S. aureus*-derived membrane vesicles (MVs) contribute to worsening of AD. HaCaT cells were treated with *S. aureus* MVs and analysed for the expression of pro-inflammatory cytokine genes. Immunopathology and cytokine gene profiles were analysed in the AD-like skin lesions of mice after topical application of *S. aureus* MVs. Intact *S. aureus* MVs delivered their components to keratinocytes and stimulated pro-inflammatory cytokine gene expression. However, MVs with a disrupted membrane did not stimulate cytokine gene expression. A knockdown of TLR2 or NOD2 by using siRNAs suppressed IL-8 gene expression. Topical application of *S. aureus* MVs to AD-like skin lesions in the mouse model induced infiltration of inflammatory cells and the resulting eczematous dermatitis. This inflammatory reaction was associated with a mixed Th1/Th2 immune response and enhanced expression of chemokine genes in AD-like skin lesions. Our study provides evidence that *S. aureus* MVs trigger an inflammatory response in AD-like skin lesions. MVs produced by *S. aureus* colonizing or infecting AD skin lesions may be responsible for worsening of AD.

OF24.5

***Legionella pneumophila* outer membrane vesicles are potent pro-inflammatory stimulators for macrophages**

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²Institute for Virology, Philipps-University Marburg, Marburg, Germany

The formation and release of outer membrane vesicles (OMVs) is a phenomenon of Gram-negative bacteria. This includes *Legionella pneumophila* (*L. pneumophila*), a causative agent of severe pneumonia. Upon its transmission into the lung, *Legionella* primarily infects and replicates within macrophages. Here, we analysed the influence of *L. pneumophila* OMVs on macrophages. To this end, differentiated THP-1 cells were incubated with increasing doses of *Legionella* OMVs, leading to a TLR2-dependent classical activation of macrophages with the release of pro-inflammatory cytokines. Inhibition of NF- κ B signalling reduced the induction pro-inflammatory cytokines. Furthermore, treatment of THP-1 cells with OMVs prior to infection reduced replication of *L. pneumophila* in THP-1 cells. Blocking of TLR2 activation or heat denaturation of OMVs restored bacterial replication in the first 24 h of infection. With prolonged infection-time, OMV pre-treated macrophages became more permissive for bacterial replication than untreated cells, dependent on NF- κ B signalling, and showed increased numbers of *Legionella*-containing vacuoles and reduced pro-inflammatory cytokine induction. Additionally, miRNA-146a was found to be transcriptionally induced by OMVs and to facilitate bacterial replication. Accordingly, IRAK-1, one of miRNA-146a's targets, showed prolonged activation-dependent degradation, which rendered THP-1 cells more permissive for *Legionella* replication. In conclusion, *L. pneumophila* OMVs are initially potent pro-inflammatory stimulators of macrophages, acting via TLRs, IRAK-1 and NF- κ B, while at later time points, OMVs facilitate *L. pneumophila* replication by miR-146a-dependent IRAK-1 suppression. OMVs might thereby promote spreading of *L. pneumophila* in the host.

Room: Willem Burger
 Caris Diamond Sponsor Session
 Chairs: *Marca Wauben and Aled Clayton*

3:15-4:15 p.m.

Adaptive Dynamic Artificial Poly-ligand Targeting (ADAPT) of Plasma Exosomes: a Highly Multiplexed Non-Invasive Diagnostic Discovery Platform

Mark R. Miglarese¹, Valeriy Domenyuk¹, Zhenyu Zhong¹, Jie Wang¹, Adam Stark¹, Xixi Wei¹, Patrick Kennedy¹, Brandon Toussaint¹, Radhika Santhanam¹, Symon Levenberg¹, Nianqing Xiao¹, David Halbert¹, George Poste², Michael Famulok^{3,4}, Günter Mayer⁴ and David B. Spetzler¹
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Introduction: Understanding individual biomarkers and multi-molecular complexes in their native states represents a major hurdle in the development of systems biology platforms and requires multiplexing capabilities many orders of magnitude greater than what is currently available. This knowledge is especially important in diseases like cancer, where perturbations in signalling pathways lead to the initiation and propagation of a vast range of molecularly heterogeneous phenotypes. Ideally, this information would be gathered non-invasively from peripheral compartments. We report ADAPT as an unbiased discovery method to identify native proteins and multi-

molecular complexes in plasma exosomes. **Methods:** ssDNA libraries of 2x10¹¹ unique sequences were enriched towards association with exosomes of plasma pools from breast cancer patients (n = 60) or from a control cohort of women without breast cancer (n = 60). Two thousand ODNs were selected from the enriched library and used to profile 500 plasma samples: 206 breast cancer biopsy positive (BC+), 177 biopsy negative (BCi-) and 117 self-declared healthy (H) samples. **Results:** Random Forest Modelling (RFM) was used to build classifiers for BC+ versus both BC- and H, BC+ versus BC- and BC+ versus H, with AUC values of 0.64, 0.64 and 0.73, respectively. Out-of-bag validation was used for this analysis. Notably, 10-fold cross-validation, repeated 20 times, resulted in ROC AUC values of 0.58 for BC+ versus both controls, 0.59 for BC+ versus BC-, 0.62 for BC+ versus H. This difference between the out-of-bag and cross-validation results suggest that the study is underpowered to define an algorithm capable of handling the heterogeneity found in this set of breast cancer patients. **Conclusions:** The ADAPT platform uses ultra-high complexity libraries to improve the probability of identifying ODNs which recognise biomolecules in their native state. This unbiased approach quantifies differences between plasma from women with breast cancer and women without breast cancer, likely due to perturbations in molecular pathways. An ADAPT-derived breast cancer test may find utility as a diagnostic tool in clinical practice.

Room: Jurriaanse

Symposium Session 25 - EVs, viruses and viral infections

Chairs: Jennifer Jones and Matias Ostrowski

4:15-5:00 p.m.

OF25.1**MiR-9-mediated synaptodendritic alterations in HAND: role of extracellular vesicles**

Guoku Hu, Yu Cai, Lu Yang, Yeonhee Kook and Shilpa Buch
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Introduction: Synaptodendritic injury (SDI) has emerged as an important phenotype and correlate of HIV-associated neurocognitive disorders (HAND). Furthermore, persistent inflammation has been implicated as a major underlying factor in the progression and pathology of HAND. Intriguingly, similar to HIV+ subjects on antiretroviral therapy, SIV-infected rhesus macaques also demonstrate SDI, increased glial activation and dysregulation of various signature miRNAs (miRs) including miR-9. The goal of the present study was to understand how HIV protein Tat mediates SDI via the release of miR-9 in EVs. **Methods:** Using qPCR we demonstrated increased expression of miR-9 in the frontal cortex of HIV/SIV-infected humans/macques. Transmission electron microscopy and western blots were used to characterize EVs. The functional end point of SDI was assessed by spine density assays. **Results:** HIV Tat exposure resulted in increased release of miR-9 in the EVs isolated from astrocytes. MiR-9-enriched EVs, in turn, were taken up by the neurons resulting in SDI. This correlated with downregulated expression of PDGF-CC, NLGN2 and MCP1P1, key players regulating normal synaptic function in neurons, in SIV+/HIV+ brains compared to uninfected controls. This was validated by infection of rat primary cortical neurons with lentivirus-miR-9, which resulted in decreased expression of each of these proteins. Interestingly, neurons exposed to EVs isolated from conditioned media of Tat-treated astrocytes also demonstrated decreased expression of miR-9 target proteins. **Summary:** HIV Tat-exposed astrocytes upregulate the expression and release of miR-9 in EVs, which upon uptake by the neurons, leads to SDI via downregulation of its target genes. Therapeutic strategies aimed at maintaining the levels of miR-9 in the neurons, could be developed for treatment of SDI in HAND.

OF25.2**Sensing of latent EBV infection through exosomal transfer of 5'pppRNA**

Dirk Michiel Pegtel¹, Monique Aj Van Eijndhoven¹, Serena Rubina Baglio¹ and Danijela Koppers-Lalic²

¹VUmc, Department of Pathology, Exosomes Research Group; ²VU University Medical Center, Department of Pathology, Exosomes Research Group, The Netherlands

Complex interactions between large DNA herpes viruses and host factors determine the establishment of a life-long asymptomatic latent infection. The lymphotropic Epstein-Barr virus (EBV) seems to avoid recognition by innate sensors despite massive transcription of immunostimulatory small RNAs (EBV-EBERs). Here, we demonstrate that in latently infected B cells EBER1 transcripts interact with the La ribonucleoprotein, avoiding cytoplasmic RNA sensors. However, in co-culture experiments we observed that latent-infected cells trigger antiviral immunity in dendritic cells through selectively release and transfer via exosomes. In ex vivo tonsillar cultures, we observed that

EBER-loaded exosomes are preferentially captured and internalized by human plasmacytoid dendritic cells (pDCs) that express the TIM1 phosphatidylserine receptor, a known viral- and exosomal target. Using an EBER-deficient EBV strain, enzymatic removal of 5'ppp, in vitro transcripts and co-culture experiments, we established that 5'pppEBER1 transfer via exosomes drives antiviral immunity in non-permissive DCs. Lupus erythematosus patients suffer from elevated EBV load and activated antiviral immunity in particular in skin lesions that are heavily infiltrated with pDCs. We detected very high levels of EBER1 RNA in such skin lesions as well as EBV-microRNAs but no intact EBV-DNA, linking non-cell autonomous EBER1 presence to skin inflammation in predisposed individuals. Collectively our studies support the idea that virus-modified exosomes have a physiological role in the host-pathogen stand-off and may promote inflammatory disease.

OF25.3**Exosomal incorporation but not interaction with major histocompatibility class II molecules is a conserved feature of alpha herpes virus glycoprotein B**

Andrea D. Lipinska, Kinga Grabowska, Magda Wachalska, Vasil B. Krapchev, Paula Mazurek, Michal Rychlowski and Krystyna Bienkowska-Szewczyk
Department of Virus Molecular Biology, Intercollegiate Faculty of Biotechnology, University of Gdańsk, Gdańsk, Poland

Herpes viruses represent one of the most widespread pathogens. Recent studies indicate intersections between exosome biogenesis pathway and herpes virus assembly during infection and the role of exosomes in the formation of antiviral immunity. Glycoprotein B (gB) is a conserved in all herpes viruses entry mediator. Human herpes simplex 1 (HSV-1)-encoded gB can additionally divert the major histocompatibility (MHC) II molecules to exosomes, thus here we aimed at assessing whether exosomal incorporation of gB and its interaction with MHC II are conserved in other alpha herpes viruses. gB homologs from HSV-1, bovine herpes virus 1 or pseudorabies virus was introduced to human melanoma MJS as well as species-specific cells (bovine MDBK, swine SK6, ST) using retroviruses. Extracellular vesicles (EVs) were isolated from cell culture supernatants by size-exclusion chromatography or ultracentrifugation followed by discontinuous iodixanol or Ficoll density gradients. EVs were characterized by TEM or NTA and their content by immunoblotting. Subcellular localization of gB and exosomal markers was analysed by confocal microscopy and gB-MHC II interactions were also determined by immunoprecipitation. N-glycosylation was analysed with endoglycosidases, surface MHC II by FACS. EVs isolated from all stable cell lines were enriched in gB. We could detect in EVs only the mature, fully glycosylated form of gB. gB co-localized with exosomal marker CD63 and mature MHC II, but only HSV-1 and BHV-1 homologs co-precipitated MHC II. Their expression resulted in downregulation of surface MHC II levels. This feature was not shared by PRV-encoded gB which had no significant influence on MHC II. Our data show that utilization of exosome biogenesis pathway by gB seems to be conserved in alpha herpes viruses. gB released in EVs could potentially act as a decoy for virus-neutralizing antibodies. MHC class II-modulatory property is shared by HSV-1 and BHV-1 gB, but it may differ in other alpha herpes viruses.

Room: van Weelde

Symposium Session 26 - EVs in cellular differentiation and development

Chairs: *Eva Rohde and Vasiliki Machairaki*

4:15-5:00 p.m.

OF26.1**Osteogenic differentiation of human mesenchymal stem cells using exosomes derived from mesenchymal stem cells undergoing osteogenic differentiation**

Xiaoqin Wang, Omar Omar, Forugh Vazirisani, Karin Ekström and Peter Thomsen

Department of Biomaterials, Sahlgrenska Academy, University of Gothenburg, and BIOMATCELL VINN Excellence Center of Biomaterials and Cell Therapy, Gothenburg, Sweden

Introduction: In a bone trauma, human mesenchymal stem cells (hMSCs) migrate and home to injury sites and further contribute to bone regeneration. However, the mechanism how hMSCs communicate with each other to promote osteogenic differentiation is still not clear. In this study, we aimed to explore whether exosomes derived from hMSCs undergoing osteogenic differentiation induced hMSCs to differentiate and further investigated the possible underlying mechanism. **Materials and methods:** hMSCs were cultured with osteogenic additives in exosome-free media for 21 days (D). Conditioned media (CM) were collected every 3 days. Exosomes (Exo) were isolated from CM by ultracentrifugation and added to undifferentiated MSCs. The osteogenic differentiation of Exo-treated MSCs was evaluated by ALP assay after 14D, calcium/phosphate assay and Alizarin red staining after 21D. Exosomal RNA was extracted and microRNA expression profile was detected on Qiagen miRCURY LNATM Universal RT microRNA PCR Human panel I. **Results:** ALP level was significantly increased in all groups of Exo-treated MSCs compared with untreated group. In contrast only D15-, D18- and D21-Exo induced significant increase of calcium and phosphate, which indicates mineralization, in Exo-treated MSCs. Exosomes from expansion (Exo-Exp), early differentiation (Exo-D3) and late differentiation (Exo-D21) showed altered microRNA expression profiles. There were 10 and 11 microRNAs having significant change when comparing Exo-D21 with Exo-Exp and Exo-D3, respectively. Interestingly, microRNAs related to osteogenic differentiation, such as miR-31 and miR-10b, were found among these microRNAs. **Conclusions:** The present observations indicate that exosomes derived from hMSCs undergoing osteogenic differentiation promote a variable degree of osteogenic differentiation and mineralization of MSCs. Moreover, the delivery of osteogenic specific microRNAs from the exosomes is a possible mechanism for the observed differentiation.

OF26.2**Human iPS-derived cardiac progenitors secrete cardioprotective extracellular vesicles**Nisa Renault¹, Anaïs Kervadec¹, Valérie Bellamy¹, Nadia el Harane¹, Hany Nematalla^{1,2}, Mathilde Lemitre¹, Florent Dumont³, Alexandre Colas⁴, Marie Mercier¹, Albert Hagège^{1,2}, Mark Mercola³, Jean-Sébastien Silvestre¹ and Philippe Menasché^{1,2}¹Institut national de la santé et de la recherche médicale, Paris, France;²Assistance Publique-Hôpitaux de Paris, France; ³Institut Cochin, Paris, France;⁴Sanford Burnham Prebys Medical Discovery Institute, La Jolla, California

Introduction: Extracellular vesicles (EV) have been reported to mediate the cardioprotective effects of cell therapy for the treatment of chronic heart failure (CHF). However, a direct comparison of their effects with those of their parent cells has not been reported. We explored whether human induced pluripotent stem-cell derived cardiac progenitors (hiPS-Pg) and their EV were effective in the treatment of CHF, and whether these effects were mediated by microRNA (miR). **Methods:** hiPS-Pg were cultured in serum-free media and total EV were collected by ultracentrifugation. EV number and size distribution were determined by dynamic light scattering. iPS-Pg and EV miR contents were determined by chip. EV pro-survival and proliferation potential on cultured cardiomyocytes (CM) were deter-

mined in vitro. In parallel, the therapeutic effects of hiPS-Pg and their EV were examined by injection of cells (n=17 mice), EV (n=19) or PBS (vehicle control; n=17) into the infarct border zone of nude mice with CHF. Six-weeks later, blinded echocardiographic assessment of heart function was performed. **Results:** A large number of EV were isolated (732 EV/iPS-Pg; mode: 184 nm). EV increased CM viability in serum-free media by 3.3 times and CM proliferation in a dose-dependent manner, with a 2.5 fold average maximum increase. More than 100 miR, whose main targets are currently under investigation, were identified that were highly enriched in EV as compared to hiPS-Pg. EV significantly improved cardiac function in CHF mice, with decreased left ventricular (LV) volumes (LVESV: -11%, p < 0.001; LVEDV: -4%, p=0.002) and increased LV ejection fraction (+14%, p < 0.0001) compared to baseline values. Surprisingly, EV out-performed the iPS-Pg cells which showed no significant improvement in LV volumes. **Summary/Conclusion:** hiPS-Pg secrete EV which are effective in the treatment of CHF, possibly, in part, by promoting the survival and proliferation of host CM through the transfer of specific miR.

OF26.3**Breast milk-derived extracellular vesicles influence the development of the epithelial and immunological gastrointestinal barrier**Marijke I. Zonneveld^{1,2}, Martijn J. C. van Herwijnen², Marije Kleinjan², Marit de Groot³, Marcela Fernandez-Gutierrez⁴, Alice Sijts³, Michiel Kleerebezem⁴, Leonie S. Taams⁵, Johan Garssen^{1,6}, Esther N. M. 't-Hoen², Frank A. Redegeld¹ and Marca H. M. Wauben²¹Division of Pharmacology, Department of Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht, The Netherlands; ²Department of Biochemistry & Cell Biology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands; ³Department of Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands; ⁴Host Microbe Interactomics Group, Department of Animal Sciences, Wageningen University, Wageningen, The Netherlands; ⁵Centre for Molecular and Cellular Biology of Inflammation, Division of Immunology, Infection and Inflammatory Disease, King's College London, London, UK; ⁶Nutricia Research Centre for Specialized Nutrition, Utrecht, The Netherlands

Introduction: The gastrointestinal barrier is both a physical barrier and an immunological site, which needs to mature in neonates. Breast milk reduces intestinal permeability and supports neonatal immunity and oral tolerance. However, the specific contribution of the different milk components in these processes is not fully understood. Here, we investigated the potential role of breast milk-derived extracellular vesicles (EV) on components of the innate and adaptive immune system, as well as on the epithelial barrier. **Methods:** We developed a density gradient-based (optiprep) protocol for isolation of naturally occurring EV from breast milk. The function of breast milk EV was assessed in vitro by determining their effects on activation and cytokine production of adult and neonatal CD4+ T cells, on activation of Toll-like receptor (TLR) reporter cell lines and downstream signalling, and on migration/proliferation of epithelial cells in a wound-healing assay. **Results:** Breast milk EV strongly inhibited aCD3/aCD28-induced T cell activation and proliferation and resulted in an overall reduced cytokine production. In this assay, high levels of CD45RA were maintained on T cells, indicating a more naïve and unresponsive state, while no increase of FoxP3 regulatory T cells was observed. EV also inhibited triggering of intracellular TLR while activation of extracellular TLR2 and TLR4 was not inhibited. Finally, epithelial cells showed a substantially increased wound-healing capacity in the presence of breast milk EV. **Summary/conclusion:** Breast milk EV reduces immune activation by inhibiting both T-cell responses and endosomal TLR activation, while stimulating epithelial cell proliferation and migration. Taken together, our data suggest a role for breast milk EV in both the development of the intestinal barrier and the immune system.

Room: Willem Burger
 Symposium Session 27 - Cell biology of EVs II
 Chairs: *Bernd Giebel and Guillaume van Niel*

4:15-5:00 p.m.

OF27.1
KRAS signalling regulates Argonaute 2 sorting into exosomes

 Alissa Weaver¹, Andrew Mckenzie², Daisuke Hoshino³, Diana Cha⁴, Robert Coffey^{5,6,7}, James Patton⁴ and Jeff Franklin⁸
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Introduction: Secretion of miRNAs in extracellular vesicles (EVs) is a novel form of intercellular communication. However, the mechanisms governing miRNA and miRNA-binding protein secretion into exosomes remain largely unknown. Recently, mutant KRAS was shown to regulate the content of RNA-binding proteins in exosomes. Based on these data, we hypothesized that KRAS may control the secretion of key RNA-binding proteins into exosomes. **Methods:** We performed biochemical and confocal imaging experiments in isogenic cell line models to investigate whether KRAS signalling controls the secretion of Argonaute 2 (Ago2) in extracellular vesicles. **Results:** We find that Ago2 is present in exosomes but not microvesicles purified from our cell lines. Furthermore, KRAS signalling controls the secretion of Argonaute 2 (Ago2) into exosomes. Ago2 phosphorylation downstream of KRAS leads to decreased association of Ago2 with multivesicular endosomes (MVE) and a concomitant decrease in Ago2 secretion in exosomes. Mechanistically, inhibition of MEK1/II reverses the KRAS-dependent phosphorylation of Ago2 and leads to increased Ago2-MVE association and Ago2 secretion into exosomes. Moreover, inhibition of Ago2 phosphorylation rescues the secretion of KRAS-dependent miRNAs in exosomes. **Summary/conclusion:** These data demonstrate regulation by KRAS signalling of Ago2 secretion into exosomes. Furthermore, they suggest a potential mechanism for regulating miRNA secretion into exosomes downstream of KRAS.

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OF27.2
Syntenin exosomes mediate SRC function in cell-cell communication

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We previously showed that the small PDZ-adaptor protein syntenin supports the biogenesis of exosomes and the loading of these vesicles with cargo. We showed that this syntenin function depends

on its direct interaction with the ESCRT accessory component ALIX, and on mechanisms involving syndecans and heparanase, on the luminal side, and the small GTPase ARF6 and its effector PLD2, on the cytosolic side (reviewed in Friand et al., 2015). Interestingly, in a model of SRC dependent colon carcinogenesis, SRC was shown to increase the tyrosine phosphorylation of syntenin and ALIX (Sirvent et al., 2012). Here, we investigated the role of SRC in the syntenin exosomal pathway. By gain and loss of function studies, we established that SRC and SRC kinase activity strongly regulate the exosomal levels of syntenin, ALIX, syndecans and CD63 but not that of CD9 or flotillin-1. We provided evidence that SRC impacts on syntenin exosomes by controlling endosomal budding and endocytic events upstream of ARF6. We showed that SRC requires syntenin to work in trans, via exosomes, to control the migration of endothelial cells. Our study highlights that SRC functions in cell-cell communication by controlling syntenin-dependent exosome biogenesis, and reveals a mechanism most probably supporting tumour-host transcellular signalling.

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OF27.3
Exosomes surf on filopodia to enter cells at endocytic hot spots, and shuttle with endosomes to scan the endoplasmic reticulum – a highway to the cell

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Exosomes are nanovesicles released by virtually all cells which can act as intercellular messengers by transfer of protein, lipid and RNA cargo. Their quantitative efficiency, routes of cell uptake and subcellular fate within recipient cells remain elusive. Here, we will describe a quantitative characterization of exosome cell uptake at the single cell – single vesicle level using an automated high content screening assay, single particle tracking in live cells as well as transmission electron microscopy. We reveal that exosomes are recruited as single vesicles to the cell body by surfing on filopodia, as well as filopodia grabbing and pulling motions to reach endocytic hot spots at the filopodial base – highly reminiscent of viruses and pathogenic bacteria. Following internalization, exosomes shuttle as intact vesicles within endocytic vesicles to scan the endoplasmic reticulum before being sorted into the lysosome as their final intracellular destination. Our data quantify and explain the efficiency of exosome internalization by recipient cells, establish a new parallel between exosome and virus-host cell interaction, and suggest unanticipated routes of subcellular cargo delivery.

Poster Presentations

Room: Willem Burger Foyer/Van Der Mandele and Arcadis Zaals

Poster Session 1 - Non-cancer biomarkers

Chairs: *Joanne Welton and Malene M. Jørgensen*

5:00-7:30 p.m.

PF1.01

Urinary extracellular vesicles number and diameter as biomarkers of renal disease in diabetic patients

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Background: About 30–40% of diabetic patients develop renal disease, although not all of these will progress to renal failure. Extracellular vesicles (EVs) reside in various body fluids, including urine. Their amount or molecular content are specific with disease progression and tissue damage. **Aim:** Finding the relationship between the urinary EVs number and size distribution and the progress of renal disease in type 2 diabetic patients (T2DM). **Patients and methods:** T2DM patients were enrolled to this study: UD – poorly controlled; CD – properly controlled diabetes. HbA1C below 7% was a threshold for diabetes treatment. Patients were further divided on two groups: NRF – without renal failure and RF – with renal failure, according to the estimated GFR below 60 mL/min/1.73 m². First morning urine specimens were collected for analysis. Number and diameter of EVs were determined by Tunable Resistive Pulse Sensing technology, which was used in qNano system. **Results:** RF patients had reduced number of EVs compared with NRF ones (2.57*E10 vs. 8.73*E10 count/mL; $p < 0.05$). We observed a positive correlation between the number of EVs and level of serum glucose in controls ($R = 0.6$; $p < 0.05$) and a negative correlation in UD ($R = -0.38$; $p < 0.05$) and RF ($R = -0.66$, $p < 0.05$) patients. In RF, we found also a negative correlation between EVs number and creatinine concentration ($R = -0.73$; $p < 0.05$). The size distribution study showed that CD patients had larger EVs (mode) than UD patients (114.5 vs. 108.67 nm; $p < 0.05$), nevertheless the mean MV diameter was smaller in controls than in the CD group (123.28 vs. 133.86 nm; $p < 0.05$). **Summary:** Our results indicate that number and size of urinary EVs can be considered as renal failure biomarkers. The lower amount of EVs corresponds with deteriorating and worsening renal function in diabetes in routine biochemical parameters.

Funding: This study was funded by the NCN grant (2012/07B/NZ5/02510).

PF1.02

Discovery of a Urinary exosome mRNA signature for the diagnosis of human kidney transplant rejection

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Introduction: Patients with end-stage renal disease usually undergo transplantation, but acute rejection carries a risk of 10–15%. Methods for monitoring clinical rejection including increases in serum creati-

nine and urinary protein excretion may not reflect subclinical rejection. Similarly, repeat biopsy may result in increased complications and cost. An accurate non-invasive method would allow for earlier diagnosis and minimize the amount of immunosuppression needed. Nucleic acids and proteins extracted from urinary pellets are currently the main source for such biomarker studies. Extracellular vesicles such as exosomes are released by cells and carry the parent cells' surface proteins and nucleic acids. Exosomes can be isolated and their nucleic acid profile interrogated. In the transplanted kidney, exosomes will originate from glomerular podocytes, renal tubular cells and from immune cells, generated during rejection. **Methods:** Urine samples were collected from patients undergoing a transplant kidney biopsy for clinical indications. A total of 28 urine samples (14 rejections, 14 non-rejections) were collected from 24 patients. RNA from both the urinary cell pellets and exosomes were isolated for expression profiling. We characterized 586 mRNAs associated with inflammation and 21 endogenous controls on the OpenArray(r) system. **Results:** Two samples were excluded from analysis due to low RNA yield. We detected the expression of 207 (34%) to 521 (86%) genes. Seven control genes with robust detection were used for normalization. Analysis of mRNA expression in urinary pellets and exosomes identified genes that were differentially regulated. The exosome samples identified 23 significantly differentially expressed genes. The genes identified from exosomal RNA performed significantly better in correctly differentiating between rejection and non-rejection compared to the cell pellet RNA. We are now validating these markers from exosomes in an independent patient cohort.

PF1.03

The content of microRNA is modulated in plasma extracellular vesicles in patients with end-stage chronic kidney disease

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Background: On-line haemodialtration(OL-HDF) was shown to improve intra-dialytic haemodynamics and cardiovascular outcome in randomized clinical trials. Despite potential explanations for such benefit, the biological mechanism/s is still unclear. In this study, we evaluated whether an evolved form of ON-HDF, the so-called mixed OL-HDF (mOL-HDF), may modulate the release and the phenotype of plasma extracellular vesicles(EVs), as known mediators of cell-to-cell-communication. **Methods:** A total of 30 bicarbonate haemodialysis (BHD) patients were randomized to continue BHD ($n = 15$) or to switch to mOL-HDF ($n = 15$). Plasma concentration, size, cellular origin and miRNA content of EVs was evaluated for 9 consecutive months and compared with EVs from healthy controls ($n = 10$). EV-induced angiogenesis and apoptosis of endothelial cells (HUVEC) and osteoblast mineralization of vascular smooth muscle cells (VSMC) were evaluated. **Results:** Endothelial cell-derived EVs were highest in BHD patients with no difference in number or size. However, anti-angiogenic miR-223 was increased in BHD in respect to healthy subjects and mOL-HDF. In vitro EVs from BHD reduced angiogenesis and increased endothelial cell apoptosis and VSMC calcification in respect to EVs from health subjects. Of note, EVs from mOL-HDF significantly increased angiogenesis and reduced endothelial apoptosis and VSMC calcification. The involvement of mRNA-223 was evidenced in vitro by cell transfection with mRNA-223 or antagomiR. **Conclusions:** mOL-HDF significantly reduced miRNA-223 expression in plasma-EVs, increased angiogenesis and decreased VSMC calcification. These data may give a biological

rationale to the clinical benefit of OL-HDF treatments. Further studies are needed to confirm these observations on large cohorts of patients and to draw a relationship with clinical end points.

PF1.04

RNA profiles in exosomes from kidney transplantation preservation fluid are associated with post-transplantation kidney function

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Introduction: Kidney transplantation is the preferred option for end-stage kidney disease patients, but shortage of donor kidneys is an increasing problem. Zero-hour biopsies analysis can assess kidney quality and have predictive value for short- and long-term transplantation outcome but require an invasive procedure and may not reflect the entire organ. Alternatively, non-invasive and more representative methods to assess graft quality are required. We hypothesized that kidney preservation fluid contains exosomes which harbour biomarkers associated with graft quality, allowing prediction of kidney function and risk for complications after transplantation. **Methods:** We developed a protocol to collect preservation fluid. Preservation fluid from kidneys from different donor types (living, donation after brain dead [DBD] or after circulatory death [DCD]) each associated with different risks for post-transplantation adverse kidney function, was analysed for the presence of exosomes by ultracentrifugation or commercial reagents followed by nanoparticle tracking, electron microscopy and density gradient analyses. Small RNA content was analysed by next-generation sequencing and qPCR array analysis. **Results:** Exosomes of around 132 nm with a density of 1.11 g/ml were isolated from the donor kidney preservation fluid. RNA was isolated and sequenced, identifying >2000 small RNAs, including miRNAs and tRNAs. Comparing exosomal small RNAs from different donor types identified 83 small RNAs that distinguish between donor types. Analysis of the most abundant small RNAs by qPCR array, comparing RNA profiles from DCD donor kidneys with delayed or immediate graft function, identified 4 small RNAs that are significantly associated with post-transplantation kidney function. **Summary/conclusion:** We present a novel exosome-based approach to assess donor kidney quality and post-transplantation function. This approach is non-invasive, easy to implement and can be easily translated to other donor organs.

PF1.05

Detection of *Plasmodium vivax*, *Trypanosoma cruzi* and *Fasciola hepatica* proteins by extensive proteomic analysis in plasma-derived exosomes

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Introduction: Malaria is caused by *Plasmodium vivax*, fasciolosis and Chagas diseases. These are three neglected tropical diseases responsible each year for millions of infections worldwide, including disease and death. Our present research effort is focused on the molecular characterization of plasma-derived exosomes from these diseases. Exosomes are nanovesicles of endocytic origin recently shown to be involved in inter-cellular communication and whose selective-cargo is being explored as novel therapeutic agents and diagnostic markers in parasitic diseases (Marcilla et al., J Extracell

Vesicles 3: 25040, 2014). **Methods:** Exosomes were obtained from plasma of infected patients (malaria and Chagas) and infected cows (*Fasciola hepatica*), as well as from healthy volunteers and animals as negative controls. Vesicle isolation was performed using size exclusion chromatography (SEC) using sepharose CL-2B, commercially available qEV columns (iZONTM). Profiling of individual fractions eluted from SEC was conducted by use of latex beads coated with antibodies against the tetraspanins CD9 and CD81, cryo-transmission electron microscopy (cryo-TEM) and Nanoparticle Tracking Analysis (NTA) to confirm their presence, size and concentration. An extensive LC-MS/MS proteomic analysis was performed in these samples comparing different pre-treatments, digestions, and mass spectrometers. **Results:** After defining the best protocol, several parasite proteins were identified in plasma-derived exosomes. **Summary/conclusion:** Plasma-derived exosomes from samples of these neglected tropical diseases contain parasite-specific proteins which could facilitate studies on mechanistic insights of pathology, antigen discovery for vaccination and disease biomarker identification.

Funded by Fundación Ramón Areces, Madrid, Spain.

PF1.06

A plasma extracellular vesicle protein signature as a biomarker for diagnosis of unstable angina

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Introduction: Annually, millions of patients enter the emergency department with chest pain suggestive of myocardial infarction (MI) or unstable angina (UA). MI can quickly be diagnosed based on elevated high-sensitive troponin. In contrast, for the diagnosis of UA (by definition troponin-negative) no diagnostic blood-based biomarkers are available. We hypothesized that a plasma extracellular vesicle (EV) protein signature can be used as a diagnostic biomarker for unstable angina. **Methods:** From the MINERVA acute chest pain cohort, 27 patients with UA and 31 with matched controls (i.e. matched for sex, age, history, risk factors and medication) were selected. LDL, HDL, remaining (REX) and total (TEX) plasma fractions were isolated using sequential precipitation and EV proteins were measured in these plasma fractions. SerpinF2, SerpinG1, SerpinC1, CD14 and Cystatin C were identified as potentially associated with UA by a mass spectrometry-based proteomics and were measured in all plasma fractions of the individual patients with an immunobead assay. Univariable and multivariable logistic regressions, as well as ROC-curves and AUCs, were used to evaluate the diagnostic performance of individual and combined biomarkers. This study complies with the declaration of Helsinki, informed consents were obtained and ethical approval was waived by the medical ethics committee. **Results:** The best individual markers to discriminate between UA and matched controls were SerpinC1 with the ratio HDL/REX and an AUC of 0.855 (95% CI 0.753–0.957); CD14 with the ratio HDL/REX and an AUC of 0.847 (95% CI 0.746–0.948) and SerpinC1 in the HDL fraction with an AUC of 0.844 (95% CI 0.741–0.947). The best combination of markers was SerpinC1-HDL+CD14-TEX+SerpinC1-LDL and showed an AUC of 0.933 (95% CI 0.865–1.000) and is being validated in the large MINERVA cohort of 2000 patients. **Summary/conclusion:** This study shows that EV proteins can be used as diagnostic biomarkers for unstable angina.

PF1.07

Impact of Age on Plasma Extracellular Vesicle Concentration and Protein Content in Humans

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The concentration and molecular composition of extracellular vesicles (EVs) in the plasma have been reported to vary in association with various disease states, and such alterations provide opportunities for the development of novel biomarkers. However, it is not known if plasma EV concentration and molecular composition might change during ageing in healthy individuals. Here, we report the results of measurements of plasma EV concentration and levels of several proteins of interest. EVs were isolated by ExoQuick from 75 males and females (2 samples/subject 30–70 years old) in the Healthy Aging in Neighborhoods of Diversity across the Life Span study. The EV concentration was measured by nanoparticle tracking and levels of selected proteins were measured by EV-array and ELISA. The uptake of fluorescently labelled EVs by leucocyte was evaluated by FACS analysis. The concentration of EVs in the plasma was negatively correlated with age and was independent of body weight, glucose levels and smoking status ($p < 0.01$ repeated measure multiple regression model). Body weight was positively correlated with EV concentration independently of age ($p < 0.01$). EV size was not affected by age. Analyses of 37 EV surface proteins revealed age-related increases in neuronal and oncogenic proteins ($p < 0.05$). Moreover, LC3, JnK, HSP70 and pTau-181 levels in EVs increased with advancing age. EVs were internalized by monocytes and B cells, but not T cells in a dose–time-dependent manner. Interestingly, EVs from old individuals were internalized by B cells significantly more than EVs from young individuals, but similar internalization was observed by monocytes. The plasma concentration of EVs declines during ageing and is positively associated with body weight. EVs from old subjects are more readily internalized by B cells, suggesting enhanced antigenicity of EVs during ageing. Our data provide insight into the role of plasma-derived EV role in ageing, and circulating immune cells.

PF1.08

Combined use of exosome miRNA, TREC and thymulin values as circulating biomarkers of immune senescence

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Introduction: The thymus is a central immune organ that shows rapid senescence with approximately 90% decline by the age of 50 years in human. However, significant individual variations exist and immunological age may diverge from chronological age. Historically, thymulin serum levels have been suggested to characterize thymic function. Currently, TREC numbers of circulating T-cells are gold-standards for evaluating thymic function. However, selective enrichment of thymic epithelial exosomes and characteristic miRNA profiling may provide further insight into age-related thymic decline. **Methods:** For the current pilot study, 5 verified healthy young (18–25 years old) and 5 verified healthy senior (55–65 years old) individuals provided peripheral blood samples following informed consent. Serum and cellular components were separated by Ficoll-gradient centrifugation. Serum thymulin values were measured by competitive ELISA using recombinant human thymulin. TREC values were obtained by TaqMan 3D PCR using PBMC genomic DNA. CD63-negative thymic exosomes were enriched then assayed for transcription levels of miR-27, miR-154, miR-200, miR-382, miR-431 and miR-465. **Results and discussion:** As expected, if single parameters of thymic age or function (like thymulin, TREC or miRNA levels) are compared between young and senior individuals, data show significant overlap, and differences lack statistical power. However, the combined use of multiple parameters may increase statistical power despite relatively low number of samples and relatively high level of individual differences. We plan to expand our research using further samples and

biomarkers to increase the statistical power of immune age profiling from peripheral blood samples.

PF1.09

Exosomal isoforms of microRNAs as a novel group of tissue-specific biomarkers

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The high resolution down to single nucleotide changes afforded by small RNA-seq has revealed a new dimension of heterogeneity during miRNA biogenesis, resulting in multiple sequence and length isoforms (isomirs) besides canonical miRNAs. Isomirs have been shown to associate with RISC and the translational machinery and can be functional by regulating miRNA targets. Similar to strand switching between -5p and -3p as the dominant miRNAs, recent studies have shown differential expression of isomirs across tissues and developmental stages. As exosomes (Exo) are key mediators of intercellular communication, we assessed holistic isomir expression profile specificity in Exo of 2 major biofluids, bovine blood and milk. Exo were isolated from calf plasma and additionally from milk by differential ultracentrifugation and purified on a sucrose density gradient. Total RNA including miRNA was isolated from Exo as well as whole milk and blood samples. Isomir variants were generated from canonical miRNA sequences obtained from mirbase and differential expression profiles were analysed via small RNA Seq and an in-house developed data analysis workflow. Breakdown of miRNA expression into isoform transcripts revealed a substantial amount of isomirs in blood and milk samples. Isomirs accounted for 45–64% of all mapped reads and, consistent with the prevailing view, showed a clear preference for miRNA alterations at the 3' end (93.8%) and not the 5' end (6.2%). Expression of isomir variants was not evenly distributed between miRNAs in both tissues with some miRNAs being almost exclusively expressed as isomirs (e.g. bta-mir-144 in milk). Significantly different isomir profiles could be detected between both biofluids (blood and milk) and between exosomes and cells of the same tissue. In summary, our findings highlight the still unexploited potential of miRNA isoforms as tissue-specific biomarkers especially in heterogeneous biofluids.

PF1.10

Comparison of circulating exosomal miRNAs during the process of liver fibrosis by chronic hepatitis B and C infections

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Introduction: Chronic hepatitis B (HBV) and chronic hepatitis C (HCV) infections are associated with activation of the hepatic stellate cells (HSCs) towards a myofibroblastic phenotype, resulting in excessive scar formation, and the development of liver fibrosis and its progression towards a cirrhotic state. Till date, the golden standard for liver fibrosis remains liver biopsy, which is however associated with pain, bleeding and sometimes even death. Other non-invasive scoring systems evade these drawbacks, but lack inter-stage specificity and are unable to detect early stages of the fibrosis process. Circulating exosomal miRNAs derived from the affected liver could be a potential stage-specific biomarker for this pathology. Research concerning the analysis of exosomal miRNAs in hepatitis B and C patients has already been conducted by some research groups but lacks significant comparison of the results of both groups, and their relationship with the stage of fibrosis progression. We aimed to verify the dynamic expression of various exosomal miRNAs in the

circulation of HBV and HCV patients and associate them with fibrosis staging. *Methods:* Blood samples were obtained from healthy individuals and HBV- and HCV-infected patients who underwent transient elastography (Fibroscan). Exosomes were purified from the plasma by Exoquick precipitation and their miRNA content was analysed by qRT-PCR. *Results/conclusion:* Obtained miRNA expression profiles were correlated with the calculated Fibroscan liver fibrosis staging. Analysed miRNAs were selected from literature based on correlation in expression patterns during HBV- and HCV-induced fibrosis, and include amongst others miR-122, miR-21 and miR-92a. Results will be discussed during ISEV 2016.

PF1.11

The utility of exosomes as a biomarker of N-methyl-D-aspartate receptor encephalitis

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Introduction: N-methyl-D-aspartate (NMDA) receptor encephalitis presents insidiously, with changes in behaviour, but subsequently develops psychotic features and disordered movement. Hypoventilation and coma can necessitate critical care support. IgG autoantibodies to NMDA receptor NR1 subunits are believed to stimulate an autoimmune neuroinflammatory state. A growing body of evidence implies a role of exosomes in immune modulation and as biomarkers of disease. This study aims to explore the potential of exosomes as a biomarker of NMDA receptor encephalitis. *Methods:* Serum from a patient with NMDA receptor encephalitis was compared with normal serum. The concentration and size of the microvesicles was determined by NanoSight tracking analysis (NTA). They were assessed for the presence of characteristic exosome markers (ALIX, TSG101, GAPDH) and NMDA receptor using western blotting. Mouse fibroblasts (LtK)-expressing NMDA receptors were cultured with pathological (containing NMDA receptor antibody) and normal serum. Media from these cultures were assessed using NTA and western blots. *Results:* The size of microvesicles in pathological and normal serum differed: peak diameter 26–32 versus 143–169 nm, respectively. Pathological serum contained 6.3 times as many particles of 50–150 nm diameter (805 vs. 128 x 108/mL). Western blotting of exosome pellets confirmed the presence of exosome markers and the NMDA receptor. Media from fibroblasts, cultured in the presence of NMDA receptor antibody, contained 3.7 times as many exosome-sized particles (4.78 vs. 1.28 x 108/mL). Exosome markers and the NMDA receptor were again present. *Summary:* This exploratory study shows a differing microvesicle population in NMDA receptor encephalitis and normal sera. They contain recognized exosome markers and NMDA receptor. The exosome population produced by mouse fibroblasts containing the NMDA receptor altered by exposing them to NMDA receptor antibody, potentially replicating the state found in disease.

PF1.12

Skeletal muscle-derived microvesicles expressing fatty acid transporters are increased by acute exercise

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Introduction: Skeletal muscle is a secretory organ important for intercellular metabolic communication. We propose that microvesicles (MVs) shed from skeletal muscle (SMMVs) may be involved. Furthermore, acute exercise (AE) induces release of SMMVs, in particular SMMVs expressing fatty acid transporter protein 4 (FATP4)

and CD36. The aim of this study was to investigate the presence of circulating SMMVs and to study the impact of AE on SMMVs and SMMVs expressing FATP4 and CD36 in particular. In addition, the influence of T2DM was explored because fatty acid uptake is increased in T2DM. *Methods:* Obese males with (N = 13) and without (N = 14) T2DM underwent a 60-min bicycle exercise test. Blood samples were collected before and immediately after AE. SMMVs were identified by flow cytometry as particles positive for phosphatidylserine and muscle-specific Sarcoglycan and either FATP4 or CD36. Study groups were compared by Mann–Whitney U test and AE-induced MV release by Wilcoxon signed-rank test. The study was approved by the local ethical committee and informed consent was obtained before inclusion. *Results:* At baseline, total MV numbers were increased by 10% in T2DM compared to controls ($p < 0.05$) and SMMVs tended to be increased, whereas SMMVs expressing FATP4 or CD36 were unaltered. In T2DM, AE increased total SMMVs by 42% ($p < 0.05$), whereas SMMVs carrying FATP4 or CD36 were increased by 130% ($p < 0.01$) and 120% ($p < 0.02$), respectively. In controls, total SMMVs and SMMVs carrying FATP4 were unaltered after AE, whereas CD36-positive SMMVs were increased by 15% ($p < 0.02$). *Conclusion:* This study presents novel data demonstrating the presence of circulating SMMVs. AE triggers release of SMMVs into the circulation, and their fatty acid transporter expression is dependent on the presence of T2DM. We hypothesize that SMMVs shed upon muscle contraction may be involved in intercellular communication and provide novel insight to the metabolic condition of their parental skeletal muscle cells.

PF1.13

Extracellular vesicles from lung epithelial cells as early indicator of paraquat exposure

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Introduction: Despite its high toxicity, the herbicide paraquat (PQ) is still widely used in many countries worldwide. Exposure of humans to PQ causes fatal lung damage due to PQ accumulation in airway cells and extensive intracellular redox cycling. Early detection and treatment initiation of PQ poisoning is yet impossible due to a lack of sensitive markers. Since extracellular vesicles (EVs) are released upon cell stress, we hypothesized that the release of EVs may indicate PQ exposure before cytotoxic effects are detectable. *Methods:* After 24 and 48 h exposure of bronchial epithelial (BEAS-2B) cells to various PQ concentrations (12.5–1000 μ M), cell viability was assessed. EVs in the cell supernatant and after isolation were quantified by flow cytometry. EVs were further characterized by tunable resistive pulse sensing (TRPS) and transmission electron microscopy (TEM). *Results:* While 24-h exposure of BEAS-2B cells to 12.5–200 μ M PQ exerted minor cytotoxicity, viability was significantly reduced by 1000 μ M PQ compared with unexposed cells. After 48 h, a clear concentration-dependent reduction in cell viability was found. EV release in the supernatant did not change upon PQ exposure for 24 h compared with unexposed cells. However, 12.5–200 μ M PQ for 48 h increased EVs in the culture medium by 1.2- to 1.6-fold compared with unexposed cells. After isolation, TEM confirmed the presence of membrane surrounded by round-shaped vesicles; flow cytometry revealed the presence of the exosomal proteins CD63 and CD81. PQ-induced EVs' diameter (83–502 nm; mode size: 116 nm) did not significantly differ from the diameter of EVs from control cells (77–578 nm; mode size: 115 nm). *Summary/conclusion:* Our data suggest that EVs indicate PQ exposure of lung epithelial cells before substantial cell damage occurs. These findings encourage to further explore the characteristics and cellular functions of PQ-induced EVs and to determine their levels in body fluids of humans exposed to PQ.

Poster Session 2 – EVs and cancer

Chairs: Janusz Rak and Valentina Minciacci

PF2.01

Induced cell damage altered proteome and miRNA profile of melanoma exosomes and interactions with tumor stroma cells

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Introduction: Increasing evidences suggest that tumor-derived exosomes have a role in promoting cancer development and metastasis formation. Recently it was also described that these exosomes may contribute to the adaptive response of the tumor cells to microenvironmental stress. The aim of this study was to investigate the effect of suboptimal conditions on melanoma cell-derived exosomes. **Methods:** Quantitative and qualitative analyses were performed to compare exosome samples produced by the B16F1 mouse melanoma cell line under normal or suboptimal conditions (cytostatic, oxidative or heat stress). Number of the exosomes were measured by NanoSight NS500 device, their miRNA content and whole proteome were analyzed by SOLiD 5500xl technology and mass spectrometry (LC-MSMS on LTQ Orbitrap Elite [Thermo] mass spectrometer), respectively. Since mesenchymal stem cells (MSCs) are important players of the tumor stroma, uptake of stress-exposed cell-derived exosomes by MSCs was also investigated by high throughput microscopy. **Results:** Under stress conditions, significant changes were observed in the number and content of exosomes. Diversity of exosomal miRNAs increased under oxidative stress, but decreased in the two other cases. All type of stress conditions caused significant changes in qualitative and quantitative properties of exosomal proteome and miRNA profile. Kinetics of the exosome uptake by MSCs was also different in each case. **Conclusion:** Based on our results, we hypothesize that the microenvironmental stresses affect the intercellular communication between the tumor cells and the stromal cells via altered exosome profile. Induced alterations could have influence for metastatic potential of tumor cells and might predict the efficiency of different type cytostatic effects, e.g. chemotherapy, oxidative stress or heat induced cell damage. This research was funded by OTKA PD 84064, OTKA K 112493, TAMOP-4.2.2-A-11/1/KONV-2012-0025 and IPA HUSRB/1203/214/230.

PF2.02

Extracellular vesicles from mesenchymal stromal/stem cells transfer miRNAs 205 and 31 and inhibit breast cancer metastasis by regulating UBE2N/Ubc13

Krishna Vallbhaneni and Radhika Pochampally
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Extracellular vesicles (EVs) derived from mesenchymal stem/stromal cells (hMSCs) have recently gained attention for their tumour-supportive characteristics. Based on our previous studies characterizing EVs from stressed hMSCs (hMSC-EVs) and their role in primary tumours, we investigated the role of hMSC-EVs in breast cancer metastasis using metastatic MDA-MB-231 (231) and organotrophic sub-lines (231BrM-2a,

231LM-4175, 231BoM- 1833). We show that hMSC-EVs significantly suppressed the metastatic potential of parental cell lines (MDA-MB-231) when compared with their organotrophic sublines that colonize bone (231BoM- 1833), brain (231BrM-2a) and lungs (231LM-4175) in vivo. miRNA expression assays revealed that the parental MDA-MB-231 cell line expressed significantly lower levels of miR-205 and miR-31 compared with their organotrophic sublines. When MDA-MB-231 cells were incubated with hMSC-EVs, the expression levels of miRNAs 205 and 31 were increased. Further bioinformatic assays demonstrated that miRNAs 205 and 31 were capable of modulating UBE2N/Ubc13 – a known metastasis regulating gene. Silencing UBE2N/Ubc13 expression significantly suppressed migration, invasion and proliferation of MDA-MB-231 cells demonstrating its role in tumour metastasis. Studies described here suggest that hMSC-EVs support primary breast tumour progression in both non-organ-committed and organotrophic cells. However, hMSC-EVs suppress the metastasis of breast cancer cells that are not organ-committed through the UBE2N/Ubc13 pathway.

PF2.03

Exosomes from human colorectal cancer induce a tumour-like behaviour in colonic mesenchymal stromal cells

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Introduction: Cancers release high levels of nanovesicles called exosomes, acting as vehicles for biochemical messages towards neighbour or far cells. Mesenchymal stromal cells (MSCs), as ubiquitous architects of the microenvironment, play a key and contradicting role in tumour progression. Colorectal cancer cell-derived (CRC) exosomes may affect both neighbour and distant cells because they freely circulate in the bloodstream. We thus investigated the effect of human CRC exosomes on tissue-specific colonic (c) MSCs that we recently isolated. **Methods:** cMSCs, obtained from colonic mucosa, were exposed to primary or metastatic CRC exosomes and we analysed them by light and electron microscopy, proliferation assays in 2D and 3D cultures, migration and invasion assays, and Western blot and confocal analysis for carcinoembryonic antigen (CEA) and vacuolar H⁺-ATPase. **Results:** CRC exosomes express high levels of CEA and induce in cMSCs: i) atypical morphology (i.e. microvilli, pseudopods, vesicles) and functions (i.e. higher proliferation, migration, invasion) usually present in highly activated or transformed cells; ii) production of large 3D spheroids; iii) an acidic extracellular environment associated with iv) increased expression of CEA and a plasma membrane redistribution of vacuolar H⁺-ATPase. Colon cancer-derived MSCs recapitulate the changes observed in colonic MSCs exposed to CRC exosomes, forming peculiar umbilicated spheroids and therefore mimicking in vitro the events occurring in vivo upon exposure to native exosomes in the cancer cell mass. **Summary/conclusion:** CRC exosomes are able to induce morphological and functional changes in colonic MSCs, which may in turn favour tumour formation and the malignant progression, recapitulating the in vivo exposure to native exosomes inside the cancer cell mass. Our results suggest that local and/or systemic control of CRC exosomes release could represent a novel approach and a major improvement in cancer management.

PF2.04

EV transfer of the proinflammatory signal from cells carrying lymphoma-associated MyD88 mutation

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Introduction: Innate immune system protects organisms against pathogens and endogenous damage. This is however a double-edged sword as unregulated inflammation may lead to pathology, including autoimmune disease and even cancer. Toll-like receptors, interleukin-1 receptor and inflammasomes are pivotal in these processes. Another discovery in oncology is the realization of the role of extracellular vesicles (EVs) in cancer development and dissemination of their signals to the surrounding tissue. The level of EVs in peripheral blood is elevated in patients with cancer and their role in cancer has up to now been focused mainly on the miRNAs; however, proteins of the signalling pathways might also strongly affect the cellular phenotype. **Materials and methods:** EVs were isolated from overexpressed HEK293 cells and lymphoma cell line by ultracentrifugation. Confocal microscopy was used for detection of signalling complexes. Dual luciferase test was used for detection of cell activation. **Results:** Activated lymphoma cells affect other non-transformed cells within the organism through secretion of inflammatory cytokines. We investigated an alternative mechanism via EVs. We showed that signalling molecules per se can be transferred from overexpressed HEK293 cells and activated lymphoma cells via EVs to other cells. Signalling molecules formed active complexes and triggered activation of the inflammatory pathway in recipient cells thus propagating the inflammation. **Conclusions:** This mechanism should be most relevant for the lymphomas harbouring mutated constitutively active MyD88, where the cancer cells could spread inflammatory signal and might provide the necessary survival signal needed for the development of cancer systemically to the non-cancer cells.

PF2.05

The role of extracellular vesicles in the intercellular response to stress
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Introduction: When cancer cells are treated with ionizing radiation, the resulting DNA damage can also be observed in neighbouring, non-irradiated cells. We have previously shown that this spread of deleterious effects from irradiated to non-irradiated cells, which is known as the bystander effect, is mediated by extracellular vesicles (EVs). We also showed that uptake of EVs released by irradiated cells leads to a long-term genomic instability that also appears to involve the activity of specific non-coding RNAs. However, it is unclear whether this EV-mediated phenomenon is a peculiar phenomenon restricted to the effects of ionizing radiation or whether it is a more general response to stressful situations. We therefore tested whether other stressors including heat shock and cytotoxic drugs can also induce an EV-mediated bystander effect. **Methods:** Human MCF-7 breast carcinoma cells were exposed to elevated heat levels or treated with the cytotoxic drug cisplatin. Following treatment, the cells were allowed to condition the media for 24 h and EVs were extracted by differential centrifugation before being placed onto fresh MCF-7 cells. Levels of DNA damage and apoptosis were measured using comet assays and nuclear staining with DAPI, respectively. **Results:** Our preliminary data reveal that heat stress and cisplatin treatment caused the release of vesicles capable of inducing bystander effect. Further analysis of the bystander cells reveals changes in activity of a number of signalling pathways and alterations in gene expression patterns. **Conclusion:** The results of these experiments suggest that the bystander effect mediated by EVs is not limited to the consequences of ionizing radiation but may represent a fundamental mechanism by which cells respond to many forms of stressful stimuli. This work offers insight into the processes of cells during normal homeostasis and the response to supraphysiological conditions.

PF2.06

The volatile anaesthetic agent sevoflurane influences cancer-related miRNAs in circulating extracellular nanovesicles
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Introduction: There is evidence that volatile anaesthetic gases (VAGs) administered during surgical resection of solid tumours may enhance cancer cell growth and the risk for metastasis. Extracellular nanovesicles (EVs) may be released from the tumour during surgery. Their nucleic acid cargo is known to mediate tumour progression and could be influenced by VAGs. **Methods:** We assessed the impact of the VAG sevoflurane on EV miRNA in patients undergoing non-cancer surgery (n = 6). Informed consent was obtained and the study was approved by the local IRB. miRNAs were isolated from serum EVs and from blood cells at the awake state and after 40 min of anaesthesia. The presence of EVs was confirmed by transmission electron microscopy and nanoparticle tracking analysis. Unbiased profiling of miRNAs from EVs and blood cells was performed by small RNA-Seq. For data analysis and miRNA target scanning, a commercially available gene expression and pathway analysis software was used. Only miRNAs meeting predefined cut-off values (base mean = 50, log2foldchange = 11 and Padj < 0.05) were analysed and only experimentally confirmed relationships were considered. **Results:** The VAG influenced only EV-encapsulated miRNAs but had no significant effect on miRNA expression in blood cells. Among the EV-derived miRNAs, the software identified cancer, cellular development and cellular growth/proliferation as top regulated networks. Four miRNAs were identified as primarily cancer-related (miR-26a-5p, miR-409-3p, miR-493-5p, miR-205-5p, p-value range: 4.01E-02–8.27E-05). In addition, miR-26a-5p and miR-205-5p (p-value range: 6.29E-03–9.69E-04) were associated with cell cycle/cellular development and miR-493-5p (p = 2.92E-05) with cell death and survival. **Conclusion:** Given the important function of circulating EVs in cell-cell communication and cancer, our data suggest that VAG-induced changes in the nucleic acid cargo of EVs could be a mediator of this anaesthetic technique's effects on long-term survival of cancer patients.

PF2.07

Transfer of multidrug resistance between leukaemia cells via extracellular vesicles

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Introduction: Chemoresistance of tumour cells is a major issue for the treatment of acute myeloid leukaemia (AML). This chemotherapeutic resistance is established by clonal selection of resistant leukaemic cells. However, a horizontal transfer of chemoresistance has recently been discovered between tumour cells via extracellular vesicles (EVs). Several researches demonstrated a transfer of multidrug resistance proteins, miRNA and mRNA, from multiresistant cells to sensitive ones via EVs. The aim of this research is to investigate the role of EVs in chemoresistance in AML. For this purpose, two strains of the promyelocytic leukaemia HL60 cell line have been analysed: the sensitive one (HL60) and a multiresistant one (HL60/AR). The latter overexpresses multidrug resistance – associated protein 1 (MRP1) conferring resistance to daunorubicin among others. **Methods:** The production of EVs by HL60/AR (EVs/AR) was first investigated by transmission electron microscopy. The chemoresistance transfer via EVs/AR was then analysed by treating HL60 with EVs/AR isolated by ultracentrifugation. The interaction between HL60 and EVs/AR was measured by flow cytometry. The viability of HL60 treated with EVs/AR after daunorubicin treatment was assessed by a MTT cytotoxicity assay. The daunorubicin retention and the expression of MRP1 were finally evaluated by a flow cytometry drug retention assay. **Results:** HL60/AR spontaneously produces EVs/AR that

can interact with HL60. This binding or incorporation of EVs/AR confers an advantage in terms of survival to HL60 after a daunorubicin treatment. EVs/AR-treated cells also begin to express MRP1. *Conclusion:* This study demonstrates a real benefit of EVs from resistant cells on sensitive cell viability. This resistance transfer could be due to a direct transfer of MRP1 by EVs/AR or to a transmission of nucleic acids (microRNA or messenger RNA) modifying target cells' phenotype. These hypotheses will be tested in the future experiments.

PF2.08

Detection, characterization and function of extracellular vesicles in resistant melanoma

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Extracellular vesicles are nano-sized structures that are released by all cell types under both physiological and pathological conditions. It has been shown that different diseases can induce specific changes in the levels of extracellular vesicles secretion so that different amounts and/or different content profiles can be studied and potentially used as biomarkers. As extracellular vesicles can be released by "donor" cells and taken up by "recipient" cells, it has been hypothesized that they are important players in influencing key biological functions by delivering and transporting cytokines, growth factors, proteins, mRNAs and microRNAs. Recently, extracellular vesicles have also been identified as new messengers in transferring drug resistance to still sensitive or "drug-naïve" cells. In melanoma patients, drug resistance is a pressing clinical problem. Despite the promising initial results obtained with vemurafenib and dabrafenib (BRAF kinase inhibitors) in the clinic, it soon became evident that these molecules were not able to provide durable responses, as resistance to treatment soon develops within months in almost all patients. The aim of my project is to understand the potential involvement of extracellular vesicles in the "spreading" of drug resistance. The content of the extracellular vesicles released by melanoma cells and their corresponding cells that have been made drug resistant will be analysed. Preliminary results show that sensitive melanoma cells acquire the drug resistant phenotype if co-cultured with extracellular vesicles released by resistant cells. This suggests an involvement of extracellular vesicles in propagating important tumourigenic properties.

PF2.09

D-type CpG oligonucleotide encapsulating and antigen harbouring exosomes improve therapy of tumours

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Please see OPW3.3

PF2.10

Oral delivery of the chemotherapeutic drug paclitaxel via bovine milk exosomes

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Introduction: Chemotherapeutics, given intravenously, result in spikes in blood level and toxicities. We recently showed that bovine milk-derived exosomes can be delivered orally as nano-carriers for therapeutic applications. Here, we show that a widely used chemotherapeutic drug paclitaxel (PAC) can be given orally in exosomal (Exo) formulation to enhance its efficacy, and that it lacks toxicities. *Methods:* PAC was incubated with the exosomes in the presence of ethanol: acetonitrile (1:1) for drug loading. Anti-proliferative activities against human lung (A549) and ovarian (A2780) cancer cells were assessed by MTT assay, and antitumour activity was determined against lung and ovarian cancer xenografts in nude mice. Exo-PAC was administered by oral gavage and compared with the free drug given i.p. – three doses weekly each at 2.7 mg/kg against ovarian and 4 mg/kg against lung cancer; control animals received vehicle alone. Potential toxicities of Exo and Exo-PAC were determined in wild-type mice. *Results:* PAC was loaded at levels of 10–20%. Exo-PAC exhibited significantly improved anti-proliferative activity against the A549 cells compared with the free drug; higher efficacy of Exo-PAC was also observed against the ovarian cancer cells. Oral administration of Exo-PAC showed enhanced growth inhibition of lung tumour and the response was dose dependent. Higher efficacy of Exo-PAC was also observed against the ovarian tumour growth; the efficacy of Exo-PAC functionalized with folic acid (68%) was higher than Exo-PAC (41%). Finally, daily oral dosing of mice for 15 days with Exo and Exo-PAC showed no systemic toxicity and also reduced PAC-associated toxicity based on analysis of cytokines and inflammatory markers. *Conclusion:* This is the first demonstration of effective oral delivery of the chemotherapeutic drug, PAC, as an Exo formulation against lung and ovarian cancers.

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for CD73. Co-cultured NK cells with HUVECs or EXs alone showed similar results. CD73, which is negative on inactive NK cells, was observed after a co-culture with EXs alone. Furthermore, a diminution of NK cell cytotoxicity was observed after a co-culture with EXs. *Summary/conclusion:* Our results showed that CD73 acquisition on NK cells after contact with EXs carrying this nucleotidase could have an immunosuppressive effect on these cells.

PF3.06

Contact-dependent and -independent co-cultures of allogeneic human immune cells yield heterogeneous vesicle phenotype but conserved cellular phenotype

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Introduction: Extracellular vesicles (EVs) can be employed by the immune system to orchestrate immune responses. A new functional phenotype can be induced in the EV-recipient cell, which is linked to the molecular content of the EVs. Hence, a molecular profile of EVs is interesting for functional indications. Here, an extensive phenotyping of a membrane sub-proteome of EVs from an in vitro-induced immune response was performed, comparing any effects of the presence/absence of physical contact between the two interacting cell populations. *Methods:* Human peripheral blood mononuclear cells (PBMCs) were isolated from two individuals with mismatched tissue types. One PBMC population was irradiated. A 6-day co-culture of the allogeneic PBMCs was created with or without physical contact between the two cell populations. EVs from the cell culture media were captured on the EV Array containing antibodies against 24 different markers, including general EV and immune markers. EV detection was performed with a cocktail of antibodies against CD9, CD63 and CD81. The cellular phenotype of activated CD4+ T cells was determined by flow cytometry, focusing on regulatory markers. *Results:* Comparable levels of general vesicle-associated markers were observed for EVs from all culture combinations and across individuals (n = 3; biological). However, a prominent heterogeneity was found for the remaining investigated markers. In contrast, the cellular phenotype of the activated CD4+ T cells was conserved and reproducible, showing marker enrichment, when physical contact was present between the two cell populations. *Summary/conclusion:* Primary immune cells produce EVs during in vitro culturing and activation. The detected phenotype of these EVs was very heterogeneous, while the investigated cellular phenotype was conserved. This could suggest a more adaptable role for EVs in immune responses, while the requirements for the cellular phenotype are less flexible.

PF3.07

Immune-stimulatory properties of serum exosomes from (pre-) diabetic mice

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Introduction: Type 1 diabetes (T1D) is a chronic autoimmune disease that results from the destruction of insulin-producing pancreatic β -cells by autoreactive T-lymphocytes. Serum exosomes (EXO) are more abundant in non-obese diabetic mice (NOD) with spontaneous diabetes raising the question of their contribution to the immune deregulation leading to the disease. Here we studied the influence of serum exosomes on immune cell activation ex vivo. *Methods:* EXOs were isolated from serum drawn from pre-diabetic versus sponta-

neously diabetic NOD mice through size exclusion chromatography. Phenotypical changes of EXOs were analysed by nanoparticle tracking analysis, automated electrophoresis and detection of surface markers by cytometric bead assays. Biological activity of (pre-) diabetic serum EXOs on murine spleen cells was assessed in vitro using flow cytometry, confocal microscopy and ELISA analysis. *Results:* Serum EXOs from both pre-diabetic and diabetic (T1D-EXOs) mice expressed CD63+ CD9+ tetraspanins and were approximately 100 nm in size. Spleen cells readily internalized serum EXOs that co-localized with the early endosomal antigen 1 marker. T1D-EXOs reduced TNF α and IL-10 cytokine secretion in comparison with pre-diabetic EXOs. Flow cytometry data on surface and activation markers of T1D-EXO target cells will be presented. *Summary/conclusion:* In this study, we thought to elucidate the role of serum EXOs in T1D immune imbalance. We demonstrate that T1D serum EXOs modulate cytokine secretion and phenotype of murine immune cells. This evidence suggests that serum EXOs may fuel activation of diabetogenic effectors and might be a useful target in the development of T1D therapeutics.

PF3.08

Phenotyping of leukocytes and leukocyte-derived extracellular vesicles

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Introduction: Extracellular vesicles (EVs) have demonstrated an involvement in modulating the immune system. It has been proposed that EVs could be used as biomarkers for detection of inflammatory and immunological disorders. Consequently, it is of great interest to investigate EVs in more detail with focus on immunological markers. *Methods:* Five major leukocyte subpopulations were isolated from peripheral blood mononuclear cells from healthy donors and cultured separately for 2 days. Subsequently, the leukocyte-derived EVs were phenotyped for a panel of 34 immunological and vesicle-related markers using the EV Array. For comparison, the phenotype of EVs present in plasma from healthy donors was also investigated. For the leukocyte monocultures, the cellular expressions of CD9, CD63 and CD81 were determined by flow cytometry and compared with the expression pattern observed for leukocytes present in whole blood. *Results:* The majority of the lineage-specific markers, used for identification of the parent cell types, could not be detected on EVs released from monocultures of the associated cell type. In contrast, the vesicular presentation of CD9, CD63 and CD81 correlated to the cell surface expression of these markers, however, with few exceptions. In addition, the leukocyte-derived EVs displayed phenotypic differences in the 34 markers investigated. Regarding the cellular expression of CD9, CD63 and CD81, a variation in the expression of these markers were observed between the leukocytes present in whole blood and the cultured leukocytes. *Summary/conclusion:* The current data demonstrate that the cellular and vesicular presentation of selected lineage-specific and vesicle-related markers may differ, supporting the accumulating observations that sorting of molecular cargo into EVs are tightly controlled and might not represent a mirror image of the parent cell.

PF3.09

Impact of pre-eclampsia-associated extracellular vesicles on the monocyte-macrophage system: an in vitro analysis

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Introduction: Pre-eclampsia is a serious, pregnancy-specific multi-system disorder. Both maternal and foeto-placental factors are

involved in its pathogenesis, including immunologic intolerance between the foetus and mother and the imbalanced activity of the maternal monocyte–macrophage (Mo–Mph) system. The aim of our study was the characterization of the effects of pre-eclampsia–associated circulating EVs (P-EV) on the monocytes. *Methods*: THP-1 cell line was used as a monocyte model system. The EV-binding capacity of THP-1 cells as well as the EV-induced migratory and adhesive properties and phagocytic activity were monitored. Adhesiveness of THP1 cells was characterized by both multicolour immunophenotyping of cell surface adhesion proteins (CD44, integrin α 2, integrin β 1, integrin β 7) and also by an impedance-based real-time method. The effects of P-EVs on the migratory activity of THP-1 cells were documented by holographic microscopy. The uptake of PKH-labelled P-EVs by phagocytosis was evaluated by flow cytometry. EVs isolated from the plasma of healthy pregnant women (HP-EV) were used as biological controls. *Results*: Although P-EVs and HP-EVs bound to THP-1 cells equivalently, THP-1 cells phagocytosed significantly lower amounts of P-EVs. This may be explicable by the significantly higher expression levels of CD47 “don’t eat me” signal and the significantly lower externalization of the phosphatidylserine, an “eat me” signal on P-EVs. Furthermore, by reduced exofacial CD44 and integrin α 2, P-EVs may have a significant impact on adhesiveness and change the migration activity of the THP-1 cells. *Summary*: Circulating P-EVs induce a different functional pattern of cellular responses in THP-1 cells, which is reflected by i) a differential cell surface adhesion protein distribution, ii) a reduced cell motility and iii) decreased phagocytosis. Consequently, circulating pre-eclampsia–associated EVs may have a pathogenic role by modifying the Mo–Mph system.

PF3.10

Placenta-derived extracellular vesicles induce differentiation of monocyte into decidual macrophage-like phenotype

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Introduction: Macrophages comprise 20–30% of the leukocytes in the pregnant uterus and their cell number remains constant throughout pregnancy. The monocytes migrate into the decidualized endometrium in the pre-implantation period and differentiate into decidual-specific macrophages upon exposure to the local microenvironment. Decidual macrophages are located close to the invaded extravillous trophoblasts and contribute to placental development. The placenta produces large quantities of extracellular vesicles during pregnancy which are involved in foetomaternal communication. In this study, we hypothesize that placenta-derived extracellular vesicles polarize the differentiation of human monocytes into decidual macrophages. This action in turn regulates placental remodelling at the foetomaternal interface. *Methods*: First trimester human placentas were obtained with informed consent from patients who had undergone termination of pregnancy. Placenta-derived extracellular vesicles were isolated from the conditioned medium of the placental villous tissue explants culture using ultracentrifugation. Human peripheral blood monocytes were differentiated into macrophages by treatment with macrophage colony-stimulating factor in the presence or absence of placenta-derived extracellular vesicles during the differentiation process. The phenotypes and biological activities of the differentiated macrophages were compared. *Results and Summary*: Placenta-derived extracellular vesicles drove the differentiation of macrophages with a higher expression of decidual macrophages phenotypic markers. These macrophages also had an altered cytokine secretion profile, which induce trophoblast invasion. In sum, placenta-derived extracellular vesicles polarize the differentiation of macrophages towards a decidual macrophage-like phenotype, which could regulate placental development.

Poster Session 4 - EVs in the tumor microenvironment

Chairs: *Pilar Martin-Duque and Jason Webber*

PF4.01

Gastric cancer cell–derived extracellular vesicles modulate E-cadherin expression, invasion and migration of normal cells from the tumour microenvironment

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PF4.02

Functional analysis of extracellular vesicles in scirrhous type gastric cancer microenvironment

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Introduction: Scirrhous type gastric cancer (GC) is highly metastatic and is characterized clinically by rapid disease progression and poor prognosis. Therefore, better understanding of the pathological and biological basis of scirrhous type GC is necessary to improve the diagnosis and the treatment. Extracellular vesicles (EVs) are small membrane vesicles that are secreted by various cell types including cancer cells and surrounding stromal cells. Several lines of evidence support that some components of EVs derived from cancer stroma, including microRNAs (miRNAs), function to promote the progression, growth and spread of cancer. However, the precise mechanisms controlling cancer stromal interaction are not fully understood. In this study, we aimed to investigate the difference of miRNA expression in EVs between cancer stromal fibroblasts (CaFs) and normal fibroblasts (NFs) and identify the specific miRNAs of CaF-derived EVs. **Methods:** To check the differences of EV number between NFs and CaFs, we performed nanoparticle tracking analysis (NTA). To confirm the presence of EVs obtained by ultracentrifugation, western blot analyses were performed. Furthermore, we also investigated the effect of EV derived from NF and CaF on the invasive activity of HSC-44PE scirrhous type GC cell line by invasion assay. To identify specific miRNAs in CaF-derived EVs, miRNA microarray analysis was performed. **Results:** EV numbers did not differ between NF and CaF. CD9 and CD63 proteins, as markers of EVs, were detected on each fibroblast-derived EVs. There is no difference in these marker expressions between NF-derived EVs and CaF-derived EVs. However, CaF-derived EVs could impact on GC cell invasion but not NF-derived EVs. Microarray analysis identified several miRNAs that were upregulated in CaF-derived EVs as compared with those derived from NF-derived EVs. **Conclusion:** These data suggested that CaF-derived EVs contribute to the invasion activity of scirrhous type GC.

PF4.03

The role of anaplastic large cell lymphoma–derived exosomes in the tumour microenvironment

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Introduction: Lymphoma, one of the most common types of blood cancer, manifests itself mainly as Hodgkin lymphoma or non-Hodgkin lymphoma (NHL). Anaplastic large cell lymphoma (ALCL) is an aggressive type of T-cell NHL and represents the most frequent large cell lymphoma in childhood. The current WHO classification recognizes two distinct types of systemic ALCL based on the presence of the anaplastic lymphoma kinase (ALK), the ALK-positive and the ALK-negative ALCLs. In the former type, expression and activation of ALK is the result of chromosomal translocations involving the alk gene locus, the most frequent being the t(2;5) leading to the chimeric NPM. ALK oncogenic kinase. The disease disseminates in the lymph nodes, spleen, bone marrow, skin as well as other extranodal sites. Although, there have been many studies on the mechanisms of ALCL lymphomagenesis, as well as therapeutic strategies, very little is known about the bidirectional crosstalk between the microenvironment (TME) and ALCL. **Methods:** Exosomes derived from various ALCL cell lines have been isolated and characterized by nanoparticle tracking analysis, transmission electron microscopy and western blotting for their size, shape and protein composition. Stroma cells have been co-cultured with the lymphoma cells or have been educated with exosomes and the effects have been investigated. **Results:** We have studied the role of the ALCL exosomes in activating the tumour stroma (fibroblasts and mesenchymal stem cells) as well as being responsible for the horizontal transfer of oncogenes and growth promoting signalling molecules. We have also tested the ability of the TME to either protect or sensitize the lymphoma cells upon treatment. **Conclusions:** Better understanding of the interactions between the ALCL cells and their microenvironment will shed light on the mechanisms of resistance to targeted therapy that would lead to more efficient therapeutic approaches for the ALCL patients.

PF4.04

Stress response signalling in leukaemia cells modifies composition of proteases in extracellular vesicles what supports invasion

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Introduction: Secretome, which bypasses regulatory signals via secreted proteins, lipids and nucleic acids, plays an essential role in crosstalk between leukaemia and bone marrow stromal cells supporting growth, self-renewal and drug resistance of leukaemia cells. Previously, we proved that BCR-ABL1 oncogene in CML cells leads to activation of unfolded protein response [Kusio-Kobialka Cell Cycle 2012]. As a consequence, enhanced phosphorylation of eukaryotic initiation factor 2 alpha subunit (eIF2a) allows adaptation of translated mRNA to stress conditions. The aim of the current study was to verify if eIF2a phosphorylation has an impact on the composition of extracellular vesicles (EVs) secreted by CML cells and how it influences vesicles properties supporting cells invasiveness. **Methods:** EVs were obtained by ultracentrifugation of conditioned media of bone marrow stromal fibroblasts (HS-5) or CML cells (K562) of wild type expressing eIF2a non-phosphorylatable protein. The influence of EVs on proliferation/apoptosis was assessed using flow cytometry. Cells motility and invasiveness were verified in matrigel invasion assay. Antibody arrays and zymography were used to compare proteases profile and their digestive activity in vesicular fraction. **Results:** Direct interaction of fibroblasts with CML EVs facilitated motility and invasiveness in the matrigel invasion assay, accompanied by

stimulation of matrix gelatin digestion by CML vesicles. This attribute of EVs was dependent on eIF2a-P level in CML cells. We found that eIF2a-P in CML cells modified the efficacy of synthesis and release of proteases in the vesicular fraction. **Conclusion:** Altogether, our results indicated that enhanced activation of UPR pathway in CML cells that accompanies the cancer progression has an impact on extracellular vesicles and so the intercellular communication.

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PF4.05

Role of Extracellular Vesicles in Acute Myeloid Leukaemia evolution Inna Tzoran

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Introduction: Acute myeloid leukaemia (AML) is characterized by rapid growth of leukaemic blast cells. Extracellular vesicles (EVs), shedding from various cells, express antigens, reflecting their cellular origin. **Aim:** The current study was aimed to explore the role of circulating EVs as potential biomarkers of AML activity and predictors of thrombogenicity in these patients. **Method:** Blood samples were collected from healthy controls and patients with newly diagnosed AML at three time points: diagnosis, nadir and remission. EV concentration, cell origin and expression of coagulation proteins were characterized by fluorescence-activated cell sorting (FACS). EV cytokine contents were evaluated by protein array. Procoagulant activity was assessed using Factor Xa chromogenic assay. MicroRNA EVs profile was screened by Nano string technology and validated by RT-PCR. **Results:** Forty-two AML patients were enrolled in the study. Total EV numbers were higher in patients in first remission compared with controls, while blast EV counts were higher in patients at diagnosis compared with controls and patients in remission. Blast EV levels were found to be significantly lower in patients who achieved remission and were alive at 3 years of follow-up compared with their succumbed counterparts. Percentage of endothelial EVs was higher in patients compared with controls at all three time points. EV procoagulant activity was elevated at diagnosis and in remission, and unlike controls' EVs, patients' EVs increased endothelial cell thrombogenicity. We found significant changes in several micro RNA in EVs obtained at diagnosis compared with remission which are involved in myeloid differentiation. **Conclusion:** AML patients' EVs express membrane proteins of blast cells and might serve as biomarkers of leukaemia dynamics and for minimal residual disease. Increased levels of endothelial EVs and their procoagulant activity may indicate a vascular injury associated with a hypercoagulable state in AML.

PF4.06

The role of microvesicles in multiple myeloma progression

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Introduction: Multiple myeloma (MM) is an incurable, genetically heterogeneous malignancy of plasma cells. MM is characterized by bone marrow infiltration, cytopenia and increased rate of thrombotic events. Microvesicles (MVs) are involved in thrombosis and cancer. However, the effect of MM-MVs on disease progression is unclear. **Aim:** To characterize MM-MVs and investigate their effects on the bone marrow (BM) microenvironment cells model. **Method:** MVs were isolated from MM cell line, untreated or treated with bortezomib and from peripheral blood (PB) and BM of MM patients (n = 13) and healthy controls (n = 14). MM-MV size, concentration and cell origin were measured by Nanosize and FACS. Protein content was evaluated by protein array. Coagulation and proteasome activity

were assessed by chromogenic assays. Effects of MVs on migration, proliferation and cell-signalling on BM-mesenchymal and endothelial cells (ECs) were analysed. **Results:** MM cells exhibited high MV shedding rate, which further increased with exposure to bortezomib in MM patients. MM-MVs expressed membrane MM markers, coagulation and growth factors and displayed procoagulant and proteasome activity. MM-MVs penetrated cells and affected their function. MVs of untreated cells and patient MVs increased EC and mesenchymal cell migration and EC proliferation, while MVs obtained from bortezomib-treated cells decreased these effects. MVs of untreated cells increased ERK1/2 and c-Jun phosphorylation while bortezomib-treated cells reduced c-Jun phosphorylation in ECs. **Summary:** MM cells are characterized by high shedding rate of MVs. They are pro-coagulants and increase EC thrombogenicity, suggesting their involvement in MM-related thrombosis. MVs contain high levels of angiogenic factors that affect mesenchymal and EC. MVs exposed to bortezomib display lower levels of angiogenic factors, which limit proliferation and migration. MVs reflect the efficacy of therapy and involved in MM dynamics.

PF4.07

The role of lung cancer-derived extracellular vesicles in tumour progression

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Introduction: Extracellular vesicles (EVs) have been shown to play important roles in many diseases including tumour. However, the role of EVs in lung cancer is still not fully understood. In this study, we tried to find out the biological functions of EVs in lung cancer. **Methods:** EVs were isolated from culture supernatants, serum and malignant pleural effusion (MPE) using ultra-centrifugation and ultra-filtration and then evaluated by TEM, cryo-EM and NanoSight. The biological functions of EVs were analysed in both in vitro cell line model and in vivo animal model. **Results:** EVs could be isolated from culture supernatants, serum and MPE samples using these two methods with different capacity revealed by EM and NanoSight. Specific EV markers including Alix, CD63 and Tsg101 were detected in the isolated EVs which carried various RNA species that small RNAs were enriched in EV portion compared with the cytosolic portion. Furthermore, the EVs could be uptaken by lung cancer cells and trigger oncogenic signals such as Stat3 and Akt in an autocrine/paracrine fashion. Previously, we have shown that IL-6/Stat3/tissue factor (TF)/vascular endothelial growth factor (VEGF) pathway plays an important role in lung cancer angiogenesis and metastasis as well as in the formation of MPE. Here, we showed that EVs from lung cancer samples carried high level of VEGF and TF and triggered vascular permeability changes in both in vitro and in vivo models. **Summary/Conclusions:** Using these methods, we isolated EVs not only from culture supernatants but also from various lung cancer-associated clinical samples and showed that the EVs triggered oncogenic signals and increased vascular permeability in an autocrine/paracrine fashion. These results may help the understanding of the biological functions of EVs in lung cancer and also the discovery of novel biomarkers and potential drug targets.

PF4.08

Stressors alter intercellular communication and exosome profile in nasopharyngeal carcinoma cells

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Introduction: Head and neck cancers comprise the sixth most common type of cancer worldwide. One of the most remarkable malignancies of the head and neck is the cancer of the nasopharynx, with a strong metastatic tendency already in the early stage. Beside the conventional pathways of metastasis formation, the information content of exosomes produced by the cancer cells may play a key role in metastatic transformation. The aim of this study was to investigate how stressors (cytostatic therapy or oxidative stress) alter the characteristic of tumour-derived exosomes. **Materials and Methods:** In our experimental model, we compared the quantity and content of exosomes produced by a nasopharyngeal carcinoma cell line (5-8F) under conventional (chemotherapy) and alternative (AgTiO₂-catalyzed reactive oxygen species generation) cytostatic treatments. After isolation, exosomes were identified by atomic force microscopy and quantified with NanoSight N5500 device. Their microRNA content was analysed using SOLiD 5500xl technology. The sequences were annotated in CLC Genomics Workbench version 5.5.1. **Results:** Beyond the classic chemotherapeutic agent (doxorubicin), AgTiO₂ in a photo-catalytic process also showed cytostatic activity. Tumour cell damage induced by the cytostatic treatments significantly altered the quantity of exosomes and their miRNA content. **Conclusion:** The increased quantity of exosomes and diversity of miRNAs stimulated by stressors may potentiate the information transfer from tumour cells to the surrounding stroma cells. A high rate of tumour suppressor miRNAs can lead to an important second-line anti-proliferative and a metastasis inhibitory effect of cytostatic treatments.

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PF4.09

EVs from human ovarian cancer drive the normal fibroblasts shift to "cancer associated fibroblasts"-like phenotype

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Introduction: It is well known that cancer cells release extracellular vesicles (EVs) able to modulate the tumour microenvironment. Thus, our study considered how human ovarian cancer derived-EVs affect normal fibroblasts, in order to evaluate if they are induced to acquire the properties of cancer-associated fibroblasts (CAFs). **Methods:** EVs were isolated from conditioned culture media of human ovarian cancer cell lines, CABA 1 and SKOV-3 (the latter being more aggressive than the former), through differential ultracentrifugation. Normal human fibroblasts were then cultured with EVs according to the scheme: i) Single treatment (1–50 µg/ml). After 72 h, fibroblast proliferation rate was evaluated through XTT colorimetric assay, while after 24 h their motility was estimated using the Boyden chamber. ii) Daily treatment, up to 5 days (1 µg/ml). Proliferation was evaluated 48 h after the end of the treatment. Meanwhile, FBS-free supernatants were assessed for gelatinases (MMP-2 and -9) and plasminogen activators (PA) expression by means of zymography assays with appropriate substrates. **Results:** EVs do not encourage

fibroblast proliferation but increase their migration. The expressions of pro-MMP-2 and of high molecular weight urokinase-type plasminogen activator (HMW-uPA) were up-regulated in EVs-treated fibroblasts; SKOV-3 EVs induced a more marked effect than CABA 1 EVs. In turn, the conditioned media of stimulated fibroblasts promoted the migration capacity of ovarian cancer cells. **Summary/conclusion:** EVs were able to affect fibroblasts' behaviour, stating their importance in the intercellular crosstalk between ovarian cancer cells and normal fibroblasts in order to establish a tumour microenvironment favourable to cancer progression. The present results highlighted that EVs, even if not able to induce fibroblast proliferation, can promote their migration and an increased expression of proteolytic enzymes needed for tumour microenvironment invasion.

PF4.10

GBM extracellular vesicles: mediators of invasion and brain stromal modulation

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The tumour milieu in Glioblastoma Multiforme (GBM) plays an important role in therapeutic resistance and facilitates tumour progression through cell-to-cell communication mediated by extracellular signalling. Extracellular vesicles (EVs) are cell-derived nanovesicles which have been shown to contribute to cancer hallmarks, such as invasion and angiogenesis, through autocrine and paracrine signalling. We hypothesized that GBM EVs facilitate cancer stem cell-like invasion in a spheroid migration assay and that GBM conditioned media (CM) can facilitate an angiogenic response through stromal modifications that drive pericyte expression of alpha smooth muscle actin (αSMA). EVs were isolated from monolayer cell culture supernatant of a primary GBM cell line by ultracentrifugation. Characterization of EV size was done with NTA and protein quantification with BCA; vesicle morphology was examined with immuno-TEM. To investigate the invasive potential of GBM EVs (derived from monolayer culture), cells were grown as spheres, added to Geltrex matrix and treated with EVs in two concentrations (low and high). Total area of invasion was measured by brightfield microscopy for 5 days along with appropriate controls. The angiogenic potential of the EVs was examined using primary rat pericytes as a stromal model. Pericytes cultured as a monolayer were treated with CM and analysed for the expression of αSMA. EV size distribution was in accordance with the literature and appeared to be round in shape as observed with TEM. In the spheroid invasion assay, GBM EVs increased the rate of cell invasion into the matrix and the sphere core was dispersed compared with controls. The CM from primary GBM cells elicited an expression of αSMA in the pericytes mirroring the activity of TGF-β regulation of αSMA. The results of this study provide evidence that the EVs alone can facilitate cell migratory and invasive properties of GBM and that CM stimulates primary pericytes to induce the expression of αSMA.

PF4.11

Exosomal miRNAs play an important role in cell-cell communication between bladder cancer cells and tumour microenvironment

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Introduction: Interaction of tumour cells and the tumour microenvironment (TME) plays an important role in tumour development and progression. miRNAs, packed in exosomes (EV), can affect cell–cell communication at the site of origin as well as the TME. Our aims are the identification of a specific exosomal miRNA expression pattern from EV of urinary bladder cancer (UBC) cell lines depending on the invasiveness of parental cells and characterization of their functional role in reprogramming fibroblasts. **Methods:** EV were isolated from invasive (T24,253J-BV,J82) and non-invasive (RT112,5637) UBC cells and characterized by Western Blot, NTA and electron microscopy. miRNA-expression analysis in UBC cells and their EV was performed by microarray and qPCR. Uptake of labelled UBC-EV by naive fibroblasts was shown by laser scanning microscopy. EV-mediated miRNA transfer between UBC cells and fibroblasts was shown by transfection of UBC cells with cel-miR-39, EV isolation, transfer to fibroblasts and qPCR analysis of recipient fibroblasts. After treatment of fibroblasts with UBC-EV, proliferation, invasion and migration assays are performed. **Results:** Isolated EVs exhibited a size between 61–81 nm and typical EV markers. EVs of invasive UBC cells are characterized by a specific miRNA signature of 15 miRNAs. Six significantly differently expressed miRNAs clearly separate both invasive and non-invasive UBC cells and their EV. Labelled EV of UBC cells and exosomal miRNA were taken up by naive fibroblasts. After co-cultivation of EV and naive fibroblasts, high proliferation rates were detected in fibroblasts. **Conclusion:** EV secreted by UBC cells exhibit a specific miRNA signature depending on the invasiveness of parental cells. For the first time we demonstrated that EV-mediated transfer of miRNAs between UBC cells and naive fibroblasts leads to an increase of fibroblast proliferation. These results emphasize the role of exosomal miRNAs for the interaction between tumour cells and the TME.

PF4.12

Differential expression of ligands for NKG2D and DNAM-1 receptors by epithelial ovarian cancer-derived exosomes and their influence on NK cell cytotoxicity

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Tumour-derived exosomes down-regulate the immune response of cancer patients to gain an immune privilege for the primary tumour and its metastases. Epithelial ovarian cancer (EOC) is a highly malignant gynaecological tumour and a great exosome producer. We studied the immunosuppressive ability of EOC exosomes in two cytotoxic mechanisms – the NKG2D receptor–ligand pathway, known to be a major cytotoxic mechanism in anti-tumour defence, and the accessory DNAM-1–PVR/nectin-2 mediated pathway. EOC exosomes were isolated from the EOC cell lines, OVCAR-3, SKOV-3, and from tumour explant cultures and ascitic fluids from EOC patients after ethical permission and informed consent. The expression of NKG2D- and DNAM-1 ligands on/in EOC exosomes and their effect on in vitro cytotoxicity was studied. We show that NKG2D- and DNAM-1 ligands are carried by EOC exosomes in a differential way – while NKG2D ligands were constitutively expressed on the exosomal surface, the DNAM-1 ligands were less frequently expressed and present only inside the exosomes. The EOC exosomes significantly down-modulated the expression of the NKG2D receptors on NK and cytotoxic T cells whereas the exosomal DNAM-1 ligands had no influence on the DNAM-1 receptors. The down-regulation of NKG2D receptor expression led to inhibition of NKG2D receptor–mediated degranulation and cytotoxicity as measured in vitro with OVCAR-3 and K562 cells as targets. Acting as a decoy, the EOC exosomes impaired the NKG2D-mediated cytotoxicity while the DNAM-1 receptor pathway was not affected. We conclude that in EOC, NK cells kill tumour cells

mainly through the DNAM-1 signalling pathway and that the NKG2D receptor–ligand pathway probably only plays a complementary role. Our results contribute to dissect and understand the complex tumour exosome-associated network of immunosuppressive mechanisms to be able to prevent facilitation of tumour escape, booster patients' anti-tumour defence and find novel therapies to combat cancer.

PF4.13

The role of extracellular vesicle–mediated signalling in the oral cancer microenvironment

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Introduction: Oral cancer mortality rates have increased by 10% in the past decade. Efforts to reverse this are hampered by a limited understanding of the underlying molecular complexity of the disease. Recently, interest has grown in the contribution of extracellular vesicles (EVs) to cancer pathogenesis. Since the discovery of mRNA and miRNA in EVs, they have been considered a signalling system capable of exerting behaviour-changing effects on local or distant cells. Developing tumours exist as a complex milieu comprising multiple cell types, each capable of producing a range of different EVs with pleiotropic functions. The aim of this work is to explore the role of EVs, and their cargoes, in oral cancer progression. **Methods:** EVs were extracted from the culture media of oral cancer cell lines by ultracentrifugation or size-exclusion chromatography and characterized by transmission electron microscopy (TEM), tunable resistive pulse sensing (TRPS) and western blotting. Next generation sequencing and iTRAQ mass spectrometry were used to identify the molecular contents. Fluorescence assays were used to examine the ability of EVs to transfer between cell types. Proliferation and viability assays determined the impact of this transfer on the recipient cell behaviour. **Results:** EVs were isolated from cell lines representative of the stages of oral cancer development (dysplastic, carcinoma and metastatic) and their presence confirmed by western blot, TEM and TRPS showing them to be between 50–200 nm in diameter and bearing common markers including CD63. EV production increased with oral cancer stage. Fluorescent labelling visualized the transfer of EVs derived from cancer cell lines to other cell types. **Conclusion:** EVs were readily isolated from dysplastic, carcinoma and metastatic cell lines, suggesting a role for EVs in oral cancer progression. We demonstrate here that these EVs are able to transfer their molecular contents to cells of the oral cancer microenvironment.

PF4.14

Modulation of exosomes secretion during NaBu induced differentiation of HT29 colon cancer cells, and effects of exosomes administration on normal and cancer cells

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Introduction: Exosomes are involved in inter-cellular communication. Sodium butyrate (NaBu) has been shown to stimulate differentiation of colon cancer cells. We previously reported that NaBu-induced differentiation of HT29 colon cancer cells is associated with a reduced expression of CD133. **Aims:** To analyse the effects of NaBu administration on the release of exosomes and to study the effects of the administration of exosomes to both normal and cancer cells. **Methods:** Exosomes were prepared using differential centrifugations. MicroRNA and mRNA expression levels were evaluated by RT-PCR.

Cell proliferation rate was assessed by MTT assay and confirmed with the Electric Cell-substrate Impedance Sensing while cell motility was assessed using the scratch test and confirmed by Confocal Microscopy. *Results:* NaBu-induced differentiation of HT29 colon cancer cells increased the levels of exosomes, and their expression of CD133 when compared to untreated cells. HT29 cells differentiation and the decrease of cellular CD133 expression levels were prevented by blocking multivesicular body maturation with NH₄Cl. The exosomes isolated from differentiated cells carried specific microRNAs at levels higher than those isolated by untreated cells. Incubation with exosomes isolated from differentiating HT29 cells increased the proliferation and the motility rate of both colon cancer cells and normal fibroblasts, increased the colony-forming efficiency of cancer cells and reduced the NaBu-induced differentiation of HT29 cells. Such effects were associated with an increased phosphorylation level of both Src and Erk proteins and with an increased expression of genes involved in EMT. *Conclusions:* These findings confirm the role of CD133 in the maintenance of stem cells properties; exosomes are used by differentiating cells to get rid of cellular components that are no longer necessary for the cells which might continue to exert their effects in tumour micro-environment.

PF4.15

HAS3 overexpression induces secretion of diverse EVs by tumour cells
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Hyaluronan (HA) is essential for maintenance of normal tissues, but it also promotes cancer progression by creating a favourable micro-

environment for growth of tumour cells. Hyaluronan is produced by HA synthases (HAS) on the plasma membrane. Overexpression of HAS3 is known to induce formation of different plasma membrane protrusions, disturb normal epithelization and enhance shedding of EVs. This work shows that HAS3 overexpression induces formation of EVs of diverse shape and size (50–5000 nm) by MCF-7 breast cancer cells. High-resolution imaging techniques like airyscan confocal microscopy, scanning and transmission microscopy, and correlative microscopy were used to characterize the EVs secreted by HAS3 overexpressing cells in monolayer and organotypic breast cancer cell cultures. Especially EVs originating from protrusions interacting with neighbouring cells were increased upon HAS3 overexpression, suggesting EV-mediated contact-dependent interactions between cells. Interestingly, HAS3 overexpression was associated with enhanced spheroid formation in organotypic breast cancer cell cultures. The effects of HAS3-induced vesicles on target cell behaviour like invasion potential were also studied. This study shows that in addition to previously described vesicle shedding mechanism from tips of thin protrusions, activity of HAS3 induces formation of vesicles of variable size originating from membrane blebs, retraction fibres and other actin-dependent protrusions. It can be assumed that active synthesis of HA on the plasma membrane creates hydrostatic forces that promote disruption of the cortical actin cytoskeleton. This creates membrane bulges that give rise to different protrusions and blebbing of the plasma membrane, resulting in enhanced shedding of EVs. Additionally, HA carried on the surface of EVs modifies the extracellular niche of tumour cells and modulates the surface properties of EVs, regulating the interactions of EVs with target cells.

Poster Session 5 - Mesenchymal stem cells and tissue regeneration and morphogenesis

Chairs: *Eva Rohde and Magdalena Lorenowicz*

PF5.01

Biological effects of different extracellular vesicles (EVs) population on reversal of marrow cells radiation damage

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Three different fractions (10k pellet, 100-10k pellet and 100k pellet) of murine/human mesenchymal stem cell (MSC)-derived vesicles were isolated by differential centrifugation. FDC-P1 cells were exposed at 500cGy irradiation and cultured with 3 different preparations of MSC-derived vesicles for 7 days. A significant increase in cell proliferation was observed after 3 different vesicle fractions treatments. However, exosomes were clearly inferior, microvesicles and the combined exosomes and microvesicles population were superior. The capacity of human MSC-derived vesicles of different fractions on reversal of murine bone marrow damage was also evaluated *in vivo*. The combined fractions shown a significant increase donor chimerism in bone marrow at 6 months post-transplant with 5x's the level of engraftment compared to irradiation control. The 10k and 100-10k fractions showed intermediate healing. Exposure to vesicles in irradiated FDC-P1 cells downregulated phosphorylated H2AX, the DNA damage marker and decreased apoptosis marker, PARP cleavage. The effect of murine marrow MSC-derived vesicles on the gene expression in peripheral blood cells from radiation damaged mice was investigated. 28 genes were altered by irradiation; 22 showed partial or complete reversal of these alterations. The characterization of three different fractions of vesicles derived from murine bone marrow cells and human MSC were evaluated by miRNA real-time PCR array (total 750 miRNA). Several MSC-EV-associated miRNAs responsible for the reversal of radiation damage were evaluated. Our preliminary data shown that miR-221, miR654-3p and miR210-5p could partially reverse radiation damage in FDC-P1 cells. There are different biological effects in different EV populations. For reversal of radiation toxicity, the most effective vesicle population would include both smaller (exosomes) and larger vesicles (microvesicles).

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PF5.02

Mesenchymal stromal/stem cell-derived extracellular vesicles- towards a novel therapy for osteoarthritis

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Introduction: Osteoarthritis (OA) is a rheumatic disease leading to chronic pain and disability with no effective treatment available. Recently, allogeneic human mesenchymal stromal/stem cells (MSCs) entered clinical trials as a novel therapy for OA. Increasing evidence

suggests that therapeutic efficacy of MSC depends on paracrine signalling. Here, we investigated the role of MSC-derived extracellular vesicles (EVs) in cartilage repair. **Methods:** MSC-EVs were added to regeneration cultures of OA chondrocytes in fibrin glue and to monolayers of OA chondrocytes treated with TNF- α . MSC-derived conditioned medium (CM) and EV-depleted CM were used as controls. COX2 gene expression and PGE2 protein levels were measured in the monolayer cultures and culture supernatant, respectively after 48 h. The regeneration cultures were analysed after 4 weeks for glycosaminoglycan content by DMMB and paraffin sections of the regenerated tissue were immunostained for proteoglycans and type II collagen. **Results:** We show that MSC-EVs promote cartilage regeneration *in vitro*, as addition of MSC-EVs to the cultures of OA chondrocytes-induced production of proteoglycans and collagen type II by these cells. MSC-EVs also inhibit the adverse effects of inflammatory mediators on cartilage homeostasis. When co-cultured with OA chondrocytes treated with TNF- α , MSC-EVs inhibit the expression of COX2, the pro-inflammatory enzyme important for production of prostaglandins and known to inhibit proteoglycan and collagen synthesis. To understand the mechanism, by which MSC-EVs perform these beneficial effects we analysed their RNA content. We found 206 mRNAs and lncRNAs enriched in MSC-EVs. Importantly, among the genes enriched in MSC-EVs were putative regulators of cartilage regeneration, factors important for immunomodulation, cell growth and differentiation. **Conclusions:** Our data indicate that MSC-EVs are important regulators of cartilage repair and hold a great promise as a novel therapy for OA.

PF5.03

Immunomodulatory impact of mesenchymal stem cell-derived extracellular vesicles on peripheral blood cells *in vitro*

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Human mesenchymal stem cells (MSCs) have been administered in more than 500 NIH-registered clinical trials to patients suffering from various diseases including myocardial infarction, stroke and graft-versus-host disease (GvHD). Initially, MSCs were thought to replace lost cells in damaged tissues. Despite controversial reports regarding the efficacy of MSC-treatments, MSCs seem to exert their beneficial effects rather by secretion of immunosuppressive factors than by cell replacement. In this context, extracellular vesicles (EVs), such as exosomes and microvesicles, were identified to execute the MSCs' therapeutic effects. Our recent successful treatment of a steroid-refractory GvHD patient and the demonstration of pro-regenerative effects in a murine stroke model confirmed the MSC-EVs' therapeutic potential. Due to the fact that MSCs provide a heterogeneous cell entity, we compared the MSC-EV fractions obtained from MSCs of 20 healthy donors in immune modulatory *in vitro* assays. Our ongoing results confirm quantitative and qualitative differences within these MSC-EV fractions. A critical step in comparing and interpreting the obtained data is the normalization procedure. Initially, we used cell equivalents for normalization. However, upon learning that a huge proportion of EVs get lost during ultracentrifugation, which we use as a final step to concentrate PEG precipitated MSC-EVs, cell equivalents and processed media volumes, were found as inappropriate parameters for normalization. For EVs prepared with the PEG method – in contrast to EVs prepared with the differential centrifugation method – normalization to the protein amount of applied MSC-EVs revealed reliable data. Critical issues in the normalization

process and challenges of the applied purification method, especially the ultracentrifugation step, will be discussed.

PF5.04

Hyaluronan-coated extracellular vesicles are secreted by human mesenchymal stem cells

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Hyaluronan (HA) is the most abundant polysaccharide of the extracellular matrix (ECM), promoting growth and healing of tissues. HA is synthesized by specific plasma membrane-bound enzymes, HA synthases (HASs) that induce shedding of HA-coated EVs (HA-EVs). EVs can transfer information between bone marrow stem cells and damaged tissue, suggesting that stem cells can repair tissue injuries without directly replacing parenchymal cells. Human mesenchymal stem cells (MSCs) secrete high levels of HA, which may associate with the EV secretion of the MSCs. To confirm this hypothesis, we characterized the EVs secreted by the bone marrow-derived MSCs and their relationship with HA synthesis. The EVs secreted by the MSCs were characterized by NTA, immunoblotting and qPCR. Their HA content, morphology and budding mechanisms were inspected by live cell confocal microscopy. LPS and RNA interference were utilized to study the mechanisms regulating the EV secretion and their association with HA. It was found that the MSCs have extremely long HA-coated plasma membrane protrusions typical for cells with an active HA secretion. Additionally, they secreted high numbers of HA-EVs, which carried also mRNAs for CD44 and all HAS isoforms. HAS silencing inhibited and LPS increased the HA-EV secretion, suggesting that their secretion is associated with the activity of HA synthesis. The EVs that carry HA on their surface are potential vehicles in preparing suitable niches for tissue regeneration. The HA-EVs and signals within them may participate in ECM remodelling directly or indirectly by interacting with ECM-producing cells. Moreover, HA coating may facilitate EV binding to target cells via HA-CD44 interactions. These features make the HA-EVs potential therapeutic tools to heal the damaged ECM in the injured tissues. The results of this study suggest that secretion of the HA-EVs is a general process, which explains many unsolved questions on the mechanisms of HA-mediated tissue regeneration.

PF5.05

Paracrine effect of membrane vesicles released by mouse mesoangioblast stem cells

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Introduction: Mouse mesoangioblasts are vessel-associated multipotent progenitor stem cells, which are able to differentiate into different mesodermal cell types. In our previous paper, we have demonstrated that mesoangioblasts are able to shed in the extracellular environment membrane vesicles (EVs), which contain both structural proteins and biological factors such as FGF2 and the two gelatinases MMP2/9. We investigated whether these EV interact in a paracrine way with other cell types different from mesoangioblasts, and eventually the effects of this interaction. **Methods:** Mesoangioblast EVs were collected from conditioned media by ultracentrifugation. Total mRNAs from mesoangioblasts and from two preparations

of EVs were used to perform microarray. Different concentrations of mesoangioblast EV were used to evaluate in vitro migration modulation on human ECV304 cells by wound healing assay or study their influence on capillary-like structures formation. Murine RAW 264.7 macrophages were cultured with or without mesoangioblast-derived EVs to investigate their effect on cytokines secretion profile and RTK phosphorylation levels by array. **Results:** We observed that mesoangioblast EV contained a huge amount of mRNA; most of the transcripts are co-expressed in EV and cells, whereas 20 mRNA were accumulated within EV and absent in the cells. Gene ontology analysis showed that the common mRNA could be involved in cell survival and differentiation. We demonstrated that mesoangioblast EV interact with endothelial ECV304 cells by positively influencing their migration capability. Moreover, they induce in vitro the ECV304 differentiation versus capillary-like structures depending on their concentration. Mesoangioblast EVs are also able to interact with RAW 264.7 cells inducing dramatic changes in cytokines secretion and in RTK phosphorylation levels. **Conclusions:** We demonstrated that mesoangioblast EV interact with endothelial cells and macrophages and influence on their behaviour.

PF5.06

CD47 regulates the reprogramming of endothelial cells by breast cancer stem cell-derived extracellular vesicles

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CD47 is a ubiquitously expressed signalling receptor in human cells that is also present in extracellular vesicles. CD47 is often highly expressed in tumour cells and is associated with a poorer prognosis. A microarray analysis of human umbilical vein endothelial cells (HUVEC) treated with MDA-MB-231-derived extracellular vesicles showed global changes in mRNA expression. To examine whether extracellular vesicles derived from breast cancer stem cells (bCSCs) mediate these changes in mRNA expression we isolated MDA-MB-231 and MCF7 bCSCs. Quantitative PCR and ELISA analyses revealed that the expression of vascular endothelial growth factor (VEGF) and its receptor (VEGFR2) phosphorylation were downregulated in the presence of MDA-MB-231-derived extracellular vesicles but not MCF7-derived extracellular vesicles. VEGF expression and VEGFR2 phosphorylation in HUVEC were also inhibited by thrombospondin-1 (TSP1) or the CD47 blocking antibody B6H12 in presence or absence of bCSCs. The downregulation and inhibition of VEGF signalling we observed might be part of the mechanism by which cancer cell extracellular vesicles co-opt healthy cells and perturb normal endothelial cell signalling. Endothelial-to-mesenchymal transition is often categorized as a specialized form of epithelial-to-mesenchymal transition (EMT). MDA-MB-231-derived extracellular vesicles altered β -Catenin, Slug and LYVE1 markers as compared to normal HUVEC. This indicates that extracellular vesicles may reprogramme HUVEC into mesenchymal cells. MDA-MB-231 breast cancer cells are more metastatic and more aggressive than MCF7, and extracellular vesicles from MDA-MB-231 bCSCs preferentially impart these phenotypes into endothelial cells. Future studies will reveal how TSP1 and CD47 play a role in EndMT and their potential as candidates to inhibit the crosstalk between cancer-derived extracellular vesicles and healthy cells.

PF5.07

Extracellular vesicles released by induced pluripotent stem cells modulate cellular function of recipient cardiac endothelial cells

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Please see OPW1.7

PF5.08

Extracellular vesicle-delivered bone morphogenetic protein (BMP): a novel paracrine mechanism during heart development

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Introduction: The closely related morphogens BMP2 and BMP4 (BMP2/4), secreted by endodermal and ectodermal cells, respectively, have a dominant role in early cardiac progenitor cell induction and later heart formation. Recently, it has been shown that the morphogens Wnt3a and Hedgehog are transported by extracellular vesicles (EVs). We hypothesized that EVs might also be involved in transporting BMPs and thereby regulate cardiogenesis. **Methods:** A new method to isolate EVs in vivo from zebrafish embryos based on differential ultracentrifugation was established and used to purify zebrafish EVs at the onset of cardiac progenitor cell induction (bud stage, 10 h post-fertilization). Using western blotting and OptiPrep-based gradient preparation, we verified the presence of BMP2/4 in zebrafish EVs. BMP signalling activity was measured applying a dual luciferase assay as 'BMP-Responsive Element'-Luciferase reporter transfected HEK293 cells treated with EVs. **Results:** Using our own zebrafish EV isolation protocol, we can show that EVs released at the onset of cardiac progenitor cell induction in vivo contain mature and various premature forms of BMP2/4 peptide. To narrow down the origin of EV-delivered BMP2/4, we examined the endoderm as a possible source. Strikingly, using the endodermal cell line End2, we can show that endoderm-derived EVs contain BMP2/4, establishing the endoderm as a natural source for EV-delivered BMP. Additionally, besides BMP peptides, we demonstrate the presence of BMP Type II receptors being co-transported in EVs derived from zebrafish embryos. Importantly, EVs isolated from zebrafish embryos and from End2 cells are able to significantly activate a BMP-dependent transcriptional response, indicating biological activity. **Summary/conclusion:** EV-delivered BMP2/4 and BMP Type II receptors secreted partly by the endoderm represent a new paracrine signalling mechanism possibly involved in cardiogenesis.

PF5.09

Depleting extracellular vesicles from fetal bovine serum affects muscle cell proliferation and differentiation

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Introduction: We have tested the role of fetal bovine serum (FBS) extracellular vesicles (EVs) on myoblast proliferation and differentiation using the murine cell line C2C12 and confirmed the data with rat L6 myoblasts and human primary muscle cells. **Methods:** EVs were removed from sera by ultracentrifugation (18h). Myoblasts were grown either in classical DMEM or EV depleted-DMEM (EDDM). Differentiation was induced by replacing the culture medium with either normal media or EV-depleted media. qRT-PCR of relevant genes and miRNAs for myogenesis, energy metabolism and EVs secretion were performed. **Results:** Removal EVs from serum delays C2C12 and human myoblast proliferation and committed cells to differentiate precociously. In addition, the expressions of important myomiRs for myogenesis were significantly decreased (i.e. miR-1, miR-206 and miR-133a). When constantly grown in EDM during proliferation and differentiation, myoblasts formed less myotubes

than cells grown in classical serum. Conversely, L6 myoblasts were positively affected and formed more and larger myotubes when grown in EDM compared to normal growth condition. In fact, EDM induced myostatin in C2C12 and human myotubes, a negative regulator of muscle cell size. As L6 does not express myostatin, induction of early differentiation markers during proliferation by EDM medium resulted in large hypertrophic myotubes. **Conclusions:** We can recapitulate the crosstalk between myoblasts and myotubes during myogenesis (Forterre 2013, PlosOne) by using bovine serum-EVs. This result implies that bovine-EVs can transfer specific signals to cells from unrelated species and thus that part of serum EVs composition is evolutionarily conserved (e.g. miR-133a and miR-1 are detected in FBS-EVs). Generally speaking, these results suggest that EVs in body fluids could have an unsuspected function during embryogenesis and in regulation of cellular processes that lead to hypertrophy and hyperplasia.

PF5.10

Extracellular vesicles (EVs) from human cardiac-derived adherent proliferating cells (CardAPs) – a comparative characterization

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Introduction: Human cardiac-derived adherent proliferating cells (CardAPs) isolated from endomyocardial biopsies own an extraordinary phenotype and a proven cardiomyocyte protective potential with substantial pro-angiogenic and anti-inflammatory effects in vitro and in vivo. In this study, we phenotypically characterized EVs from CardAPs in comparison with CardAP cells and further investigated the ability of EVs to interact with either murine cardiomyocytes (HL-1) or human endothelial cells (HUVECs). **Methods:** EVs were isolated from CardAP conditioned medium via ultracentrifugation following removal of apoptotic bodies. BCA assays and Nanoparticle Tracking Analysis detected protein concentration, particle size and concentration. EVs were coupled to 4 µm aldehyde/sulphate beads and analysed by flow cytometry to evaluate surface markers. Interaction with target cells was visualized by microscopy and flow cytometry of co-cultured PKH26-labelled EVs with DiD-labelled HL-1 or HUVECs. **Results:** Our data indicate that CardAP EVs (n = 6, 3 donors) have a modal particle peak size of 95.7 (± 4.7) nm with a mean particle concentration of 8.05 (± 1.81) *10⁸ mL⁻¹ and a mean protein concentration of 147.4 (± 18.6) µg/mL. No significant differences in particle peak size or particle and protein concentrations were measured between different passages of CardAPs. EVs possessed CD73, HLA-ABC, CD29 and the tetraspanins CD9, CD81 and CD63 on their surface. In contrast to the originating CardAP cells, CD90, CD54, CD144 and CD105 could not be detected on EVs. Labelled isolated EVs are co-stained with DiD+ target cells already 30 min after co-culture and further accumulate over time. **Summary/conclusion:** Based on the tetraspanins detected and the modal peak size of particles, we can conclude that CardAP-derived EVs are composed of exosomes and microvesicles. Furthermore, EVs show similar but not identical surface protein expression as CardAP cells and are able to interact with relevant target cells.

PF5.11

Hematopoietic differentiation of embryonic stem cells by stromal cell-derived nanovesicles

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Introduction: Culturing embryonic stem cells (ESCs) on a stromal cells has long been used for differentiating ES cells to haematopoietic

lineage cells. ES/stromal co-culture system induces differentiation of ES cells through physical contact and soluble factor. However, the co-culture system results in low differentiation yield and requires separation of the differentiated cells from the mixed cell population. We generated nanovesicles using stromal cells. These stromal cell-derived nanovesicles had similar membrane and cytosolic components of stromal cells, thus ESCs treated with the stromal cell-derived nanovesicles (ESC/nanovesicle) would imitate the co-culture system. The ESC/nanovesicle culture method showed increased efficiency of differentiation when higher concentration of nanovesicles was treated. This new method is free from active stromal cells and, therefore, the differentiated cells can be used for further analysis without an additional separation process. **Materials and methods:** To make nanovesicles, OP9 stromal cells were extruded through two different micro-sized filters which were enclosed in a mini-extruder. OP9 nanovesicles (1 ~ 200µg/ml) were treated to B6 ESCs every 3 days for 3 weeks. Expression of differentiation markers was analysed using a flow cytometry. **Results:** The size of OP9 nanovesicles was ~100 nm and they enclosed mRNAs and proteins of stromal cells with intact membrane proteins. When the ESCs were treated with nanovesicles, they formed haematopoietic colonies with cobblestone-like appearance and expressed haematopoietic stem cell markers. The treatment of high-dose nanovesicles showed 5–8 times increased haematopoietic marker (CD45, c-Kit, SCA-1) compared to stromal cell co-culture samples. **Conclusion:** ESCs/nanovesicle culture method shows increased efficiency of differentiation and did not require additional separation steps, thus nanovesicles are expected to substitute co-cultures in various cell–cell interaction studies.

PF5.12

The Effect of Exosome-Mimetic-Nanovesicles on Mesenchymal Stem Cell Proliferation.

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Introduction: Mesenchymal stem cells (MSCs) are widely used in regenerative medicine, but low proliferation rate and loss of multipotency were limitations of MSCs. Exosomes may become good materials for the cell proliferation because they can stimulate the signal transduction and deliver the genetic materials. However, low yield and complex isolation have made it difficult to obtain the enough amount of exosome for exosome-related research. We fabricated the exosome-mimetic nanovesicles (NVs) from living cells to substitute the exosome. The NVs were generated from ruptured cell membrane which can form NV due to self-assembly ability. The NVs were applied to mouse MSC (mMSC), and enhancement of proliferation was verified. **Methods:** Murine embryonic stem cells (ES) were used for source of NVs, and large-scale generation system was used to generate the NVs. To confirm the effect of NVs on proliferation, NVs were applied to mMSCs after 24 h of serum-starving. After 24 h treatment, the cell number was counted by haemocytometers. BrdU were shown by fluorescence microscopy image, and proliferation-related proteins (PCNA and MAPK) were shown by western blotting. To identify the characteristic of mMSCs

after treatment, immunophenotyping and differentiation were performed. **Results:** The ES NVs were ~100 nm in diameter and contained cellular contents like natural exosomes. The treatment of ES NVs induced the enhancement of mMSCs proliferation (1.5–2 times) and phosphorylation of MAPK regulating proliferation. The repeated NV treatments with proper dose (50 µg/ml) enhance mMSC proliferation steadily without detrimental damage. Also, the NV-treated mMSC showed well osteogenic and adipogenic differentiation. **Conclusion:** The ES NVs had similar characteristic of natural ES exosome and enhanced the proliferation of mMSCs. The treatment of NVs did not influence to characteristics and multipotency of mMSC. Thus, the NVs generated from living cells are expected to contribute to regenerative medicine.

PF5.13

RNA profiling of naive and differentiated MSC-derived EV: towards miRNA-based release criteria for future therapeutic application of EV in clinical trials

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Introduction: Mesenchymal stromal cells (MSCs) of various tissue origin display differential therapeutic effects despite their overall similar surface marker expression. Similarly, extracellular vesicles (EVs) that largely mediate the paracrine effects of MSC differ with respect to their protein and RNA content. Here, we analysed the EV of MSC from three different sources [bone marrow (BM), adipose tissue (AT) and umbilical cord (UC)] as well as from naive and differentiated BM-MSC-derived EV to evaluate whether miRNA profiling may serve as a release criterion for future EV-based therapies. **Methods:** EV were purified from either naive or osteogenically differentiated MSC by differential ultracentrifugation and characterized by NTA and western blotting. Agilent Bioanalyzer-based total RNA profiling and miRNA next-generation sequencing were employed to identify differences in the EV RNA content. **Results:** RNA profiling revealed that EV from UC-MSC contained significantly larger amounts of total RNA and miRNAs than EV from the other two MSC tissue sources. Comparison of naive and osteogenically differentiated MSC EV showed a significant shift towards larger RNA species in the latter. miRNA sequencing results confirmed the increased content of miRNAs found in UC-MSC-derived EV. However, the most abundant miRNA in EV from all three naive tissue sources was miR-146a, which is involved in immunosuppressive processes. This miRNA was strongly reduced in EVs from osteogenically differentiated BM-MSCs, which were enriched in miRNAs involved in promoting osteogenic differentiation. **Conclusions:** Immunosuppressive miRNAs packaged into EV from naive MSC may reflect their immunomodulatory potential. In contrast, EVs released by differentiated MSC contain miRNAs as potent transducers of differentiation signals. miRNA profiling may thus be employed to define the targeted therapeutic activity in MSC-derived EV to promote future preclinical and clinical trials.

Poster Session 6 - Novel technical developments in EV characterization

Chairs: Uta Erdbrügger and Sten Libregts

PF6.01

Characterization of extracellular vesicles by flow cytometry

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Please see OPW1.3

PF6.02

Optimized staining protocol for extracellular vesicle characterization by dedicated flow cytometry

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Background: Flow cytometry is commonly used to detect and characterize single extracellular vesicles (EVs). However, conventional staining protocols were developed for flow cytometers with an EV detection limit around > 500 nm. Dedicated flow cytometers detect smaller EVs, but these EVs have lower numbers of antigens and their labelling has not been optimized. Therefore, we developed a new labelling protocol to increase the number of fluorochromes per EV and improve antigen detection of smaller EVs. In addition, we determined the minimal detectable number of fluorochromes per EV. **Methods:** Platelet-derived EVs, obtained from platelet-depleted supernatant of outdated platelet concentrates, were diluted 10-fold in PBS/citrate. Samples (22.5 µL) were labelled with the conventional staining protocol, that is, 62.5 ng/mL CD61-PE or 90 ng/mL CD9-PE and incubation for 15 min. In addition, the sample was labelled with higher concentrations (ranges of CD61-PE: 62.5-625 ng/mL or CD9-PE: 90-900 ng/mL) and incubated for 15 or 60 min. IgG1-PE isotype control was used in identical conditions. PBS/citrate (200 µL) was added after incubation. Samples were analysed on a dedicated flow cytometer (Apogee A50). Quantibrite beads (BD Biosciences) were used to extrapolate the minimal detectable number of fluorochromes per EV. **Results:** With the conventional staining protocol, 5.7×10^8 CD61+ and 8.8×10^7 CD9+ EVs/mL were detected, whereas incubation of EVs with the highest antibody concentrations during 60 min resulted in a 5- and 18-fold increase in CD61+ and CD9+ EVs, respectively. Extrapolation of the fluorescence signal of Quantibrite beads showed that approximately 100 PE molecules are required to score a single EV as CD61+ or CD9+. **Conclusion:** A 10-fold increase in antigen concentration and a 4-fold longer incubation time resulted in a 5- to 18-fold increase in detected CD61+ or CD9+ EV. This increase is likely due to more EV exceeding the detection threshold of approximately 100 PE molecules/EV.

PF6.03

Flow cytometry for determining size distribution of microvesicles and variation in the lipid composition of individual liposomes

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Recently, the need for a standard to determine the sizes of extracellular vesicles, including microvesicles, by flow cytometry has

been emphasized. We suggest using artificial vesicles as calibrators to estimate the size of microvesicles from the side scattering (SSC) measured by flow cytometry. We prepared fluorescently labelled liposomes with different maximum sizes defined by the pore size (200, 400, 800 and 1000 nm) of the membrane used for the extrusion. The fluorescence strengths from the largest liposomes pertaining to each pore size enabled us to verify the correlation between the SSC from a liposome and the corresponding size. We propose that artificial vesicles are more accurate size calibrators compared with the commonly used polystyrene calibrator beads illustrated by the SSC from 110 nm polystyrene beads corresponds to the scattering from ~400-nm-sized vesicle-like particles. We also evaluated the size resolution of this technique. In addition, we used flow cytometry to reveal the variation in the lipid composition of individual liposomes based on the ratio of the fluorescence intensity of two different fluorophore-labelled lipids. We show for the first time that the degree of variation in the lipid composition of the liposomes is about 20-fold larger than predicted by a binomial distribution and show in a quantitative way that this variation decreases as the size of the liposome increases. The size-dependent variation in lipid or in principal protein composition of microvesicles could potentially be used as new disease biomarkers.

PF6.04

Development of a bead-assisted flow cytometry method for the semi-quantitative analysis of EVs

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Because of the under resolution size of extracellular vesicles (EVs), most experimental approaches commonly employed for the characterization and quantitation are time consuming, require expensive instrumentation or are rather inaccurate. Our main aim for this project was to assess whether we can circumvent all these caveats by the use of general and specific markers in bead assisted flow cytometry, to provide a semi-quantitative measure of EV content in a given sample. EVs were isolated from cells in in vitro culture (both tumoural cell lines and primary T-lymphoblasts) and from biological samples such as urine. Isolation was performed by classical ultracentrifugation and size exclusion chromatography. EVs were coupled to aldehyde/sulphate latex beads to provide the sample with an appropriate size to be detected by standard flow cytometers. We performed serial dilutions of the samples and we quantified their EV content using BCA, NanoDrop and NanoSight. By comparing these results with Mean Fluorescence Intensity values using flow cytometry, we demonstrated that differences in EV concentration using flow cytometry, we demonstrated that differences in EV concentration linearly correlated with MFI values. Linearity and detection limits of this technique were determined using a panel of EV-general markers. EV classical markers such as CD81, CD63 and the complement regulatory molecule CD59 gave a higher sensitivity in EV detection, when mean fluorescence intensity was plotted against sample dilution. Thus, we provide evidence that this bead assisted flow cytometry method is a fast, accurate and reliable method for the semiquantitative bulk analyses of EVs, which could be easily implemented in a laboratory routine.

PF6.05

Label-free detection of extracellular vesicles in human breast milk compared to infant formula

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Please see OPW1.2

PF6.06

Using F-NTA for characterizing exosomes

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Introduction: NTA is a valuable technology within the field of exosomes research providing both high-resolution-sized data and number concentration of exosomes. It is often extended to fluorescence labelling for better insight into the samples (F-NTA). However the procedures for carrying out such measurements can be found to be challenging because several variables need to be investigated and optimized. Here, we show data for membrane labelling of exosomes populations and for labelling specific surface markers with antibodies, sharing our insight into the factors that influence the chance of labelling success. **Methods:** Commercially available membrane labelling dyes were used to label human exosomes. Dye: exosomes concentration, incubation times, volumes, temperature, etc., were investigated in order to optimize labelling conditions. Using a similar approach, QDots were used to label exosomes' surface markers via commercially available antibodies. Instrument configurations and settings were investigated to ensure optimum sample measurement. In addition, appropriate controls were taken to measure size distribution and concentration with NTA. **Results:** Both membrane dyes and antibody labelling successfully labelled a population of exosomes in the samples. Various issues were identified during the optimization process, such as disturbing the stability of the sample by the addition of dye formulation buffer, impact of labelling kit preparation, understanding about the risk of labelling contaminants that may co-purify with exosomes and the impact of excess reagents. **Conclusions:** F-NTA has been shown to be an effective and rapid way to characterize and identify the presence of membrane containing particles, while the use of antibodies and quantum dots allowed exosomes exhibiting specific epitopes to be measured. Using this approach, we were able to characterize exosomes of interest from the total population while membrane labelling provided confidence as to the purity of the sample.

PF6.07

Simulation study of optical trapping of vesicles with a dual-waveguide trap

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There is increasing interest in extracellular vesicles (EVs) as biomarkers for diseases such as cancer. As a result, efforts direct towards developing techniques to characterize EVs. We develop a dual-waveguide trap for optical trapping and Raman of single and multiple vesicles, to obtain the composition of the trapped vesicle(s). In this device, two opposing waveguides emit counter-propagating beams into a microfluidic channel that supplies the EVs. The beams create a strong light concentration for trapping and Raman. In this contribution, we report on the capability of the device to stably trap spherical particles with a homogeneous refractive index mimicking EVs. We perform FDTD simulations of the optical field between the waveguide facets, which have a separation of 5 μm . The simulated

field is inclusive the EV. Water is the fluid surrounding the EV, with a refractive index $n(\text{H}_2\text{O}) = 1.33$. The wavelength of the light is 785 nm, a common Raman wavelength. The waveguides have a composite structure which is very suitable to generate confined beams. The waveguide cross section is $1 \times 1 \mu\text{m}^2$. The refractive index is $n(\text{EV}) = 1.37$. Diameters are $d = 1000, 500$ and 200 nm . The forces are calculated using the Maxwell stress-tensor method, inputting the simulated field. The forces are calculated on the axes perpendicular to the waveguide direction. Trapping potentials follow from integrating force curves. For an optical power of 20 mW we get normalized trap stiffnesses of 140, 22 and 0.05 pN/nm/W for $d = 1000, 500$ and 200 nm , respectively. The corresponding normalized potential well depths U/kT are 205, 28 and 3, respectively. Using the criterion $U/kT > 10$, the diameters 1000 and 500 nm are stably trapped, while 200 is not. Raising the power to 75 mW, the $d = 200 \text{ nm}$ EV is stably trapped as well. The powers we find suffice for Raman spectroscopy. In conclusion, our simulations indicate that a TripleX dual-waveguide device can stably trap EVs with diameters down to at least 200 nm.

PF6.08

Optimal EV measurement in complex biological samples using nanoparticle tracking analysis

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Introduction: Nanoparticle tracking analysis (NTA) allows a quantitative estimation of extracellular vesicle (EV) size, size distribution and concentration. For routine NTA analysis of biological samples, containing polydispersed EVs, it is essential to capture sufficient data to facilitate more accurate quantification. Currently, NTA analysis is performed by capturing consecutive recordings over a 1–5 min period. We sought to determine if an increase in total recording time would increase the reproducibility of EV quantification from plasma and from the supernatant of activated purified platelets, the platelet releasate (PR). **Methods:** Consecutive 1-min NTA recordings were captured for 5, 10, 15, 20, 25 and 30 min for three technical replicates of both plasma and PR isolated from three healthy donors. Statistical analysis was performed in R Studio 3.2.3. Following this, 5 and 15 min of NTA recordings of the plasma and PR isolated from a larger cohort of 32 healthy donors were then compared. **Results:** When comparing technical replicates of both plasma and PR, we found increased correlation between donor samples with increasing NTA capture time, concomitant with a reduction in relative standard error (RSE). We propose 15 min to be the optimal recording time, with average correlation coefficients between technical replicates found to be 0.93 and 0.95 and a decrease in RSE by 42 and 41% for plasma and PR, respectively. Decreased %RSE was also observed when directly comparing 5 and 15 min NTA capture time across 32 healthy donors, with differences between individual donor measurements falling outside the 95% confidence interval especially in the exosomal size range. **Summary/conclusion:** Increasing NTA capture time thereby enables a more accurate and reproducible characterization of EVs in complex biological samples, especially in the exosomal size range, and will facilitate future clinical diagnostic studies investigating circulating EVs.

PF6.09

Physical characterization of extracellular vesicles using surface-based sensing

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The enquiry to unravel different modes of biological action of extracellular vesicles (EVs) is limited by the availability of methods able to characterize EV multifaceted profiles which include their biomolecular composition, their physicochemical properties and their concentration in biological fluids. In this work, we explore the potential of two surface-based sensor methods, namely surface-plasmon resonance (SPR) and optical waveguide scattering and fluorescence microscopy (OWSFM) for physical characterization of EV suspensions. Here, EVs isolated from culture media of HMC-1.2 cell line through density gradient were characterized. SPR operated in mass transport regime was used to determine the bulk concentration of EVs carrying CD63 exosomal marker. For this approach to yield reliable quantification, both the size of EVs and the average thickness of the film they form on the sensor surface must be known. The latter parameter was determined by operating SPR in dual-wavelength mode and the average size of CD63-positive HMC-1.2 EVs was obtained using fluorescence-based nanoparticle tracking analysis. The analysis demonstrated that this particular sub-population of EVs corresponds to 10% of the entire EV content and that its mean diameter (~50–80 nm) is significantly lower than that of the average entire EV suspension (~140 nm). OWSFM was utilized to compare refractive index and lipid content of two sub-populations of HMC-1.2 EVs of different buoyant densities at the single EV level using combination of scattering and fluorescence observations. This study revealed a higher lipid content for low density EVs while the analysis of the scattering intensity suggested a low average optical density for both low and high density EVs. In summary, this work highlights new surface-based sensing tools for the characterization of physicochemical properties of different EVs at the single particle level and improves concentration determination of subpopulations of EVs.

PF6.10

Use of surface plasmon resonance and Raman spectroscopy for the detection and characterization of exosomes

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Introduction: Exosomes have been proposed as potential biomarkers of multiple diseases. The quantity of exosomes released from cells and their composition changes according to the physio-pathological condition of the tissue of origin, mirroring their direct involvement in specific pathogenic mechanisms, including neurodegenerative processes like those of Alzheimer's disease. In order to better understand their biological function, an effective method of detection and characterization of exosomes is still needed. We propose herein two label-free approaches: surface plasmon resonance imaging (SPRI) that allows the study of the interactions of exosomes with biomolecules, and Raman spectroscopy (RS), suitable for exosomes characterization. **Methods:** Commercial exosomes from plasma of healthy donors were injected with a low flow on a SPRI chip conveniently treated: the chip was coated with a self-assembled monolayer, used to perform the conjugation of antibodies and to reduce unspecific binding. SPRI signals were collected to evaluate the interaction of antigens on exosome membranes with the antibodies. RS was used for the chemical characterization of exosomes extracted by ultracentrifugation from different human cell cultures, such as mesenchymal stem cells and SH-SY5Y neuroblastoma cells. **Results:** Taking advantage of the multiplexing capability of SPRI technology, different biomarkers have been analysed simultaneously, revealing the pre-

sence of selected antigens on the surface of exosomes. The collected Raman spectra showed the chemical composition of exosomes, with Raman peaks related to cholesterol, phospholipids, proteins and nucleic acids. **Conclusion:** Our preliminary results suggest that the SPRI technology has the potential to detect exosomes and to provide information about the interaction with biomolecules, paving the way for a better understanding of their biochemical role. Moreover, SPRI coupled with RS represents a valid and fast approach for exosomes characterization.

PF6.11

Combined lipid and protein analysis of extracellular vesicles with surface plasmon-enhanced fluorescence spectroscopy

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Extracellular vesicles (EVs) emerged as a new class of biomarkers, but simple and reliable methods for their analysis are still lacking. In 2014, plasmonic biosensors appeared as EV analysis tools using surface plasmon resonance for detection of EVs captured by specific antibodies to sensor surfaces. Surface plasmons can also be employed to enhance fluorescence emission of fluorophores in their proximity, which is used in surface plasmon-enhanced fluorescence spectroscopy (SPFS). Furthermore, such sensors are capable of detecting exosomes which are typically hard to detect due to their small size. We developed an SPFS biosensor for EV analysis based on combined lipid and protein binding. For EV isolation, cell culture supernatant of the cell lines CaOV3, HCT8 and BJ-1 biological samples, that is, ascites and plasma of ovarian cancer patients, was filtered and ultracentrifuged. The purified vesicles were analysed with flow cytometry examining the surface markers CD63, CD9 and PS as general EV makers and EpCAM and CD24 as markers for ovarian cancer cell-derived EVs. A biosensor employing SPFS for EV analysis was built, where Annexin-V was used as capturing molecule on the sensor surface and fluorescently labelled Annexin-V or specific antibodies were used for detection. EVs purified by ultracentrifugation were characterized by flow cytometry and the general EV markers were present on EVs of all sources. The ovarian cancer-specific markers were only present on EVs from CaOV3 cell culture supernatant and ascites, but not from plasma of ovarian cancer patients, indicating that the concentration of cancer-derived EVs in plasma might be below the detection limit. The SPFS model system could detect standard vesicles with a size of about 50 nm down to fM concentrations. Combining the specificity of molecular binding, the sensitivity of SPFS and the simplicity of plasmonic sensors, SPFS can provide a cheap and simple platform for EV analysis for scientific and clinical application.

PF6.12

A microfluidic chip system for profiling individual extracellular vesicles

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Introduction: To accelerate the application technologies of extracellular vesicles (EVs), their accurate characterization is important. Multi-parameter single-EV profiling using diameter, zeta potential and surface protein levels can provide reliable information of EVs. However, it is difficult for conventional equipment to measure several parameters of individual EVs simultaneously. We have been devel-

oping a microfluidic chip system for profiling individual EVs within 10 minute. In this study, we present the simultaneous measurement of individual EVs of breast cancer cells using the prototype. *Methods:* The system for profiling EVs comprises a microcapillary electrophoresis (mCE) chip and a laser dark-field microscope with a CMOS camera. Individual EVs with a diameter of 50 nm or smaller can be visualized. EV samples were extracted from supernatant of human breast cancer MDA-MB-231 and Sk-Br-3 cell lines after cultivation with a serum-free MEM medium for 48 h. After the differential centrifugation, a final pellet was suspended in phosphate-buffered saline (PBS). *Results:* Firstly, to examine a detection range of concentration of our prototype, dilution series of standard silica beads of 100 nm were introduced into microchannels, and area density of bright spot was evaluated. A liner relationship between the area density of bright spot and concentration was observed between 10^8 and 10^{10} particle/ml. For obtaining detection ranges of concentration of EVs, the measurement is now studying. Secondly, to examine the applicability of our system to EV analysis, EVs of breast cancer cells were evaluated. Mean diameter and zeta potential of EVs were 229 nm and -2.1 mV (MDA-MB-231) and 170 nm and -1.5 mV (Sk-Br-3), respectively. Since surface protein expression level of EVs can be evaluated by immunoelectrophoresis, protein expression level of each EV can be obtained.

PF6.13

Cloud-hosted tools for the analysis of extracellular RNA and the construction of the exRNA Atlas

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As part of the Extracellular RNA Communication Consortium, we developed tools for the analysis of extracellular RNAs and are employing them to construct a comprehensive atlas of exRNA species found in human body fluids. The tools were deployed using the Genboree framework and include Data Analysis and Data Submission Pipelines, and exRNA Metadata Tracking tools. Data analysis tools such as the exceRpt small RNA-seq pipeline, RSEQtools long RNA-seq pipeline, Pathway Finder and Target Interaction Finder are available in the Genboree Workbench. The exceRpt pipeline generates a variety of sample-level quality control metrics, produces abundance estimates for various small RNA species and makes available detailed alignment information for visualization and validation. The FTP data submission pipeline has been implemented for submitting small exRNA-seq datasets (with associated metadata) for processing through exceRpt, sharing via the exRNA Atlas and deposition into dbGaP, GEO and SRA archives. The GenboreeKB-hosted exRNA Metadata Tracking System allows for submission, tracking and editing of associated exRNA metadata for biosamples, donors, experiments, runs, studies and analyses. The public exRNA Atlas is available via the ExRNA Atlas link in the quick links section of the exRNA Portal. As of 15 January 2016, it includes 519 small exRNA profiles derived from about 6.4 billion reads uniformly processed using the exceRpt pipeline. Faceted filtering and data navigation tools are enabled by rich metadata standards developed by the consortium and metadata annotations contributed by the data producers. Uniform data quality metrics agreed by the ERCC were applied to all datasets. Users can browse, select, filter and download processed results and raw data files of open access datasets available in the exRNA Atlas. All links to data analysis tools, the exRNA Atlas and learning materials can be found in the exRNA Portal.

PF6.14

Developing quality control metrics for Extracellular RNA Communications Consortium data

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We present the current progress towards developing quality control (QC) standards for data being generated by the Extracellular RNA Communications Consortium (ERCC). In particular, we will focus on the QC metrics for small exRNA-Seq data which form the majority of the data that is currently being produced by members of the consortium. Metrics such as, for example, mapped read counts and fraction of reads mapping to categories, such as annotated miRNAs or potential laboratory contaminants. The metrics presented here were developed using data from seven different ERCC labs that were shared with the DMRR for the purposes of this analysis. Many of the sample-level QC metrics discussed here are already built in to the exceRpt small-RNA pipeline, which automatically reports detailed QC information for each sample. However, there is a clear desire amongst the international exRNA community to understand and interpret the quality of their RNA-seq samples. We therefore intend to include automatic QC evaluation of any sample submitted by any user based on the current set of 'gold-standard' exRNA samples provided by ERCC members. We discuss the various QC metrics, and acceptable tolerances, for what we define as 'high-quality' RNA-seq data from an extracellular preparation.

PF6.15

Size and refractive index determination by flow cytometry to discriminate between extracellular vesicles and lipoprotein particles in human plasma

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Introduction: Flow cytometry is the most widely used method to study single extracellular vesicles (EV). However, flow cytometry data have arbitrary units, which hamper data interpretation and comparison. Moreover, identification of EV requires fluorescent labelling, which is expensive, laborious and prone to artefacts. Here we present a label-free method to obtain the diameter and refractive index (RI) from the light scattering signals of a flow cytometer. Besides the obvious advantage of relating scattering signals in arbitrary units to comparable measurement units, we hypothesized that RI detection can discriminate between EV (RI 1.36-1.42) and lipoprotein particles (RI 1.45-1.60). *Methods:* Mie theory and reference beads were used to obtain the relation between the measured forward and side scattering of a flow cytometer (A50-Micro; Apogee, UK) and diameter and RI of a particle. We validated this approach by determining diameter and RI of (1) a mixture with 5 populations of silica and polystyrene beads <500 nm, and (2) EV and lipoproteins from the platelet-depleted supernatant of outdated platelet concentrates (centrifuged 3-fold, 1,550 g, 20 min). *Results:* For each population in the beads mixture, d and RI were determined with

measurement errors on the mean <8% and <2%, respectively. For the platelet-depleted supernatant, two populations with different RI were clearly discernable. Using fluorescent labelling, we confirmed that the population with RI = 1.42 contains EV and the population with RI > 1.42 contains lipoproteins. *Summary/conclusion:* We have developed the first method that converts the scattering signals of a commercial flow cytometer to the comparable measurement units of dimension and refractive index. We have demonstrated that this method is accurate and that it allows label-free discrimination between EV and lipoprotein particles in plasma.

PF6.16

Biological reference materials to standardize measurements of extracellular vesicles

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Most current studies on extracellular vesicles (EVs) use synthetic reference materials (SRM) to increase the comparability of EV measurement results between instruments, institutes and over time. As the physical properties of SRM, such as silica and polystyrene beads, differ from EV, the use of SRM as size references for EV may lead to erroneous inclusion of EV subpopulations and/or non-EV particles. Here, we present an overview of: 1) the optimal physical properties of biological reference materials (BRMs) as surveyed from people working with EV, 2) choices for such BRMs based on a literature search and 3) characterization of the physical properties of a promising potential erythrocyte-derived BRM, nanoerythrocytes (NEs). To discover the user preferences of the physical properties of BRM, a questionnaire was sent to 46 laboratories working with EVs (44% reply rate). According to the survey response, the most used method to measure EV is flow cytometry (90%), and biochemical resemblance to EV was considered to be the most important property of BRM. The results of literature search of BRM could be divided to three categories: 1) naturally occurring BRM (e.g. isolated EV populations, marine bacteria), 2) BRM from purified biochemical components or membranes (e.g. liposomes and oil droplets) and 3) miscellaneous biological particles (e.g. lipoparticles). Taking into consideration the desired properties for BRM and the feasibility for a large scale production, NEs, particles created by (mechanical) disruption of erythrocytes, were chosen for further studies. Large-scale production of NEs was tested by three different methods. Two important physical properties, that is, the size distribution and refractive index, of the produced NEs were comparable to EVs satisfying some of the key aspects of an optimal BRM. Further investigation of the BRM choices, novel and those reported in the overview, will be needed to find BRMs which match multiple standardization properties of the EV.

Poster Session 7 - Late breaking poster session 2

Chairs: *Aris Panaretakis and Alicia Llorente*

PF7.01

On-chip biodetection, analysis and nanometrology of platelet-derived vesicles

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Introduction: Extracellular vesicles (EV) are small vesicles released from different type of cells upon their activation or apoptosis. They play an important role in multiple biological processes and can serve as biomarkers of various health disorders. Thus the detection and characterization of EV in the large spectrum of size are of primary importance. Flow cytometry is conventionally used for the EV analysis, however due to its detection limit a large fraction of smaller particles (<300 nm) remains uncovered. Our work aims to use the potential of Surface Plasmon Resonance (SPR) technique to detect and qualify EVs specifically and without labeling and perform their nanometrology by using Atomic Force Microscopy (AFM). **Methods:** The on-chip EVs characterization is based of the SPR method. EVs are immunocaptured on the biochip surface using specific ligands. For the quantification of EV component we use two calibration standards of known size and concentrations: virus-like particles (VLP – diameter 50 nm) and modified melamine resin beads (diameter 920 nm). EVs captured on the biochip are further subjected to AFM investigation in order to identify their subpopulations in size and shape. **Results:** We have demonstrated that capture level of calibration standards is linearly related to their concentration in the sample. Dynamic concentration ranges vary from 8×10^{10} to 2×10^{12} particles/mL for the VLPs and from 3.25×10^7 to 2×10^8 particles/mL for melamine beads. We have adjusted this analytical platform to qualify microparticles released from resting platelets (PMP). We demonstrate that MPs with diameter below 300 nm represent up to 90% of the PMPs samples. Moreover differential analysis of MPs released from resting and activated platelets have been performed leading to significant size variation profiling that we aim to correlate with proteomic profiling. **Summary:** Our strategy enables simultaneous determination of EVs cell origin and size subpopulation in the large ranges.

PF7.02

Surface protein profiling on exosomes by proximity barcoding assay

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Proteomic profiling of exosomes provides valuable knowledge about their species and functions. Previous studies have shown structural and proteomic heterogeneity among exosomes secreted from a certain organ or isolated from a specific cell type. However, most of the current methods only measure the total protein content of a mixture of exosomes, thus fail to characterize proteomic heterogeneity on individual exosome level. Here we propose a novel method, proximity barcoding assay (PBA), to analyze the surface proteins on individual exosomes in the same sample. In PBA, distinct oligonucleotides with different Protein-SpecificTags (ST) were conjugated to each of 24 different antibodies, directed against 24 different potential proteins targets, expressed on the surface of exosomes. Clusters of antibody-conjugated oligonucleotides are brought in proximity when the antibodies bind proteins on the surface of the same exosome and will hybridize and extend on part of a DNA

concatemer of identical copies of a DNA strand carrying a unique Complex-ST, generated by rolling circle amplification (RCA). Thus clusters of oligonucleotides on the same exosome will incorporate the same Complex-ST. Through next-generation sequencing, we will be able to identify the protein composition on individual exosome by analyzing the Protein-ST and Complex-ST from each sequencing read. We applied PBA to analyze surface proteins expressed on individual exosomes purified either from seminal fluid (prostasomes) or isolated from different cell lines (Colo1, HCT116, MMK7, Kat0III). Among the complexes identified by PBA, we are able to find a series of surface protein combinations specific for each type of exosomes isolated from different body fluid or cell lines (specificity >95%). This method can be potentially used to identify exosomes from a mixture sample, e.g. human serum as biomarkers for diagnostics.

PF7.03

Search for the optimal dye for EV characterization

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Extracellular vesicles (EVs) are currently explored as biomarkers, cell communicators and therapeutic tools. A rigorous characterization is needed to understand their correct biology. AnnexinV (AnV) binding is commonly used as a general membrane marker for detecting EVs, however, electron microscopy studies have shown that the majority of vesicles, e.g. <500 nm, do not bind AnV. The purpose of this study was to find a membrane dye that would label the majority of EVs, not form micelles and help to distinguish EVs from cells and other particles. In addition, we investigated the possibility to use these dyes for labeling liposomes and nanoparticles for use as reference particles for standardization of EV measurement. We studied different lipophilic dyes and carboxyfluorescein succinimidyl ester (CFSE). The lipophilic dyes are water soluble, non-toxic, and show fluorescence only after membrane incorporation. CFSE is a membrane-permeable fluorescent dye that binds to intracellular/intravesicle amines as a way to fluorescently label cells and vesicles. All dyes were titrated for labeling of EVs generated from PPP, liposomes and polystyrene beads (PS). Dyes were also added to buffer as control. Fluorescent signals of particles were detected with imaging flow cytometry (ImageStreamX MKII, Amnis). As expected, CFSE did label EVs but not liposomes or beads. The lipophilic dyes labeled all particle types: EVs, liposomes and PS, the latter believed to be through charge interactions. Use of CFSE in comparison to the other lipophilic dyes resulted in a good separation of larger and smaller EVs. Of note, not all CFSE positive EVs were positive for AnV. CFSE and lipophilic dyes can be used to label the membrane of EVs and might offer a more complete analysis of EVs than it is possible with use of AnV. The lipophilic dyes also label liposomes and PS, which may be useful as standards/calibrators. A comprehensive analysis of these dyes is crucial to improve characterization of EVs.

PF7.04

Rapid isolation of intact exosomes and extracellular vesicles

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Introduction: We previously demonstrated the use of a new membrane affinity-based procedure in spin column format for fast and efficient isolation of vesicular RNA from exosomes and other extracellular vesicles (EVs) in serum and plasma [1]. Here, we demonstrate a new protocol, using the same principle for isolation of intact EVs from different biological fluids. **Methods:** Morphology and integrity of isolated vesicles were assessed using cryo-electron microscopy. Protein content of isolated vesicles was examined using SDS-PAGE. Physical characterization of EVs was performed by Nanoparticle Tracking Analysis (NTA). RNA content was analyzed quantitatively using real-time RT-PCR on selected mRNA and miRNA targets. Uptake of isolated EVs into different target cells, including primary mouse fibroblasts was examined using fluorescent labeling (bodipy-ceramide). Performance was compared to alternative methods, including ultracentrifugation and precipitation reagents. **Results and Conclusions:** EVs isolated using the new method are within expected size range and morphologically intact when viewed by cryo-electron microscopy. By analyzing total protein content of the isolated EVs, we also demonstrate efficient separation of EVs from highly abundant plasma proteins, compared to alternative methods. Consistent with this observation, particle counts determined using NTA in precipitated material appear to be inflated due to the presence of particulates other than vesicles, whereas counts obtained using the new method are more representative of EVs. Finally, we show efficient uptake of isolated EVs into target cells, further confirming recovery of intact, biologically functional vesicles.

Reference

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PF7.05

Comparative analyses of differential miRNA expression profiles between extracellular and intracellular components of SCN2.2 cells

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Suprachiasmatic nuclei cells (SCN2.2) are crucial in mammalian clock pacemaker, and secret soluble factors that drive oscillation of peripheral tissues or cells. The mechanisms of the synchronization are not fully understood, however, extracellular vesicle-mediated communication has been implicated in the intercellular transferring of miRNAs in circadian clock. Thus, this study explored the miRNA expression profile between extracellular matrix, including SCN2.2 cell-derived exosome and exosome-depleted conditioned media (EDCM), and intracellular components of SCN2.2 cells. We have assembled a quantitative miRNA expression profiles between intracellular (SCN2.2 cells) and extracellular (SCN-derived exosomes and EDCM) through the use of small RNA deep sequencing. The profiling data shows that expression profiles of extracellular miRNAs in both exosomes and EDCM are significantly different from their parental cells. We have identified 71 unique exosomal miRNAs and 270 unique miRNAs in EDCM by differential expression (DE) analyses. In particular, miR-34c-5p, miR494-5p, miR142, and let-7 in exosomes have been reported to be involved in circadian gene regulation. Additionally, several DE miRNAs in EDCM including miR-122, miR-155, miR-219, miR-200 and miR-375 were reported to target circadian clock genes such as *Per2*, *Bmal1&2*, *Clock*, and *Cry1&2*. Interestingly, both miR-155 and miR-29, which were significantly higher in exosomes and EDCM than in SCN2.2 cells, have been reported to directly involve in circadian clock

system. Through the comparison of the miRNA DE profiles in extracellular fluids, including exosomes and EDCM, and intracellular part of SCN2.2 cells, we have identified several candidate miRNA which are associated with in the regulation of circadian clock system. More work need to be done to explore the pathway and functional role of extracellular miRNAs, which regulate circadian rhythmicity in peripheral tissues by facilitating cell to cell communication.

PF7.06

Increased recovery of CD44+ extracellular vesicles from synovial fluid of healthy joints by incubation with hyaluronidase prior to isolation

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Introduction: Synovial fluid (SF) extracellular vesicles (EVs) are investigated for their role in joint disease and articular tissue repair. To understand EV-driven (patho)physiological processes in joints, knowledge about EVs in healthy joints is of utmost importance. EV sedimentation from SF is challenging due to the high and differential viscosity of the fluid in normal and diseased states of the joint. Since hyaluronic acid (HA) in SF causes this viscosity, it was hypothesized that EV-recovery could be optimized by enzymatic degradation of HA by incubation of SF with hyaluronidase (HYase). We here analyzed the EV-recovery from healthy SF in the presence and absence of HYase. Since CD44 is a marker for synovial cells and it is the HA-receptor, we focused on the effect of HYase-treatment on the recovery of CD44+ EVs. **Methods:** SF from healthy equine joints was incubated with or without HYase. EVs were isolated by differential centrifugation at 1,000g, 10,000g, 100,000g and 200,000g. EVs were labeled with PKH-67 and anti-CD44 antibody, floated in density gradients and quantified by high-resolution flow cytometry. Characterization was performed with western blotting (EV markers) and cryo-TEM (morphology and size distribution). **Results:** The majority of EVs from healthy SF could be recovered at 100,000g, however, in the absence of HYase a substantial amount of EVs was only recovered after 200,000g. Healthy SF-derived EVs contain CD9, Annexin-1 and CD90/Thy1.1, and most EVs are spherical with a diameter of 20-200 nm. Importantly, an increased yield of CD44+ EVs was achieved by pre-incubation of SF with HYase. **Summary:** For body fluids which contain relatively high amounts of HA, such as SF, HYase treatment could facilitate recovery and detection of CD44+ EVs from the HA-rich extracellular matrix. Furthermore, it is recommended, especially if HYase treatment is omitted, to use a high-speed centrifugation step of 200,000g for efficient sedimentation of EVs from SF.

PF7.07

Biological properties of extracellular vesicles in bovine milk

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Introduction: Milk extracellular vesicles (MEVs) are a novel class of milk bioactives which most likely support infant development by transferring molecular messages to the infant's cells. Bovine milk is also a rich source of MEVs. As bovine milk represents a significant food source world-wide, it is of our interest to investigate the biological potential in bovine MEVs. We have investigated the cellular uptake and fate of bovine MEVs in a human intestinal cell line.

Method: MEVs from untreated bovine milk was purified using a gentle protocol developed to avoid MEV pelleting. Isolated MEVs were labeled with Alexa Fluor tagged lactadherin, which binds tightly to phosphatidylserine in membrane bilayers. Moreover, lactadherin is an abundant protein naturally associated with MEVs. A human intestinal epithelial cell line, Caco-2, was used to investigate cellular internalization of MEVs. Fluorescence microscopy and plate reader measurements were used to evaluate the qualitative and quantitative cellular uptake, respectively. Additionally, fluorescent constructs of early, late and recycling endosomal markers (Rab5, Rab7, and Rab11 respectively) will be used to evaluate localization of MEVs taken up in Caco-2 cells. **Results:** Bovine MEVs were successfully labeled with fluorescent lactadherin. Caco-2 cells incubated with labeled MEVs reveal a linear increase in total cell fluorescence over 24 hours compared to controls when measured on a plate reader. Consistently, fluorescence microscopy confirmed uptake of MEVs in Caco-2 cells. Colocalization between MEVs and Rab5, Rab7, and Rab11, which indicate internalization via endocytotic pathways, are to be performed. **Summary/Conclusion:** A successful and specific fluorescent labeling method was developed to investigate bovine MEV uptake in the human intestinal cell line, Caco-2. Cell studies indicate increased uptake over time and fluorescence microscopy is being applied to investigate uptake mechanisms and faith of MEVs in the cells.

PF7.08

First proteomic profiling of exosomes in rodent intestinal lymph

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Introduction: Exosomes are released by many cell types and can be taken up by other cells. They may play an important role in cell-to-cell communication and disease pathogenesis. Exosomes derived from plasma or urine have been extensively studied, but that of intestinal lymph has not been reported due to the difficulty in obtaining these samples. Intestinal lymph is continuously draining from the intestine and enters the veins just before the heart and lungs. These organs may therefore be directly influenced by intestinal exosomes. **Methods:** Intestinal lymph was collected from a rodent experimental model of critical illness. Exosomes were isolated from the intestinal lymph using a commercially available exosome isolation kit. Particle size and concentration were determined by Nanosight. Proteomic profiling of lymph exosomes and its changes in critical illness were analysed by LC-MS. **Results:** The size and concentration of "exosomes" isolated from the intestinal lymph did not change significantly in the critical illness. The exosome preparation contained proteins previously identified in microparticles or exosomes (e.g. inter-alpha trypsin inhibitor), but also the highly abundant plasma proteins (e.g. complement C3, albumin). Common exosomal markers (e.g. TSG101, CD63) were not detected. Instead, substantial amounts of Apo B and A-I were found, indicating presumed co-isolation of chylomicrons. Lymph chylomicrons are similar to size of "exosomes", and produced in high concentration from the intestine. Our study indicates the unexpected difficulty in isolating pure exosomes using a commercial kit in this unique fluid. **Conclusion:** This present study provides the first attempt at a proteomic profile of an exosome preparation from intestinal lymph. Collectively, multiple proteins were identified, but found to have come from both exosomes and chylomicrons. New purification methods will be needed to study pure isolates of each particle type in this unique fluid.

PF7.09

Non-phagocytic epithelial cells take up microvesicles by macropinocytosis

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Introduction: As intercellular communicative vectors, able to deliver or present a range of surface and intravesicular molecules, many of the findings in the Exosomes and Microvesicles (EMV) field depend on an initial EMV interaction with recipient/acceptor cells. The nature of EMV-cell interaction, however, remains a poorly defined area requiring further investigation. **Methods:** Microvesicles (MVs) were isolated by removing cells (HeLa/HCVepC) and cell debris (160 g/5 min; 2x 4,000 g/60 min) and spinning the resulting supernatant (11,000g /90 min). To assay MV uptake by macropinocytosis, PKH67-labelled MVs and Dextran-FITC (as control) uptake was monitored by flow cytometry. The fate of endocytosed MVs together with that of lysotracker red DND-99, was monitored by confocal microscopy. MV-cell interaction was monitored by R18 dequenching (lipid mixing) and time lapse fluorescence microscopy. **Results:** It was possible to inhibit macropinocytosis with the Na⁺/H⁺ ion exchange pump inhibitor, amiloride and a range of other inhibitors including methyl- β -cyclodextrin, dynasore, bisindolylmaleimide, cytochalasin D and nocodazole. Using dextran-FITC as positive control, MV-PKH67 were found to co-locate to late endosomes. In addition, MVs were found to undergo a degree of fusion with the recipient cell plasma membrane using a lipid mixing assay. **Summary:** This study showed microvesicles to stimulate their uptake in non-phagocytic epithelial cells by macropinocytosis. Upon uptake by macropinocytosis, MVs located to late endosomes/lysosomes. MVs were also found to fuse with the plasma membrane of acceptor epithelial cells by lipid mixing. **Conclusion:** In short, this work reveals macropinocytosis as an important route by which MVs are taken up by epithelial cells. These findings are of paramount importance, from the point of view of understanding MV involvement in disease pathology and progression, but also with the longer term aim of their use in drug delivery and therapy.

PF7.10

Characterization of RNA contained in extracellular vesicles released by primary astrocyte cultures

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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 microvesicles 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 microvesicles and their content. In particular, we showed that exosomes derived from mutant SOD1-primary astrocyte cultures are sufficient to induce selective motoneuronal death (Basso et al., J Biol Chemistry 2013). Considering the growing evidence of a role for RNA pathology in ALS, we are interested in analyzing the RNA profile of EVs derived from several models of ALS and investigate the possibility that an alteration in RNA cargos into the EVs contributes to the spreading of motoneuronal death. In order to reach our goal, we are comparing numbers of astrocytes, plating, purification protocols and RNA extraction methods to reach a consistent method to analyze the nucleotide EV content.

PF7.11

Proteomics and miRNA Analysis of Exosomes Derived from Differentiating Neuronal Cells

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Tufts University, Medford, MA, USA

Exosomes deliver their endogenous proteins, lipids and RNAs to their neighboring cells to cause functional and physiological effects. They are known to promote tissue regeneration by the delivery of various biologics, including microRNA, mRNA and proteins. However, the mechanism of exosome-promoted tissue regeneration is still not well understood. In our previous study, we showed exosomes derived from differentiating neuronal progenitor cells (PC12), drives the differentiation of mesenchymal stem cells (MSCs) into neuron. We have isolated exosomes from different differentiating stage of these cells to identify the functional contents for neuronal differentiation. Here, we discuss our results on microRNA profiling and proteomics analysis with an aim to elucidate the mechanism of exosome-assisted cell reprogramming.

PF7.12

Cytotoxic miRNAs against the cultured mesenchymal stem cells are included in human T cell-derived exosomes

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Introduction: Our group has found in murine models that CD8+ T cell-released exosomes play as an inhibitor for tumor progression including invasion and metastasis by destructing mesenchymal tumor stromal cells in part micro (mi) RNA-mediated manner. In this study, we tried to identify cytotoxic miRNAs in human T cell-released exosome-dominant miRNAs against mesenchymal stem cells (MSCs) in vitro. **Methods:** Exosomes were purified from the culture supernatants of CD3- and RetroNectin-stimulated PBMCs by the ultracentrifugation after removing cells and debris. Exosome-dominant miRNAs were selected by comparative analysis of microarray data of the exosomal miRNAs with the donor T cell miRNAs, and then synthesized to examine cytotoxicity against MSCs. Cytotoxicity of each synthesized miRNA against adipose tissue-derived MSCs cells was examined after transfection by a real-time cell analyzer. **Results:** CD8+ T cells enriched in the culture process of PBMCs produced exosomes of approximately 150 nm size. The CD8+ T cell-released exosomes showed the expression of not only tetraspanins but also CD8 and HLA-I molecules. miRNAs from CD8+ T cell-released exosomes showed different distribution pattern from donor T cell miRNAs, indicating the active translocation of endogenous cellular miRNAs into exosome cargos when multivesicular bodies were generated in donor T cells. By the real-time cell analysis, we identified two exosomal miRNAs (miR-6089, miR-6090) capable of exhibiting cytotoxicity and the attenuation of growth of MSCs. The total mRNA array of cytotoxic miRNA-introduced MSCs revealed the correlation between MSC cytolysis and interferon (IFN)-related gene expressions. **Conclusion:** We demonstrated that anti-MSC miRNAs existed into human T cell-released exosomes consistent with the study of murine CD8+ T cell-released exosomes. Our study may propose pivotal insights for clinical application of exosomal miRNAs in patients with invasive and metastatic cancer.

PF7.13

Automated extracellular vesicle production and concentration from bone marrow-derived mesenchymal stem cells

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Introduction: The Quantum cell expansion system has been shown to successfully expand a variety of adherent and suspension cells in vitro all the while being functionally-closed and automated. The properties of the semi-permeable hollow fibers along with the ability

to command the system to automatically perform various tasks via the graphical user interface make the Quantum an efficient system for producing extracellular vesicles (EVs). When cells shed EVs into the fluid, EVs can be concentrated on the intracapillary (IC) side of the hollow fibers while the semi-permeable membranes allow essential nutrients to reach the cells via continuous perfusion and waste is actively removed. **Methods:** Bone marrow derived mesenchymal stem cells (bmMSC) were expanded on the Quantum with serum-containing media until confluent. The serum was completely washed out from the system and replaced with base media. EVs were concentrated on the IC side for 48 hours and then harvested. **Results:** The total protein concentration of isolated EVs from the Quantum was five-fold higher than EVs isolated from cells cultured in tissue culture flasks. The ratio of cells harvested to CD63+ EVs was nearly 1:10,000. **Summary:** EV production in the Quantum required less manual intervention due to the automation of the system and EV particles were able to concentrate on the IC side before harvesting. The number of EV particles compared to the number of cells harvested from the Quantum was exponential.

PF7.14

Extracellular vesicles in the regulation of host-pathogen interactions during malaria

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¹University of Fribourg, Fribourg, Switzerland; ²Harvard University, Boston, USA

Extracellular vesicles (EVs), including exosomes and microvesicles, are small membrane vesicles derived from multivesicular bodies or from the plasma membrane. Most cell types release EVs, which then enter the bodily fluids. These vesicles contain a subset of proteins, lipids and nucleic acids that are derived from the parent cell. Recent works demonstrated that EVs play important roles in intercellular communication, both locally and systemically, as they transfer their contents, including proteins, lipids and RNAs, between cells. EVs are involved in numerous physiological processes, and vesicles from both non-immune and immune cells have important roles in immune regulation. Therefore EVs contribute significantly to pathology during infections. We have showed that *Plasmodium falciparum* infected red blood cells (iRBCs) release vesicles, which not only regulate parasite development but also mediate the host-pathogen interactions. EVs contain RBC-derived miRNAs that regulate gene expression in the recipient cells including endothelial cells and neutrophils. We are investigating the contribution of EVs in the regulation of the pathogenesis during malaria. Our data suggest that miR451a contained in EVs is an important regulator of the immune response to the malaria parasites.

PF7.15

The role of extracellular vesicles in pathogenesis of the human pathogen *Cryptococcus gattii*

Ewa Bielska, Marta Arch Sisquella and Robin C. May
 School of Biosciences, Institute of Microbiology and Infection/School of Biosciences, University of Birmingham, UK

Cryptococcus gattii is a fungal pathogen of humans and other animals. Although typically a disease of immunocompromised patients, since 1999 an unusual lineage of this organism has been responsible for a large and ongoing cluster of infections in otherwise healthy people living in the Pacific Northwest area of North America. We recently demonstrated that the hypervirulence of this lineage results from an unusual 'Division of labour' mechanism in which a virulent strain of *C. gattii* enables a non-virulent strain to survive and proliferate inside the hostile environment of host macrophages. To achieve this, individual fungal cells must communicate in order to act as a "team", but how they do this remains unknown. Our data suggests that this communication may be mediated via extracellular

vesicles (EVs), which are known to be secreted by many organisms including bacteria, fungi and mammalian cells. EVs isolated from a virulent strain of *C. gattii* increased survival (but not phagocytosis) of a non-virulent strain inside macrophages. This effect relies on proteins within the vesicles but not on capsular polysaccharide, which is a major EV cargo. Thus our data suggest a novel mechanism of fungal virulence in *C. gattii* that involves communication via EVs and potentially represents a new avenue for therapeutic intervention.

PF7.16

Profiling and characterization of embryo and seminal fluid extracellular vesicle-associated microRNAs in *Sus scrofa*: a possible role in differentiation and developmental communication processes

Muhammad Nawaz¹, Tina Rødgaard Højboe², Marco Maugeri¹, Matin Mehryar¹, Klaus Villemoes³, Peter M. H. Heegaard², Henrik Callesen³ and Hadi Valadi¹

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Introduction: Extracellular vesicles (EVs) are nano-sized vesicles secreted by variety of cell types, contain RNAs, proteins, lipids and serve as newly described mediators of cell-to-cell communication.

Hitherto, embryonic cells and seminal fluid have been reported to contain EVs, nevertheless, their miRNA content have not been characterized. We performed a systematic evaluation of miRNAs from pig embryonic cell-derived EVs and semen EVs to identify putative miRNAs and their possible role in differentiation and development. **Methods:** EVs (exosomes and microvesicles) were isolated from embryo cells, seminal fluid and PZM-3 medium and were characterized by Zetasizer-nano, Nanosight Tracking Analysis (NTA) and Atomic Force Microscopy (AFM-Bio). Total RNA was extracted from embryo cells, embryonic EVs, seminal EVs and PZM medium by 3D-Gene™ miRNA extraction reagent (Toray, Japan) and miRNA identification was performed using microarray platforms. From microarray data, some potential EV-miRNAs were validated by high-throughput qPCR system (Fluidigm) and used for in vivo uptake to witness their effects on embryo growth. **Results:** The size of characterized EVs ranged between 40–350 nm which include both exosomes and microvesicles. In total, 79 miRNAs were identified in embryonic cells, 16 miRNAs were unique to EVs from embryonic cells and 11 unique to EVs from semen. In comparison, 10 miRNAs were common between embryonic cells and embryonic EVs and 4 common between embryonic cells and semen EVs. However, no common miRNA species were detected in embryonic EVs and semen EVs. MiRNA target analysis, gene ontology and networking analysis revealed that embryonic and semen EV-miRNAs are enriched in RNA biosynthetic regulation process, reproduction, cellular differentiation and embryonic development. **Summary/conclusion:** These data for the first time provide an insight into the role of EV-miRNAs in pig embryonic cell communication and differentiation during developmental process.

Networking Event
Location: Laurens Church

7:30 p.m.

Scientific Program ISEV2016 meeting Saturday May 7, 2016

Room: Willem Burger

Experts Meet Session 1 - Biofluids: Blood

Moderator: *Kenneth W Witwer*

8:00-8:45 a.m.

Room: van Weelde

Experts Meet Session 2 - Biofluids: Milk

Moderator: *Marca Wauben*

8:00-8:45 a.m.

Room: Ruys, van Ruckevorsel, Plate Zaal

Experts Meet Session 3 - Biofluids: Urine

Moderator: *Guido Jenster*

8:00-8:45 a.m.

Oral Presentations

Room: Willem Burger
Late Breaking Session 1

Chairs: *Alicia Llorente and Lydia Alvarez-Erviti*

9:00-10:00 a.m.

LBO1.1

Cancer-ID: strategies to identify rare extracellular vesicles in human body fluids

Linda Rikkert¹, Frank Coumans¹, Rienk Nieuwland¹ and Leon W.M.M. Terstappen²

¹Academic Medical Center, Amsterdam, The Netherlands; ²Department of Medical Cell BioPhysics, MIRA institute, University of Twente, Enschede, The Netherlands

Introduction: Extracellular Vesicles (EV) have a great potential as biomarker in cancer diagnostics. In the Dutch Cancer-ID program 8 universities and 23 companies work together to develop a platform capable of reliable identification and characterization of tumor vesicles in human body fluids. **Methods:** Methods involved are atomic force microscopy, Coherent anti-Stokes Raman spectroscopy, flow cytometry, scanning electron microscopy-Raman microspectroscopy, RNA sequencing, smart microsieves, and surface plasmon resonance imaging. To compare measurement results, EV-containing samples from prostate cancer cell lines (PC3 and LNCaP) and blood products (platelets, red blood cells, and plasma) were distributed to obtain a first assessment of specificity and sensitivity of all methods. **Results:** Of before mentioned methods, both flow cytometry and Raman microspectroscopy are operational, whereas other methods are currently being optimized for EV detection. Raman microspectroscopy is capable of capturing and measuring single EV, but a molecular fingerprint to reliably identify tumor EV will require additional experiments. Flow cytometry was able to identify tumor EV (CD63+, EpCAM+) in PC3-EV and LNCaP-EV spiked plasma samples at a ratio of 1:80,000. The spike in dilution and the lowest detectable tumor EV concentration can be used to compare sensitivity and specificity between techniques. **Conclusion:** The heterogeneity of EV in morphology, size and refractive index comprises a major challenge to develop a single platform capable of reliable identification and characterization of tumor EV in "liquid biopsies". By combining complementary detection methods, we will be able to explore the full potential of EV in cancer diagnostics. Further, a detection limit of 1 tumor EV in 80,000 EV means that the sample will need to be enriched prior to analysis of clinical samples.

LBO1.2

Chemokine receptor targeting inhibits EV uptake and EV-induced phenotype

Jordi Berenguer¹, Tonny Lagerweij¹, Xi Wen Zhao¹, Marloes Zoetemelk¹, Sophie Dusoswa², Mark C. de Gooijer³, Jacco Van Rheenen⁴, Olaf Van Tellingen³, Michiel Pegtel⁵ and Thomas Wurdinger¹

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Introduction: Extracellular vesicles (EVs) are important mediators of tumor communication. Tumor EVs have been shown to support tumor growth and induce therapy resistance. Therefore, targeting EV

uptake is a promising therapeutic strategy. **Methods:** In order to identify EV receptors, we performed a siRNA screen against a library of G protein-coupled receptors combined with PKH-67-labelled EV uptake analysis. We performed validation studies by genetic and functional inhibition. We used a Cre recombinase-based model for EV transfer studies in vivo and in vitro. In vivo tumor growth analysis was performed using bioluminescent GBM xenografts. **Results:** We have observed physiologically relevant transfer of GBM EVs within the tumor. We identified and validated a chemokine receptor responsible for glioblastoma (GBM) EV uptake, in a mechanism involving bridging by the chemokine ligand. GBM EVs induced phenotypical changes in GBM EV recipient cells such as increased proliferation rates and enhanced resistance to the chemotherapeutic agent temozolomide. Pharmacological inhibition of the newly identified EV receptor counteracted the phenotypical changes observed in GBM EV recipient cells. **Conclusion/Summary:** GBM EVs stimulate both tumor growth and therapy resistance. We have identified a chemokine receptor which can be pharmacologically targeted for inhibiting EV uptake and counteracting EV-induced effects.

LBO1.3

The role of cancer cell-derived exosomes in monocyte survival

Yanping Ding, Xiao Song, Gang Liu, Xiao Yang and Guangjun Nie
National Center for Nanoscience and Technology, Beijing, China

Introduction: Tumor-associated macrophages (TAM), an essential tumor stromal cell type, are mainly derived from recruited monocytes. In physiological environment, monocytes undergo spontaneous apoptosis to maintain the homeostasis of the immune system. However, they can survive in the inflammatory tumor microenvironment for continuous generation of sufficient TAMs. The mechanism underlying how monocytes escape apoptosis within tumor is largely unknown. **Methods:** We imitated a pro-inflammatory environment by treatment of human primary monocytes with a combination of lipopolysaccharide and interferon- γ , and a conflicting environment with lipopolysaccharide, interferon- γ and interleukin-4. Cancer cell-derived exosomes were isolated from the culture medium of various human cancer cell lines by ultracentrifugation. After treatment of exosomes in above inflammatory niche for different days, viable monocytes stained by trypan blue were counted. Key signaling molecules of the survival machinery in monocytes were analyzed by western blot. Functional molecules in cancer cell-derived exosomes were evaluated after construction of knockout clones using CRISPR-Cas9 system or treating cells with specific inhibitors. **Results:** Cancer cell-derived exosomes significantly elevated monocyte survival rate and prevented caspase cleavage in the inflammatory niche through activation of Ras and extracellular signal-regulated kinases in the mitogen-activated protein kinase pathway. Activated receptor tyrosine kinases in cancer cell-derived exosomes, such as phosphorylated epidermal growth factor receptor and human epidermal growth factor receptor 2, were indispensable for improving monocyte survival. **Conclusion:** This study demonstrated the role of cancer cell-derived exosomes in promoting monocyte survival and explored the underlying molecular mechanism, which provided evidences for the alteration of monocyte status before differentiation into tumor-associated macrophages.

LBO1.4

GABARAPL1 is required for hypoxia-induced extracellular vesicle secretion

Tom Keulers¹, Sten Libregts², Marco Schaaf¹, Hanneke Peeters¹, Kim Savelkouls¹, Hans Duimel³, Johan Bussink⁴, Barry Jutten¹, Marc Vooijs¹, Marca Wauben² and Kasper Rouschop¹

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Hypoxia is a hallmark of solid tumors and triggers secretion of pro-angiogenic factors to stimulate blood vessel formation. In part, Extracellular Vesicles (EVs) mediate the intercellular communication between tumors and endothelial cells. Here we describe the role of GABARAPL1 in the secretion of EVs that contribute to angiogenesis and tumor progression. GABARAPL1 production is increased during hypoxia. GABARAPL1 associates with vesicles and colocalizes with CD63, CD81 and CD9. Its knock-down results in reduced EV secretion

as assessed by immunoblot analysis and quantitative single vesicle-based high-resolution flow cytometry. EV analysis by Resistive pulse sensing (qNANO) revealed GABARAPL1 requirement for a subset EVs that is uniquely secreted during hypoxia. Mass spectrometry on conditioned medium of GABARAPL1 knockdown cells show a decrease of angiogenic factors like VEGF and PDGF-AA. In line, the angiogenic capacity of GABARAPL1-deficient vesicles compared to control EVs, as assessed by endothelial tube formation, is abrogated. Furthermore, cryo immuno-electron microscopy showed that GABARAPL1 is expressed on the outer leaflet of EVs, indicating that GABARAPL1 is accessible for targeting. Interestingly, anti-GABARAPL1 antibodies blocked the angiogenic capacity of GABARAPL1 EVs. Together, these data suggest that EV secretion is mediated by GABARAPL1 and that GABARAPL1+ EVs have angiogenic potential. In vivo, GABARAPL1 knockdown reduced tumor growth and is associated with decreased vessel density. Interestingly, implantation of a control tumor in the same animal resulted in growth and vessel density normalization, emphasizing the importance of circulating factors. Moreover, GABARAPL1 targeting after a single dose of irradiation (10Gy) increased tumor regrowth delay. Also these tumors displayed a decreased vessel density. In conclusion, we provide evidence that GABARAPL1 is involved in the secretion of hypoxia associated EVs that act as mediators of angiogenesis.

Room: van Weelde
Late Breaking Session 2
Chairs: Yves DeClerck and Felix Roy

9:00-10:00 a.m.

LBO2.1

EV-TRAQ: Tracking transparent reporting and quality to boost standardized extracellular vesicle research

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Introduction: While a small, dedicated part of the scientific community is struggling to determine the characteristics of all extracellular vesicle (EV) subtypes, their putative functions are featured in an increasing number of publications. This dangerous hiatus between our limited basic knowledge and extensive functions that are being attributed to EVs is emphasized by the fact that most studies do not properly report their isolation and characterization. **Methods:** We performed a PubMed search for research articles with keywords "exosomes" or "extracellular vesicles" published in 2010–2015. After manual inspection, 1256 articles were withheld for inclusion. Articles were analysed based on a matrix querying article content, aim, and EV isolation and characterization procedures. **Results:** After evaluating the quality of all study entries (n=1785) using the ISEV minimal experimental requirements, only 1 study was fully compliant. For all sample types, less than 10% of studies have a compliance of 50% or more. Isolation methods increasingly comprise commercial kits (15% in 2014) at the expense of density gradients (10% in 2014). Differential ultracentrifugation is by far the most used method (>50%), but the heterogeneity in applied centrifugation steps reveals an estimated 250 potentially different vesicle populations that were isolated. Quality controls are often omitted, with more than 2 protein markers being checked maximally in 25% and non-EV enriched proteins in only 20% of studies (dependent on sample type). We established EV-TRAQ, a database tracking EV research evolution as online resource to identify trends in methods and quality allowing for timely update of the minimal experimental requirements. **Summary:** We are confident that this meta-analysis will contribute to the maturation of EV research by pinpointing existing gaps in isolation and characterization procedures as well as allowing comprehensive evaluation of EV research methods and quality through our online repository.

LBO2.2

A combination of size-exclusion chromatography with density gradient centrifugation to purify EVs from plasma

Glenn Vergauwen^{1,2}, Joeri Tulkens¹, Pieter Mestdagh³, Jo Vandesompele³, Raija Sormunen⁴, Esther Nolte-t-Hoen⁵, Marca Wauben⁵, Kris Gevaert⁶, Hannelore Denys⁷, Geert Braems², Rudy Van den Broecke², Olivier De Wever¹ and An Hendrix¹

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Introduction: The exponential increase of EV-related research in the last decade has given rise to multiple described protocols for isolation of EVs in several sample types, including plasma. Despite these efforts, the 'golden standard' has not yet been established for

plasma. The quantity-purity trade-off is crucial and mandatory for biomarker discovery. Here we provide a protocol for high-purity isolation of EVs in plasma. **Methods:** Plasma EVs were isolated in a two-step protocol consisting of size-exclusion chromatography (SEC) followed by iodixanol density gradient ultracentrifugation (ODG). Nanoparticle tracking analysis (NTA), Western blot (WB), high resolution flow cytometry and ELISA were implemented to assess the purity of isolated EV samples. Results were validated by proteomic analysis. **Results:** SEC separates EVs from the bulk of protein contaminants such as albumin, and Ago2. The EV-rich SEC-fractions contain 31% of total particles of plasma, while only less than 1% of protein. Combinations of SEC fractions show therefore a 250-fold enrichment in EV compared to protein but still show presence of lipoprotein particles. Addition of density gradient ultracentrifugation further purifies the CD9 and flotillin-1 positive EVs from lipoproteins and Ago2 protein complexes. Spiking of plasma with exogenous GFP-positive EVs shows presence of GFP in similar SEC and ODG fractions as the endogenous EVs. Our protocol was further validated on plasma from breast, ovarian and prostate cancer patients. We were able to detect EV-enriched markers (CD9, flotillin-1) from 6ml plasma of cancer patients on WB, without contaminating Ago2 and ApoA-1. **Conclusion:** Combination of SEC and density gradient ultracentrifugation is mandatory for purification of EVs from contaminating proteins and lipoproteins in plasma. We provide hereby a protocol for isolation of high-purity EVs from plasma, a prerequisite for biomarker discovery.

LBO2.3

Raman tweezers microspectroscopy of extracellular vesicles: a pilot study on nucleic acids detection and characterization

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Introduction: Extracellular vesicles (EVs) are involved in intercellular communication providing a wealth of information on health and disease. The search for EVs-based biomarkers is an actual challenge especially for early diagnosis of cancer. In addition to «omics» techniques deciphering individual molecules, Raman Tweezers Microspectroscopy (RTM) is arising as a label-free analytic tool, providing information on global molecular composition of EVs [1]. Inspired by the discovered ability of EVs to transport nucleic acids (NAs), in this study we tested the RTM potential for NAs detection and characterization. **Methods:** RTM was used to study small EVs obtained from human urine (exosomes) and secreted by primary rat and mouse hepatocytes, as well as mixed samples of transfection agents FuGENE and oligofectamine in DNA and siRNA solutions, respectively. Bioparticles were optically trapped by a 785-nm laser beam through a microscope objective and their Raman spectra were analyzed for the presence of NAs. **Results:** The lower concentration limit for Raman detection of NAs was of the order of a few mM in base-pairs, as determined for free calf-thymus DNA in PBS. However, for optically trapped mixed samples, DNA and siRNA signals were observed even for initial NAs concentration as low as 1 μM in the surrounding solution. Exosomes from human urine did not show any NA signal, while EVs from rat and mouse hepatocytes revealed random NAs signals of variable intensity. **Summary/conclusion:** Although nano- and micromolar concentrations of free NAs are out of detection range, RTM can effectively detect and quantify high local concentration of NAs bound or incorporated into bioparticles.

Raman spectra provide specific structural information about NAs, in particular the A/B form of the helix. Possible RTM applications comprise a search for DNA-biomarkers and discrimination between small EVs and viruses in 50-100 nm size range, among others.

Reference

1. I. Tatischeff et al. JEV, 2012, 1, 19179.

LBO2.4

A Mathematical Approach to Understand the Generation of Multivesicular Bodies

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²Institute of Molecular Biomedicine, Comenius University, Bratislava, Slovakia;

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Introduction: Current theory suggests that multivesicular bodies (MVBs) are produced from endosomes through their inward budding. Thus, the intraluminal vesicles (ILVs) inside the MVBs are formed by the budding of the membrane of an early endosome (EE). Here we challenge this hypothesis, first by testing mathematically

whether this dogma can be true, and then applying the mathematical models to published data. *Methods:* The surface area (SA) of MVBs and the SA of ILVs were calculated using images produced by published studies, including electron microscope tomography. Further, electron microscope sections of MVBs were modeled to be spherical, and the SA of MVBs and ILVs were estimated based on the packing density. Mathematical operations were used to establish credible conclusion. *Results:* The numbers of ILVs within an MVB, diameters of both and the packing density varies hugely among species. One MVB in a eukaryotic cell was apx. 600nm and carried estimated 500 ILVs. In this example, the SA of the MVB is $1.1\mu\text{m}^2$ and the SA of ILVs (60nm diameter) is $5.7\mu\text{m}^2$. This example would have suggested that the EE was 1400nm in diameter. Further developing the mathematical models compared published data with estimated calculations, which suggested that there should be a negative correlation between MVB diameter and number of ILVs if the budding was uniquely inward budding. However, the published data showed a positive correlation in different cells. This argues that the EE membrane is insufficient to provide membrane for the ILVs. *Summary/Conclusion:* This mathematical modeling of the relationship between MVB diameter and number of ILVs strongly suggests that the ILVs are not only produced by inward budding of the EE membrane. Therefore, ILVs are likely to be produced by addition of membrane material prior to their release to the extracellular space as

“exosomes”. This data can explain why bioinformatics argues the presence of multiple organelle components in exosome isolates.

Room: Ruys, van Ruckevorsel, Plate ZaaIs Late Breaking Session 3

Chairs: *David Carter and Jason Webber*

9:00-10:00 a.m.

LBO3.1

Assessing the role of extracellular vesicle enclosure as non-lytic egress route for mengovirus

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Picornaviridae are a large family of non-enveloped RNA viruses that are released via induction of cell lysis. However, recent electron microscopy studies indicate that part of the virions exit the cell enclosed in extracellular vesicles (EV), thereby blurring the classic distinction between ‘enveloped’ and ‘non-enveloped’ viruses. Enclosure of virions and viral components in EV may have a large impact on antiviral immunity, and this phenomenon can thus influence the pathology of many human and animal diseases caused by the picornaviridae. Nevertheless, the relative frequency of this process and the regulating viral and/or host factors involved remain elusive. We therefore set out to characterize the composition and release dynamics of the particles excreted upon picornavirus infection, using mengovirus as model system. The release of mengovirus particles was analyzed over time using end-point titration while the onset of the lytic phase of the infection cycle was monitored via live-dead staining and release of cytoplasmic luciferase. A strong increase in extracellular virus titers could already be observed 8 hours post infection. In this time frame, however, there was no evidence of cell death or lysis, suggesting the presence of a non-lytic release pathway. Iodixanol density and velocity gradient centrifugation were used to purify particles released early in infection. The different iodixanol fractions were analyzed for the presence of viral RNA, capsid protein, infectious units and EV markers. This analysis revealed the existence of a previously undescribed membrane-associated form of mengovirus. Building upon this approach, EV composition and release kinetics during mengovirus infection will be assessed quantitatively on a single particle basis using high-resolution flow cytometry. Knowledge on the formation and release of EV-virus hybrids will aid our understanding of how EV contribute to viral dissemination and antiviral responses.

LBO3.2

Kaposi’s sarcoma-associated herpesvirus manipulates exosomes secretion to regulate its microenvironment metabolism

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Altered tumour metabolism is now generally regarded as a hallmark of cancer and presents an attractive therapeutic target. Although

most studies focus on the autonomous metabolic regulation in cancer cells and their role in tumorigenesis recently it was suggested that cancer cells can induce metabolic alteration also in surrounding cells. Kaposi’s sarcoma-associated herpesvirus (KSHV) is the etiological agent of Kaposi’s sarcoma (KS). KS is the most common neoplasm in HIV-1-infected individuals and is also induces significant morbidity in other immunosuppressed individuals and in populations where KSHV infection is endemic. KS also provides a tractable model for elucidating to delineate critical pathways involved in oncogenesis, and an entry point to study aspects of tumour development. We demonstrate that exosomes secreted from KSHV latently infected cells can strikingly reprogram metabolism in surrounding non-infected cells. We found that upon uptake of these exosomes non-infected cells reduce mitochondria biogenesis and oxidative phosphorylation, and induce aerobic glycolysis. Moreover our results suggest that this reprogramming is mediated by the KSHV encoded microRNAs which are transferred to non-infected cells via exosomes and down regulate metabolic genes. Finally our data shows that this exosomes mediated metabolic reprogramming of neighbour cells supports the growth of infected cells. Taken together our results reveal a new mechanism used by KSHV to transfer its microRNAs into non-infected cells. In this way KSHV manipulating and recruiting its microenvironment during latency without producing new viruses and exposing itself to the immune system.

LBO3.3

Exosome from Plasma of Plasmodium-infected Host Expressing MicroRNAs Inhibit Angiogenesis in A Murine Lewis Lung Cancer Model

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Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, China

Our previous studies demonstrated that malaria infection provided anti-tumor effects by inducing both anti-angiogenesis and potent anti-tumor immune responses in a murine Lewis lung cancer model. Exosomes, which are extracellular vesicles that function in inter-cellular communication, may play a key role in the dissemination of pathogen, host-derived molecules and in modulating the immune response during infection. It is reported that microvesicles isolated from the plasma of Plasmodium-infected could induce specific immune response in host, but it is unknown whether the exosomes derived from Plasmodium-infected hosts effect tumor angiogenesis or not, therefore we designed a murine Lewis lung cancer model study to answer this question. Exosomes were isolated from plasma of Plasmodium-infected mice. We found Intra-tumor injection of the exosomes significantly inhibit tumor growth in mice. Furthermore, the exosomes when co-cultured with endothelial cells markedly suppressed VEGFR-2 expression and decreased endothelial cell migration, which demonstrated the exosomes contribute to angiogenesis inhibition in vitro. Interestingly, we detected remarkably higher expression levels of micro-RNA cluster (miRNA16/322/497) in the exosomes that secreted from the plasma of Plasmodium-infected mice, which are predicted to target VEGFR-2 through bioinformatics. Overexpression of miRNA16/322/497 cluster in endothelial cells was followed with decreased VEGFR-2 expression and inhibition of

angiogenesis in vitro, whereas inhibition of miRNA16/322/497 cluster significantly alleviated these effects. These data suggest exosomes derived from Plasmodium-infected host play a specific role in anti-angiogenesis in tumor development, which provided a novel understanding of the interaction between malaria infection and lung cancer.

LBO3.4

Spinal Cord Injury Recovery Mediated by Exosomes from Mesenchymal Stem Cells (MSC)

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Introduction: Traumatic spinal cord injury (SCI) damages the cord vascular barrier and allows local accumulation of destructive inflammatory cells with release of toxic molecules that infiltrate into the cord, contributing to the neuronal loss, axon severing, and demyelination that can lead to paralysis. Intravenous infusions of mesenchymal stem cells (MSCs) can reduce the severity of SCI, but

the involved mechanisms are not fully understood. *Methods:* Groups of 8 young adult rats were subjected to a homogeneously delivered moderate contusive SCI at the T9 level. Then cell treatment was attempted by i.v. infusion of 1x10⁶ rat bone marrow derived MSCs or exosomes isolated from MSC-conditioned media, compared to media alone, at one week post-SCI. Animals then were assessed for functional recovery and vascular permeability by Evans blue dye extravasation. Distribution of DiR-labeled MSCs vs MSCexos was assessed by in vivo and ex vivo imaging of organs and by fluorescence microscopy monoclonal antibody staining of frozen sections. *Results:* SCI resulted in profound weakness and large vascular leakage that were reduced in MSC transplanted rats beginning 1 week post-SCI. However, i.v. infused MSCs were not detected within the spinal cord at any time point, but appeared to traffic just transiently to the lungs. In contrast, MSCexos produced comparable improvements and were readily detected at sites of SCI; especially localizing to M2 macrophages (Mf) associated with healing vs. pro-inflammatory M1 Mf. *Summary:* Infusion of MSCexos one week after contusive SCI reduced, like i.v. injection of MSC increased vascular permeability and improved functional recovery with strong localization at site of SCI; preferentially to local M2 Mf of healing and not M1 Mf of inflammation *Conclusion:* The data suggest that the therapeutic effect of MSCs on SCI likely is mediated by their secreted MSCexos that preferentially target healing M2 and not inflammatory M1 macrophages.

Featured Abstracts

Room: Willem Burger

Plenary Session 4 - ISEV2016 Featured Abstracts

Chairs: *Guillaume van Niel, Edit I Buzás and María Yáñez-Mó* 10:30-11:50 a.m.

PRS.1

The first massive open online course on extracellular vesicles
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Introduction: Massive Open Online Course (MOOC) is an online course that is open access via the World Wide Web and can be accessed by an unlimited number of participants. It was first introduced in 2008 and has emerged as a popular mode of learning, where filmed lectures and presentations are used. **Methods:** ISEV has decided to organize a series of educational MOOCs for students and beginners in the field of extracellular vesicles. These MOOCs will be open access, and will be produced via the platform 'Coursera' (www.coursera.org/), in collaboration between ISEV, University of California Irvine (USA), University of Gothenburg (Sweden) and Pohang University of Science and Technology (South Korea). **Results:** The first course, 'Basics of Extracellular Vesicles', is divided in five modules and will be launched during spring 2016 and aims to provide the basic knowledge about extracellular vesicles. Module 1 is an introduction to the field and will cover the nomenclature and the history of EVs. Module 2 will focus on the biogenesis, release and uptake mechanisms of EVs as well as the different EV cargos (RNA, protein, lipids). In module 3, we will focus on the collection and processing of cell culture media and body fluids such as blood, breast milk, cerebrospinal fluid and urine prior to isolation of EVs. Modules 4 and 5 will present different isolation methods and characterization/quantification techniques, respectively. Here, differential ultracentrifugation, size exclusion chromatography, density gradient, kit-based precipitation, electron microscopy, cryo-electron microscopy, flow cytometry, atomic-force microscopy and nanoparticle tracking analysis will be presented. **Summary:** This first MOOC on extracellular vesicles will be free of charge and open to everybody. It will cover the basics of EVs and will be launched in spring 2016.

PRS.3

Tracking exosomal miRNA biomarkers in prion disease
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Introduction: Prion diseases are transmissible, fatal neurodegenerative disorders in which the misfolded prion protein (PrP^{Sc}) drives transmission and intercellular spread of the disease by inducing conversion of the normal prion protein (PrP^C) into the misfolded form. Both normal PrP^C and infectious PrP^{Sc} are transported by exosomes, with PrP^{Sc} containing exosomes capable of transmitting the disease. Furthermore, exosomal miRNA cargo is altered in exosomes isolated from prion-infected cells, suggesting these changes may provide a source of biomarkers for prion disease. **Methods:** Brain tissue and serum (n = 4) were collected from three-time points in a prion mouse model, spanning from early in the infection (weeks 3 and 13 post-inoculation) to terminal disease. Disease progression was assessed by histology and western blot detection of PrP^{Sc}. Exosomes were isolated from brain tissue and characterised by electron microscopy and western blot for disease and exosome markers. Small RNA deep-sequencing was then performed on whole brain (thalamus) tissue, and brain-derived exosomes and serum to identify differentially expressed miRNA at each time point of disease. **Results:** Expression changes of several prion-associated miRNA biomarkers were detected in both the brain and serum throughout the time course of the disease. These serum exosome biomarkers may be an indicator of early pathological changes in the brain. Additional miRNAs were also detected upon clinical symptoms and terminal stage. **Conclusion:** This study is the first in vivo analysis of exosomal miRNA deregulation across prion disease progression. The comparative analysis of exosomes from brain tissue and sera allows for the identification of potential brain derived biomarkers in the sera furthering our understanding of brain derived in the periphery.

PRS.4

Neutrophil-derived microvesicles enter cartilage and protect the joint in inflammatory arthritis

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Introduction: Microvesicles (MVs) are found in high abundance within RA synovial fluid, and in particular MVs derived from neutrophils. As there is emerging evidence of pro-resolving and anti-inflammatory functions of neutrophil MVs, the aim of this study was to ascertain the role neutrophil MVs play in cartilage remodelling within the RA synovium. **Methods:** Healthy human neutrophil MVs were isolated from TNF α -stimulated neutrophils by ultracentrifugation at 100,000 g for 60 min at 4°C after removing cells and contaminating platelets/erythrocytes. MVs were immunophenotyped and enumerated using the Imagestream MKII and standard cytometry. 3D cultures of primary and immortalized chondrocytes and mouse cartilage explants were used to quantify changes in cartilage accumulation. Rat femoral head explants were used to test whether articular cartilage was penetrable by MVs. The K/BxN murine model of autoantibody-induced arthritis was used to evaluate the impact of exogenous MV administration on cartilage integrity. **Results:** Neutrophil-derived MVs were increased in concentration in RA synovial fluid compared to paired plasma and expressed the pro-resolving protein annexin A1 (AnxA1). Exogenously administered neutrophil-derived MVs migrated into cartilage and protected against its degradation during inflammatory arthritis. This was

dependent on MV-associated AnxA1 interacting with its receptor FPR2 increasing TGF β production by chondrocytes, ultimately leading to cartilage protection. *Summary/conclusion:* The present study demonstrates that neutrophil-derived MVs can migrate into cell-impermeable cartilage where they exert anabolic effects on chondrocytes by delivering AnxA1 signals via chondrocyte FPR2 and preserve cartilage integrity in models of inflammatory arthritis. We envisage that MVs, either natively or loaded with therapeutics, can be harnessed as a unique therapeutic strategy for protection in diseases associated with cartilage degeneration.

PRS.5

PAD-1 activates TAT-5 to prevent extracellular vesicle release in *Caenorhabditis elegans* embryos

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Background: Despite the pleiotropic functions of extracellular vesicles (EVs), little is known about the molecular details of EV release, especially for EVs that form by plasma membrane budding. The outer membrane of EVs contains lipids such as phosphatidylethanolamine (PE) that are normally restricted to the inner leaflet of the plasma membrane, suggesting a role for lipid asymmetry in EV

release. Previously, we showed that TAT-5 phospholipid flippase activity prevents EV release and maintains PE asymmetry in *Caenorhabditis elegans* (Wehman et al., *Curr Biol* 2011). However, it was unclear how TAT-5 activity is regulated. The yeast homolog of TAT-5 binds to a protein homologous to the large and novel protein PAD-1. Therefore, we hypothesized that PAD-1 could regulate TAT-5 activity and EV release. *Methods:* Using *C. elegans* as a genetic model system, we analysed the role of PAD-1 in EV release. We examined pad-1 mutants using transmission electron microscopy and a fluorescent membrane reporter that makes EVs visible by light microscopy. To test whether PAD-1 regulates the lipid flipping activity of TAT-5, we stained worms with the PE probe duramycin. *Results:* We discovered excess membrane labelling in pad-1 mutants and TEM analysis revealed excessive release of EVs, similar to TAT-5 mutants. Using a GFP-tagged reporter, we found that PAD-1 localizes to domains on the plasma membrane, where it is likely to interact with TAT-5. PE was exposed on the plasma membrane of PAD-1 mutants, similar to TAT-5 mutants. Thus, PAD-1 is required for the lipid flipping activity of TAT-5. We are currently testing how PAD-1 activates TAT-5 to prevent EV release. *Conclusion:* We identified PAD-1 as a new regulator for EV release in *C. elegans*, providing a better understanding of the molecular details of EV release. Our study further supports the model that lipid asymmetry regulates plasma membrane budding.

Room: Willem Burger

Special Achievement Award, Wrap-up Sessions, Oral and Poster Awards

Scientific wrap-up: Alissa Weaver

Clinical wrap-up: Peter Quesenberry

11:50-12:35 p.m.
