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ISEV

INTERNATIONAL SOCIETY FOR EXTRACELLULAR VESICLES

Third International Meeting of ISEV 2014

Rotterdam, The Netherlands, April 30th – May 3rd, 2014

Abstracts

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Scientific Program 2014 ISEV meeting
Wednesday April 30th, 2014
Oral Presentations

Registration		8:30-10:00
Welcome & Networking coffee	Arcadis Room	8:30-10:00
Arcadis Room Setting up posters (Poster Sessions 1A, 1B, 1C, 3A, 3C, 2B, 10A)		8:30-9:00
Sponsor Exhibition	Jurriaans Foyer/Mandele room	10:00-18:00
Opening Session	Willem Burger room	10:00-10:30
Welcome/Development ISEV 2013–2014		10:00-10:20

Jan Lötvall, President of ISEV

ISEV 2014 Rotterdam	10:20-10:30

Marca Wauben, President LOC

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Willem Burger room
Symposium Session 1A - EV in the tumor micro-environment
Chair: *Antonella Bongiovanni and Janusz Rak* 10:30-12:00

O1A-001

Intravital imaging of extracellular vesicle exchange between tumour cells

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Introduction: Most cancer cells release heterogeneous populations of extracellular vesicles containing proteins, lipids and nucleic acids that reflect, at least in part, the cell of origin. Accumulating data obtained in vitro has demonstrated that the molecules packaged in extracellular vesicles can be functionally transferred into a variety of recipient cells affecting their gene expression and behaviour. Vesicles released by human tumour cells have been shown to interfere with anti-tumor immunity, and it has been suggested that tumor-released vesicles play a role in autocrine signalling, the formation of a pre-metastatic niche, drug resistance, angiogenesis and stromal remodelling. However, how tumour cell behaviour is altered upon extracellular vesicle uptake in vivo is largely unknown. *Methods*: We developed a novel method to visualize the functional transfer of molecules packaged in extracellular vesicles in real-time in living mice, providing further opportunities to study in detail the distribution and biological relevance of extracellular vesicles between various cells and tissues. *Results*: Using this method, we show in living mice that local and systemic transfer of molecules carried by extracellular vesicles is a physiological process that is directly coupled to the migratory behaviour and metastatic capacity of recipient breast cancer cells. *Summary/conclusion*: The data presented here are consistent with the idea that tumour cells do not act autonomously, but can share proteins and nucleic acids with other tumour cells, locally and systemically. These results shed light on the mutual influence of cancer cells and draw a new perspective on the complexity of intercellular communication in diseases such as cancer.

O1A-002

High-resolution imaging of uptake and processing of prostate cancer-derived exosomes in living prostate epithelial cells

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Introduction: Prostate cancer (PCa) is the most common malignancy in men. Whereas overall survival of patients with early-diagnosed localized PCa is high, metastasized PCa decreases survival dramatically. Tumor cells influence their microenvironment to enhance tumour progression and metastasis. Recently it was found that tumor-derived exosomes play a role in this communication between tumour cells and surrounding stromal and epithelial cells. We aim to provide novel insight into the exosome-mediated mechanisms by which PCa cells influence their microenvironment. Using live cell confocal imaging and high-resolution microscopy, the different stages of uptake and intracellular processing of PCa-derived exosomes by prostate epithelial cells were visualized. This will be accompanied by development of approaches to block exosome function. *Methods*: Exosomes from PCa cell line DU145 are isolated by differential ultracentrifugation and labelled by a fluorescent membrane dye (e.g. PKH26/67). Uptake and further processing of fluorescently labelled exosomes by non-tumorigenic prostate epithelial cells are followed on different time scales, from seconds to multiple hours, by live cell imaging using conventional confocal microscopy and, for high-speed imaging, spinning disk microscopy. Different stages of exosome uptake and co-localization of exosomes with specific proteins are studied in more detail using structured illumination super-resolution microscopy (SIM). *Results*: Confocal time-lapse images show a rapidly initiated and continuous uptake of individual fluorescently labelled exosomes by living PNT2C2 cells. High-speed spinning disk microscopy shows that internalized exosomes are transported via microtubules. This was extended with super-resolution co-localization studies between exosomes and proteins involved in endocytosis that showed processing of internalized exosomes through endosome and lysosome pathways. *Summary/conclusion*: Different imaging approaches enabled us to visualize subsequent steps and dynamics of exosome interaction, uptake and further processing by target cells. Using high-speed imaging and super-resolution SIM allows us to further unravel the molecular mechanisms of action and the role of exosomes in PCa.

O1A-003

Hodgkin lymphoma cells dispatch shedding-sensitive signalling proteins on microvesicles to transiently interact with the tumour microenvironment

Hinrich P. Hansen[1], Adriana F. Paes Leme[2], Maria Dams[1] and Elke Pogge von Strandmann[1]
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Introduction: Hodgkin lymphoma (HL)-affected lymphoid tissue contains only a few disseminated tumour cells, which stimulate immune cells in the tumour microenvironment not to suppress but to support tumour growth. Extracellular vesicles (EVs) from the tumour cells participate in this communication (Hansen *et al.*, 2014, J Pathol). *Methods*: EVs were isolated by sequential ultracentrifugation and investigated by electron and confocal microscopy as well as bead-coupled flow cytometry. Tryptic peptides of EV proteins were analysed by LTQ Velos Orbitrap mass spectrometry. Immune cell migration was performed by chemotaxis experiments and video time-lapse microscopy. *Results*: Mass spectrometry of EVs from 4 different cell lines showed a strong expression of the sheddases ADAM10 and ADAM17, and among their potential substrates were many functional molecules, including CD30, CD44, CD166/ALCAM, Notch. Eighteen-hour incubation of isolated EVs reduced the CD30 expression to 36.8% and CD44 to 41.3% versus metalloproteinase-inhibited aliquots (BB-94, 5 μM), whereas the expression of CD40 (94.3%) and CD70 (90.6%) remained rather uninfluenced. CD30 is a HL-selective receptor of the tumour necrosis factor receptor family (TNFRSF8). We showed that EVs expressing CD30 stimulated the release of tumor-supporting chemokines in immune cells in a CD30-dependent manner. The shedding product (sCD30) lacked this function but itself served as a chemokine for polymorphonuclear leukocytes. *Summary/conclusion*: Metalloproteinases alter the functionality of EVs through selective cleavage of certain membrane proteins. As an example, the soluble ectodomain of CD30 (sCD30) and $CD30^+$ EVs showed different functionality, suggesting that metalloproteinases, by converting selected functional proteins, are able to coordinate a time-limited functionality of EVs in tumour tissue.

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O1A-004

Role of nasopharyngeal carcinoma-derived exosomes on the development of tolerogenic immature dendritic cells

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Introduction: We have shown previously that nasopharyngeal carcinoma (NPC) immune escape is closely related to the presence of regulatory T cells (Treg) recruited by NPC-derived exosomes. We investigate here the role of these tumour exosomes on dendritic cells (DCs), which are highly represented in the immature state surrounding the tumour and that are able to induce the differentiation of naive CD4 T cells into Treg. *Methods*: Phenotypic and functional assays were carried out to assess the interplay between exosomes derived from NPC cell lines (C15) and human DCs during the differentiation process. Impact of NPC-exosomes on human DC differentiation from monocytes was determined using adapted assays (FACS, Q-PCR and ELISA). *Results*: Experiments implied that during the differentiation process, the NPC-derived exosomes do not allow for a fully mature DC to form, but rather a semi-mature state. This state has characteristics of both immature and mature DCs, but is not able to function as a mature DC. These results suggest for the first time that NPC-derived exosomes could promote the generation of tolerogenic immature DCs that may induce the differentiation of Treg. *Summary/conclusion*: Understanding the functional effect of tumour exosomes on the NPC microenvironment and their interplay with the immune system will aid in the progress of immunotherapeutic approaches to cancer treatment.

O1A-005

Large oncosomes are internalized and modulate transcription factors in recipient cells

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Introduction: Rapidly migratory, ''amoeboid'' prostate cancer cells shed large (1–10 μm diameter), bioactive extracellular vesicles (EVs), termed large oncosomes, whose abundance correlates with tumour aggressiveness (Di Vizio et al. *Cancer Res*. 2009; Di Vizio et al. *Am J Pathol*. 2012). Increasing evidence supports an important role for EVs in mechanisms of communication between cancer cells and the surrounding microenvironment. EVs can activate signal transduction as well as transfer biomolecules to recipient cells, processes that may promote oncogenesis and/or enhance tumour progression. The aim of this study was to investigate the molecular mechanisms of large oncosome internalization into recipient cells and large oncosome-mediated intercellular communication. *Methods*: We used high-speed centrifugation and filtration for large oncosomes and smaller EV purification, immuno-flow cytometry with size beads for large oncosome quantitation and sorting, confocal microscopy, ELISA, microarray and western blotting. *Results*: Fluorescently labelled large oncosomes were internalized into recipient cells and maintained their stability as discrete microvesicles localized in the perinuclear space at early time points (1–36 h) and into the nucleus at later times (7 days). We then investigated whether exposure to large oncosomes altered mRNA, protein levels and/or activity of transcription factors in recipient cells, and FOXO1 emerged as a functional target of large oncosomes. A comparison in the rate of oncosome uptake demonstrated significant differences among different cell lines, including benign and cancer prostate epithelial cells, fibroblasts, endothelial and immune cells. These results suggest cell-specific affinities for target cells and, perhaps, finely regulated mechanisms underlying large oncosome internalization. Moreover the rate of large oncosome uptake was reduced by inhibition of specific pathways involved in EV internalization, indicating that large oncosome entry to the cells is mediated by an active endocytic process. *Summary/conclusion*: Our results show for the first time that internalization of intact large oncosomes might have the potential to modulate transcriptional activity in recipient cells.

O1A-006

Differentiation of tumour-promoting stromal myofibroblasts by cancer exosomes

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Introduction: The stroma surrounding carcinomas is aberrantly altered, consisting of cells with a myofibroblast-like phenotype. The onset of such cells is controlled by TGFβ. We have previously demonstrated tethering of TGFβ to the surface of cancer exosomes via the heparan sulphate proteoglycan betaglycan. Here we compare the relative importance of soluble and exosomal TGFβ in stromal activation and investigate novel ways of targeting stroma-assisted tumour growth. *Methods*: Du145 prostate cancer exosomes, isolated by flotation within 30% sucrose/D2O, were characterized by flow cytometry, nanoparticle tracking and western blot. Patient-matched normal or tumour-associated stromal cells were obtained from prostatectomy specimens. Myofibroblast differentiation was determined by the onset of a-smooth muscle actin (αSMA), and angiogenic function was assessed by co-culture with endothelial cells (HUVECs). A sub-cutaneous xenotransplantation murine model was used for in vivo assessment of stromal-assisted tumour growth. *Results*: Normal stroma underwent differentiation to a aSMA-positive phenotype in response to either exosomal or soluble TGFβ. Exosomes were unique, however, in triggering secretion of pro-angiogenic factors. This correlated with the capacity of stroma to organize HUVEC into vessel-like structures in vitro and support tumour growth in vivo. Rab27a knockdown attenuated Du145 exosome secretion and subsequent tumour-stroma interactions, failing to induce stromal differentiation or promote tumour growth in vivo. Digestion of exosomal heparan sulphate side chains did not alter exosomal-TGFβ levels, yet abrogated exosome-mediated differentiation. *Summary/conclusion*: Exosomal, not soluble, delivery of TGFβ is essential for generating diseased stroma with pro-angiogenic and tumour-promoting function. This exosome function is dependent on heparan sulphate proteoglycans such as betaglycan present on the exosome surface. These proteoglycans may therefore represent novel targets for attenuating tumour growth.

Jurriaanse room
Symposium Session 1B - Novel developments in characterization of EV
Chair: *Edit Buzás and Edwin van der Pol* 10:30-12:00

O1B-007

AFM nanoindentation reveals mechanical properties of extracellular vesicles from red blood cells

Daan Vorselen[1,2], Susan van Dommelen[3], Jack van Loon[1], Raymond Schiffelers[3], Gijs Wuite[2] and Wouter Roos[2]
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Introduction: From proteomic studies it is known that many extracellular vesicles (EVs) contain cytoskeletal proteins. These proteins could give rise to unique structural and mechanical properties of EVs, and thereby affect interaction with target cells. We use EVs from red blood cells (RBCs), which are shed from the cell membrane. Such EVs can be recovered from plasma *in vivo*, and for example are thought to play a role in communication between parasites in Malaria. *Methods*: RBC EVs were isolated from RBCs from donors by stimulating with Ca^{2+} - ionophore and subsequent differential centrifugation. We studied the mechanics of RBC EVs by performing nanoindentations using atomic force microscopy. *Results*: Liposomes with a similar lipid composition as RBC EVs show behaviour which corresponds to an elastic shell with an empty core. RBC EVs however behave more like an elastic shell with a filled core, characterized by a Hertzian force response ($\sim$1 MPa). Furthermore, indentation of these vesicles can show multiple types of breaks. They show large break events corresponding to ripping of the membrane or a collapse of the interior and small reversible break events that correspond to different structural states of the vesicle. We show that the interior of RBC EVs is dense (using CryoEM) and contains spectrin, which may explain how the interior influences the mechanics. *Summary/conclusion*: These observations suggest a contribution of a structured interior of the vesicle to the mechanical properties. Our results show that EVs differ mechanically from liposomes and inspire further experiments to investigate the role of the vesicle mechanics in the interaction with the target cell.

O1B-008

Improved and extended EV array: can it be used for intravesicular protein detection?

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Introduction: Based on the technology of the highly sensitive Extracellular Vesicle (EV) Array (Jørgensen *et al*., 2013, *JEV*), we hereby present an improved array capable of detecting up to 75 different surface markers simultaneously as well as intravesicular proteins. The quantity and molecular composition of EVs shed from different cell types differ considerably. Until now, the ''gold standard'' for quantification, characterization and phenotyping of EVs is either by western blotting or by flow cytometry (FACS) using bead capturing. These types of analyses require considerable amounts of vesicular material (20–30 μg of protein derived from approximately 10^8 cells), and it is only possible to get phenotypical data on one sample per experiment. *Methods*: The EV Array utilizes the possibilities to detect and profile EVs for several individual surface-exposed antigens simultaneously using only small amounts (1–100 μL) of starting material in a high-throughput manner. With the original EV Array it was possible to phenotype EVs for the presence of 21 antigens at the same time. The possibility of extension of this panel up to 75 antigens and the correlation with sample amount was tested. Measuring the surface markers on EVs gives valuable information of the cellular communication and cells of origin. However, it does not give any information of the cargo of proteins and other molecules inside the vesicles. Therefore, detecting intravesicular proteins was another desired use of the EV Array, and several combinations of treatments and detergents were tested. *Results*: Under well-defined conditions, it was found to be possible to detect the intravesicular proteins HSP70 and TGF-β1 as well as the intravesicular domains of HER2 and CD30. *Summary/conclusion*: These new improvements of the EV Array open the possibilities to get an extensive analysis of EVs without time-consuming purifications, western blotting or proteomics analyses.

O1B-009

On-chip size and concentration measurements of extracellular vesicles in biological fluids

Kevin Braeckmans[1], Hendrik Deschout[2], Koen Raemdonck[1], Stephan Stremersch[1], An Hendrix[1], Marc Braecke[1], Magnus Röding[3], Mats Rudemo[3], Stefaan De Smedt[1] and Kristiaan Neyts[1]
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Introduction: Fluorescence single particle tracking (fSPT) is a technique capable of measuring the size distribution and number concentration of fluorescently labelled extracellular vesicles (EVs) directly in biofluids, such as blood (1,2). However, being based on epi-fluorescence microscopy, the contrast is limited due to out-of-focus fluorescence. Here we report on a mass producible microfluidic chip with integrated light sheet illumination for fSPT size and concentration measurements of fluorescently labelled EVs directly in body fluids. The sheet of light only illuminates EVs in the focal plane of the detection lens, so that out-of-focus fluorescence is eliminated. We demonstrate accurate on-chip characterization of membrane vesicles in cell culture medium and in interstitial fluid collected from primary human breast tumours (3). *Methods*: On-chip light sheet illumination is realized by means of a planar waveguide structure consisting of three layers of SU-8 in which a microchannel of 100 μm width is created using standard photolithography. The process is carried out on a 10 cm diameter wafer, so that multiple chips are fabricated simultaneously. Laser light is coupled into the chip by means of a butt-coupled fibre. A single chip is mounted on a fluorescence microscope for acquiring fSPT movies of EVs in the microchannel for subsequent size and concentration analysis. *Results*: The contrast of fluorescent EVs was markedly improved when viewed on-chip as compared to epi-fluorescence microsocpy. On-chip fSPT measurements were performed on cell-derived EVs secreted in cell culture medium of breast cancer cells. A 4 × higher concentration was found using the microfluidic chip with a size distribution shifted towards smaller values. On-chip fSPT measurements were also successfully performed on cell-derived EVs secreted in interstitial fluid harvested from human breast cancer specimens, which was impossible using epi-fluorescence illumination. *Summary/conclusion*: The microfluidic chip presented here is simple in design, can be mass-fabricated at a low cost, and allows at the same time EV identification, as well as size and concentration measurements. Thus, this chip opens the

possibility to be used as a diagnostic tool that combines low cost, ease of use, and sensitivity.

References

1. Braeckmans K, et al. Nano Lett. 2010;10:4435–42.

2. Roding M, et al. Phys Rev E. 2011;84.

3. Deschout et al. Nanoscale. 2013. DOI: 10.1039/C3NR04432G.

O1B-010

Visualization of extracellular vesicle membrane traffic in real time

Leonora Balaj[1], Raphael Gaudin[2], Xandra Breakefield[3], Tomas Kirchhausen[2] and Emanuele Cocucci[4]

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Introduction: Extracellular vesicles (EVs) are membrane-bound structures released from living cells. Although they were first visualized by electron microscopy more than 50 years ago, only in the last decade has their role emerged in various processes, including cancer progression. In this context EVs can mediate phenotypic changes in target cells by releasing their contents (including small non-coding RNAs, as well as larger RNAs and double stranded DNA). However, for them to influence gene expression and behaviour, multiple steps are required. Indeed, the vesicles first need to be internalized in a functional state. This occurs, at least in part through access to an intracellular compartment, with sequential escape from membrane fusion. At this point the EV content is released in the cytoplasm modulating acceptor cell functions. Until now, most of the studies have been strictly qualitative, assessing the entry of EVs as an ensemble population. Since EVs are extremely heterogeneous, in terms of size and cellular origin, to obtain a clear picture of their behaviour, it is necessary to visualize single vesicles in real time. *Methods*: To visualize single vesicles in real time, we took advantage of the high sensitivity of last generation spinning disc confocal microscopy. These instruments allow the detection of a few copies of fluorophores, even using weak laser illumination, a feature which limits cell toxicity and permits the observation of living cells for relative long time periods. To obtain large amount of specific fluorescently labelled EVs, we are developing clonal cell lines, derived from primary cells of glioblastoma multiforme (GBM), overexpressing known markers of EVs (e.g. CD63, CD9, CD81) tagged with EGFP. EVs, collected by sequential centrifugation, are characterized for their fluorescence content, and then administrated to living fibroblast cells expressing markers of different intracellular compartments. *Results*: Using this approach we observed that a subpopulation of EVs interacts with the clathrin pathway, which is responsible for receptor-mediated endocytosis. This finding demonstrates that EVs can be internalized by different mechanisms and therefore be subjected to multiple fates during their journey into the acceptor cells. *Summary/conclusion*: In conclusion, high-resolution microscopic approaches are crucial to enhance our knowledge of EV trafficking and to define which vesicles are most suitable to be developed as drug carriers.

O1B-011

Analysis of antigenic composition of single nano-sized extra-cellular vesicles in blood

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Introduction: Blood extracellular vesicles (EVs) are important in normal physiology and are altered in various pathologies. EVs produced by different cells are antigenically different. Since the majority of EVs are too small for routine flow cytometry, with a few exceptions, their composition is studied predominantly in bulk by biochemical analysis of EV extracts and immunochemical analysis of EVs adsorbed on various particles, thus not addressing their antigenic heterogeneity. *Methods*: We report on flow antigenic characterization of *single* nano-sized blood EVs. This analysis is based on binding magnetic nanoparticles to EVs through one of their surface molecules, staining their other antigens with monoclonal antibodies, separating the formed complexes on magnetic columns, and characterizing stained EVs with high-throughput flow analysis. *Results*: We demonstrate that the individual blood EVs carry different sets of antigens, none being ubiquitous, and we quantified their distribution. For example, a common antigen, CD81, is associated with less than 70% of all the EVs. By specific immune-capture, it became possible to analyse EVs of minor subfractions. In particular, we immune-captured CD31-carrying EVs and showed that about half of these EVs co-expressed CD41 and another common antigen, CD63. The latter, although being highly prevalent, is nevertheless not expressed on approximately 20% of the CD31 + CD41 + EVs which thus form a separate fraction. *Summary/conclusion*: Direct analysis of the antigenic makeup on individual blood EVs will help to address their role in different pathologies and reveal physiological mechanisms of EV function. This analysis may lead to the development of new nanotechnology-based diagnostic tools.

O1B-012

Quantitative analysis of the distribution and clearance of mouse melanoma B16-Bl6-derived exosomes in mice

Yuki Takahashi, Takafumi Imai, Makiya Nishikawa, Kana Kato, Masaki Morishita and Yoshinobu Takakura

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Introduction: The development of exosomes as delivery vehicles requires understanding how and where exogenously administered exosomes are distributed in vivo. In the present study, we designed a fusion protein consisting of *Gaussia* luciferase (gLuc) and a truncated lactadherin, gLuc-lactadherin (GL), to label exosomes. By using GL-labelled exosomes, the distribution of intravenously injected exosomes was investigated. In addition, as distribution of exosomes to macrophage-rich organs including the spleen and liver was observed, the role of macrophages in the clearance of exoxomes was investigated. *Methods*: Plasmid vector encoding GL was constructed and transfected to murine melanoma B16-BL6 cells. Exosomes labelled with GL were collected from the transfected cells by an ultracentrifugation method. Macrophage-depleted mice were prepared by intravenous injection of clodronate liposomes. The GL-labelled exosomes were intravenously injected into naïve or macrophage-depleted mice, and the in vivo imaging was performed. Exosomes labelled with PKH26, a lipophilic fluorescent probe, were used to evaluate the cell type involved in the clearance of exosomes. *Results*: After intravenous injection of GL-labelled B16-BL6 exosomes in naïve mice, serum gLuc activity in the blood circulation quickly declined with a half-life of approximately 2 min. Macrophage depletion greatly increased the half-life. Imaging of GL-labelled exosomes in naïve mice revealed that the exosome-derived chemiluminescent signal was detected in the liver, spleen and lung at early time points after injection. The signal in the liver and spleen was quickly declined. Immunofluorescent staining with macrophage-specific antibody showed the co-localization of PKH26-labelled B16-BL6 exosomes with macrophages in the liver and spleen. *Summary/conclusion*: These results indicate that GL labelling is useful for tracking exosomes in vivo and that intravenously injected B16-BL6 exosomes are taken up mainly by macrophages.

Van Weelde/Mees room
Symposium Session 1C - Pathogen-derived EV
Chair: *Antonio Marcilla and Hernando del Portillo* 10:30-12:00

Introduction (15 min) ''Parasite-derived EV'' by Hernando del Portillo

O1C-013

Trypanosoma cruzi-derived microvesicles trigger distinct strain-specific pro-inflammatory activity via tlr2

Rodrigo Soares[1], Kleber Ribeiro[2], Célia Miranda[2], Paula Nogueira[1], Amanda C. Silveira[1], Olindo Martins-Filho[1] and Ana C. Torrecilhas[2]
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Introduction: *Trypanosoma cruzi*, the etiological agent of Chagas disease, presents a broad range of surface antigens able to elicit distinct patterns of macrophage activation. It has been demonstrated that GPI-mucins, the most abundant surface antigen in *T. cruzi* trypomastigotes, can be also found on the parasite-derived microvesicles (TcVes) (Trocoli-Torrecilhas et al., 2009). Previous reports have shown that *T. cruzi*-derived surface glycoconjugates (GPI-mucins) display a ''strain-specific'' ability to induce macrophage NO and cytokine production (Soares et al., 2012). However, it is still unknown if ''strain-specific'' TcVes also trigger similar immunological responses. In this study, we evaluated the in vitro pro-inflammatory properties of TcVes from Y, Yu-Yu, CL-14 and Colombiana strains on macrophage pro-inflammatory response. *Methods*: Thioglycollate-elicited peritoneal macrophages (3×10^6/well) recovered from mice (C57BL/6, TLR2 $-/-$ and TLR4 $-/-$), were primed with INF-g (25 U/ml) and exposed to strain-specific TcVes (1, 5 and 50 mg/mL) and controls (LPS, LPG and *T. cruzi* trypomastigotes 5:1). Following incubation (72 h), NO and cytokines (IL-1b, IL-6, IL-12p40, IL-12p70 and TNF-a) were measured in the supernatants by Griess reaction and CBA-multiplex flow cytometry, respectively. *Results*: Our findings pointed out a strain-specific stimulation pattern, with YuYu and CL-14 strains displaying a patent pro-inflammatory profile, supported by enhanced NO, IL-6 and TNF-a production. Moreover, it was clearly demonstrated that this phenomenon was mediated by the TLR2 pathway, similar to the purified GPI-mucins. *Summary/conclusion*: These data reinforce the relevant role of *T. cruzi* surface molecules in the pro-inflammatory events underlying the immunopathogenesis of Chagas disease.

O1C-014

Biogenesis mechanisms of bacterial vesicles

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Introduction: Outer membrane vesicles (OMVs) are heterogenous spherical proteoliposomes constitutively produced by all Gram-negative bacteria. OMVs form when buds from the outer membrane (OM) of cells encapsulate periplasmic material and pinch off from the OM. OMVs that are approximately 10–300 nm in diameter may contain enriched envelope constituents. OMVs accomplish a diversity of functional roles, and their role in virulence factor dissemination is particularly well-characterized. Inclusion of lipid and protein cargo into OMVs may impart a variety of functions. Mechanistic details of OM biogenesis and relationships between LPS structure and OMV-cargo inclusion rates shed light on new potential models for OM organization and consequent OMV budding. *Methods*: We utilized biochemical and genetic analysis to identify mechanistic parameters that are critical to bacterial vesicle formation and regulation. *E. coli* wild-type and mutant strains were analysed for vesicle production, the amount of total and peptidoglycan (PG)-crosslinked Lpp lipoprotein, the degree of PG remodelling, the induction of vesicle production and membrane integrity phenotypes. *Results*: We demonstrate that, in some cases, an inverse relationship exists between the concentration of covalent, Lpp-mediated OM-PG crosslinks and the level of OMV production. In other strains, OMV production is driven by an increase in periplasmic pressure. Periplasmic pressure results from accumulation of protein, PG fragments, or lipopolysaccharide. Additionally, we found that changes in PG structure as well as the presence of non-canonical D-amino acids correlated with altered vesiculation levels. D-amino acids are secreted by numerous bacteria at the onset of stationary phase. *Summary/conclusion*: PG structure, turnover, and covalent crosslinking between the PG and the OM play a role in OMV biogenesis and regulation. In addition, natural factors produced by the same or other species can modulate OMV production in the wild. In sum, we demonstrate that at least two pathways lead to OMV production: Lpp-PG crosslink-dependent and Lpp concentration-independent, envelope bulk-driven, and that these can be modulated by environmental factors.

O1C-015

Membrane vesicles released from uropathogenic *Escherichia coli* transport an RNA cargo

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Introduction: Bacterial membrane vesicles (MVs) are reported to be involved in stress response, nutrient acquisition and infection pathogenesis. Using uropathogenic *E. coli* (UPEC) as a model for an infectious bacterium, we have investigated the contents of their MVs and potential transfer of these vesicles and their cargo into human host cells in culture. *Methods*: MVs were isolated from concentrated UPEC 536 media by ultracentrifugation. RNA was isolated from these separating small (200 nt) fractions with a mirVana RNA isolation kit. Sequencing libraries were made using Truseq kits comprising the 15–50 nt, 50–200 nt and >200 nt RNAs. Libraries were sequenced on Illumina MiSeq and HiSeq instruments and reads linker trimmed and mapped to the UPEC 536 genome for identification. MVs were labelled for cell-tracking experiments using PKH26/67 lipid dyes and Click-iT 5-ethynyl-uridine (5-EU), which labels their packaged RNA cargo. Human bladder 5,637 cells were treated with labelled MVs and visualized using confocal microscopy. *Results*: UPEC 536 bacteria release MVs that we have successfully isolated by ultracentrifugation, as confirmed by electron microscopy and Zetasizer particle analysis. These vesicles did not contain DNA but did however have an abundant cargo of RNA. Using a novel triple-size sequencing method, we have identified the full complement of the bacterial RNA packaged within UPEC MVs and this was found to include many ribosomal RNA and transfer RNA fragments. Furthermore, membrane-staining experiments has shown that UPEC 536-derived MVs can be

transported into cultured human bladder epithelial cells. Direct labelling of the MV RNAs using 5-EU could also track RNA transfer into these cells. *Summary/conclusion*: Our data has shown that not only do infective UPEC release MVs, but we have also shown for the first time that they carry multiple sized RNAs from various RNA families. These MV bacterial RNAs were confirmed to be delivered into human bladder cells in culture.

O1C-016

Extracellular vesicle-mimetic nanovesicles derived from bacterial protoplast as next generation vaccine delivery system for effective prevention of infectious diseases

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Introduction: Bacteria-secreted extracellular vesicles (EVs) have been highlighted as acellular new vaccine platform and are being developed for use clinically in many countries like New Zealand and Cuba. However, bacterial EVs possess toxicity and productivity issues as EVs include toxic components of bacteria and are produced in low quantity, respectively. Here, we developed EV-mimetic nanovesicles derived from bacterial protoplast, without the Gram-negative bacterial outer membrane components to safely and effectively induce antigen specific humoral and cellular immunity. *Methods*: Protoplast-derived nanovesicles were prepared by extruding genetically engineered bacteria protoplast through a polycarbonate membrane with nano-sized pores. The vaccination effect of antigen-loaded nanovesicles was examined by evaluating the antigen-specific B-cell antibody response and T-cell cellular response and prevention of bacterial sepsis of mice immunized with protoplast-derived nanovesicles. *Results*: Protoplast-derived nanovesicles and EVs originated from *Escherichia coli* shared similar characteristics of size and shape but protoplast-derived nanovesicles had 15,000-fold higher production yield than EVs. Protoplast-derived nanovesicles showed no adverse symptoms when administered in high amount to mice, whereas EVs injected in 40-fold lower amount caused death by symptoms of systemic inflammatory response syndrome. In addition, protoplast-derived nanovesicles were effectively taken up by antigen-presenting cells, and antigen-specific antibodies as well as memory T-cell response were induced. Moreover, immunization with protoplast-derived nanovesicles loaded with bacterial antigen like outer membrane protein A and *Staphylococcus aureus* coagulase survived from the bacteria-induced sepsis in murine model. *Summary/conclusion*: We have newly designed protoplast-derived nanovesicles as antigen delivery system that is produced in higher quantity and is safe compared to naturally produced EVs. The results obtained herein demonstrate a proof-of-concept for novel approach of overcoming many of the infectious diseases of today using EV-mimetic nanovesicles derived from bacterial protoplast.

O1C-017

Conservation of exosome function during viral infection in *Drosophila melanogaster*

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Introduction: The innate immune system is conserved throughout invertebrates and vertebrates. Exosomes play roles in viral infection both in the innate immune response and in promoting pathogenesis. For example, Hepatitis C virus RNA is packaged into exosomes and delivered to dendritic cells, provoking an interferon response. Conversely, Hepatitis A virus hijacks the exosome pathway to gain an envelope during viral egress concealing it from antibodies. It is unclear if these mechanisms are restricted to mammalian cells or are conserved. It has been shown that *Drosophila* cells secrete exosomes that contain markers such as Syntaxin1A. Here, we examine exosomes during Cricket paralysis virus (CrPV) infection in *Drosophila* S2 cells. CrPV is a non-enveloped, single-stranded RNA virus, that is related to HAV. We address the hypotheses that exosomes may be conserved in aiding the immune system or promoting infection. *Methods*: Ultracentrifugation was used to isolate exosomes from *Drosophila* S2 cells which were examined via electron microscopy, western blots and density gradients. Dimethylation labelling followed by LC-MS/MS was used to quantify peptides between CrPV- and mock-infected samples. *Results*: Exosomes from S2 cells display cup-like morphology, densities of 1.09–1.16 g/mL, and contain RNA and protein markers (e.g. Syx1A). Quantitative proteomics of exosomes isolated from CrPV-infected cells 6 h.p.i. resulted in 180 proteins with a twofold increase and 34 proteins with a twofold decrease relative to mock-infected exosomes. CrPV-infected exosomes contained viral proteins and AGO2, a key component of RNAi. Moreover, CrPV RNA is detected in two populations in iodixanol gradients. One population co-fractionates with Syx1A, has densities similar to exosomes and is disrupted with detergents. *Summary/conclusion*: Our results suggest that CrPV may hijack exosomes to gain an envelope. Conversely, detection of AGO2 and CrPV RNA suggests that some exosomes may be delivered to uninfected cells to prime them against infection.

Networking lunch — Arcadis Room — 12:00-13:00

Poster Viewing Sessions 1A, 1B, 1C, 3A, 3C, 2B, 10A
Posters attended by authors — Arcadis Room — 12:30-13:00

Poster Walk, by chairperson, Sessions 1A, 1B, 1C, 3A, 3C, 2B, 10A
Posters attended by authors — Arcadis Room — 13:00-14:00

Willem Burger room
Symposium Session 2A - EV as indicator of (cardio)vascular disorders
Chair: *Dominique de Kleijn and Sean Davidson* 14:00-15:00

O2A-068

Store-operated calcium entry controls microparticle production in human platelets

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Introduction: Microparticles generated from platelets far outnumber those generated from other cell types, and their incidence is correlated with a myriad of cardiovascular diseases. We recently demonstrated that stimulation of gel-filtered human platelets through protease activated receptor (PAR) 4 leads to the generation of 4–5 times more platelet microparticles (PMP) than PAR1 stimulation. The purpose of this study was to determine the role of extracellular calcium entry and identify the channel in human PMP generation. *Methods*: PMP generation from gel-filtered human platelets was quantified by flow cytometry. Submicron particles positive for both CD41a and CD62p were classified as true PMPs. We used specific inhibitors and activators of store-operated calcium entry (SOCE) and non-capacitive calcium entry (NCCE) to determine their relative roles in PMP production. Data were correlated with agonist induced Ca^{2+} tracings in the presence or absence of EDTA. *Results*: Thrombin and PAR4-activating peptide (AP) stimulation induced equivalent levels of PMP production, confirming that PAR4 is the major thrombin receptor responsible for PMP generation, with PAR1 playing only a minor role. Collagen receptor stimulation with convulxin leads to a comparable level of PMP generation as thrombin stimulation. However, co-stimulation with convulxin and thrombin leads to PMP production exceeding the sum of PMP by convulxin and thrombin alone suggesting a synergistic response. Interestingly, the magnitude of the extracellular Ca^{2+} entry did not correlate with the magnitude of PMP production. It is well documented that PMP generation does not occur in the absence of extracellular Ca^{2+}. To determine the role of extracellular Ca^{2+} entry in PMP generation, we treated platelets with the SOCE inhibitor SKF 96365. Pre-incubation with SKF 96365 reduced PMP production induced by all agonist. The activator of SOCE, thapsigargin, leads to robust PMP generation, whereas the activator of NCCE, OAG, did not. Scanning electron microscopy of PAR4-AP stimulated platelets in suspension revealed extended filipodia and bag-like structures protruding from the platelet core which correlated with the size of PMPs as analysed by flow cytometry. Pre-treatment with the SKF 96365 or exclusion of extracellular Ca^{2+} prevented the formation of the microparticle-like extensions and blunted filipodia extension. Finally, confocal analysis of Orai1 staining on platelets spread on a collagen matrix and co-stimulated with PAR4-AP revealed Orai1 throughout the plasma membrane with intense staining of the microparticle-like structures. *Summary/conclusion*: These data suggest that PMP generation is nucleated by Orai1 Ca^{2+} entry and that SOCE is essential to PMP generation. However, due to the lack of correlation between Ca^{2+} responses and PMP production, we hypothesize that other signalling pathways are involved in modulating PMP production.

O2A-069

Dynamic release and clearance of microvesicles during cardiac stress

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Introduction: Circulating microvesicle levels are known to be altered in patients with cardiovascular disease. We investigated microvesicle release and clearance from the plasma during cardiac stress in individuals undergoing stress echocardiography. The differential response in those with and without cardiovascular disease was examined. *Methods*: Circulating cell-derived microvesicles were measured by a standardized flow cytometric protocol and a procoagulant assay at several time points during a stress echocardiogram protocol in over 100 individuals. Risk factors for cardiovascular disease were also recorded in all participants for association analysis with microvesicle levels. *Results*: Procoagulant, platelet, erythrocyte and endothelial-derived microvesicles were elevated immediately following a dobutamine stress echocardiogram, and decreased within 1 h. Those patients who developed stress-induced myocardial ischemia had similar baseline microvesicle levels to participants who did not develop ischaemia but, interestingly, their microvesicle levels did not change during stress. No stress-induced increase was observed in patients without inducible ischaemia, but with a history of vascular disease. A subset of patients subsequently underwent coronary angiography to confirm their diagnosis. The stress-induced microvesicle rise occurred only in those with normal coronary arteries, as determined by angiography. *Summary/conclusion*: Procoagulant, platelet, erythrocyte and endothelial microvesicles are released into the circulation during acute cardiac stress and then clear from the circulation during the next hour, suggesting an effective clearance mechanism. This stress-induced rise appears to be a normal physiological response that is diminished in those with vascular disease. Future work will examine the miRNA content of these elevated microvesicles.

O2A-070

Improvement of the endothelial-derived microvesicle detection by flow cytometry

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Introduction: Over the past decade, endothelial-derived microvesicles (EMVs) have received an increasing interest due to their role as biomarker of the endothelium activation and lesion. However, accurate measurement of EMVs in flow cytometry (FCM) is impeded by their scarcity in plasma and the low density of specific surface markers used to identify them. Therefore, our goal was to develop and validate a new protocol using a selected panel of antibody to improve the sensibility of EMVs detection by FCM in human plasma. *Methods*: To that aim, EMVs were generated from six micro- or macro-vascular human primary endothelial cells in unstimulated or TNF-α stimulated condition. Antibody clones were selected among the following specificity: CD31, CD51, CD62E, CD105, CD106, CD144, CD146, according to their capacity to bind EMVs which was evaluated by immunomagnetic capture and FCM. Cross-reactivity was tested against leukocytes-derived MVs. Sensitivity of different antibody mixes to detect EMVs was measured in cell culture medium or plasma conditions. Based on the selected antibody combination, a new FCM protocol was set up and its performances were compared to a standard methods using CD31. *Results*: Antibody binding to

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EMVs was very heterogeneous according to the antibody specificity and EMV origin. A combination of three antibodies allowed increasing the immunocapture efficiency up to 75±10% of the activated annexin V+ EMVs and detecting up to 68±8% of the same population by FCM in vitro. In the same conditions, less than 1% of leukocytes-derived MVs were measured. In human plasma, the sensitivity of the new protocol to detect artificially spiked EMVs was significantly increased by 1.5–2 fold ($p < 0.0001$) compared to CD31 alone. The lower limit of linearity was reduced from 50 to 25 EMVs/μl and the reproducibility was 25% at 100 EMVs/μl. The new strategy was also evaluated on clinical samples from patients suffering from sclerodermia ($n = 52$) and resulted in a 100±40% increased ($p < 0.0001$) EMV counts compared to the standard methodology. *Summary/conclusion*: We proposed a new FCM protocol which improved the EMVs detection in human plasma samples. This strategy needs to be challenged evaluating how it modifies the capacity of the biomarker EMVs to correlate with the vascular status of the patients and to predict vascular events.

O2A-071

Microvesicles of erythrocyte and platelet origin are increased in broncho-alveolar fluids of lung transplant recipients presenting with chronic lung allograft dysfunction

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Introduction: Chronic lung allograft dysfunction [CLAD, synonym: bronchiolitis obliterans syndrome (BOS)] remains the leading cause of mortality in lung transplant recipients (LTR) with an incidence above 50%. Prognosis is bad and treatment limited and unpredictable, as the pathogenesis remains obscure. While there is evidence for alloimmune and non-alloimmune mechanisms, existing data strongly point to an aberrant tissue repair following recurrent epithelial and subepithelial injury and inflammation. As fibrinous exudate is found in CLAD/BOS lungs and as a relevant role of intrapulmonary coagulation has been established for lung diseases like acute lung injury and pulmonary fibrosis, in which procoagulant serine proteases also lead to fibrin deposition, we hypothesized that recurrent "micro"-bleedings might be involved in this process and that microvesicles (MV) of erythrocyte (EryMV) and platelet (PMV) origin might be increased at the time of diagnosis. *Methods*: LTR routinely underwent lung function tests and broncho-alveolar lavages (BAL) every 3 months or at time of clinical complication. BAL fluid was stored at −80°C until further processing. MV concentration was determined by flow cytometry, using Trucount beads, Annexin V and antibodies against CD235a (EryMV), CD41 (PMV), EpCAM (epithelial MV), CD284 (TLR-4) and HLA-DR. *Results*: We compared MV concentrations in 24 BAL samples of newly diagnosed CLAD/BOS patients (Grad 1–3) to 37 controls of LTR without signs of CLAD/BOS. There were no significant differences between both groups regarding age, sex or days from transplant to BAL (median: 670 vs. 428). For CLAD/BOS samples, we found significantly higher concentrations ($p < 0.05$) of EryMV (mean: 158/μl vs. 57/μl) and PMV (84/μl vs. 38/μl) and a trend to a higher concentration of epithelial MV (1,444/μl vs. 435/μl), while MV levels for Annexin (4,068/μl vs. 4,893/μl), TLR-4 (2,189/μl vs. 1,838/μl) and HLA-DR (1,511/μl vs. 1,664/μl) were in a similar range. *Summary/conclusion*: These results point to a process including recurrent "micro"-bleedings rather than major immunological changes directly preceding diagnosis. Quantification of EryMV and PMV might thus be useful for early detection of intrapulmonary "micro"-bleedings, and we will follow up these results by measuring BAL samples preceding the diagnosis.

Jurriaanse room
Symposium Session 2B - EV therapeutics 1
Chair: *Julia Gross and Luc van der Laan* 14:00-15:00

O2B-072

Therapeutic MSC exosomes are derived from lipid raft microdomains in the plasma membrane

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Introduction: Mesenchymal stem cell (MSC) was previously shown to secrete lipid vesicles that when purified by high-performance liquid chromatography as a population of homogenously sized particles with a hydrodynamic radius of 5,565 nm reduce infarct size in a mouse model of myocardial ischemia/reperfusion injury. As these vesicles exhibit many biophysical and biochemical properties of exosomes, they were identified as exosomes. Here we investigated if these lipid vesicles were indeed exosomes that have an endosomal biogenesis. *Methods*: In most cells, endocytosis is thought to occur at specialized microdomains known as lipid rafts. To demonstrate an endosomal origin for MSC exosomes, MSCs were pulsed with ligands, for example, transferrin (Tfs) and cholera toxin B (CTB), that bind receptors in lipid rafts. The endocytosed ligands were then chased to determine if they were incorporated into the exosomes. *Results*: A fraction of exogenous Tfs was found to recycle into MSC exosomes. When MSCs were pulsed with labelled Tfs in the presence of chlorpromazine, an inhibitor of clathrin-mediated endocytosis, Tf incorporation in CD81-immunoprecipitate was reduced during the chase. CTB which binds GM1 gangliosides that are enriched in lipid rafts extracted exosome-associated proteins, CD81, CD9, Alix and Tsg101 from MSC-conditioned medium. Exogenous CTBs were pulse-chased into secreted vesicles. Extraction of Tf- or CTB-binding vesicles in an exosome preparation mutually depleted each other. Inhibition of sphingomyelinases reduced CTB-binding vesicles. *Summary/conclusion*: Our data demonstrated that MSC exosomes are derived from endocytosed lipid rafts and that their protein cargo includes exosome-associated proteins CD81, CD9, Alix and Tsg101.

O2B-073

Retargeting of extracellular vesicles by introduction of anti-EGFR nanobodies: lessons from the liposome field

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Introduction: Extracellular vesicles (EVs) are attractive candidate drug delivery systems with the potential to efficiently and specifically deliver biological drugs to selected tissues. Their applicability could be improved with a universal method to target them to cells other than those directed by their native tropism. In this work a novel and straightforward method is described to functionalize the EV surface with targeting ligands and thereby alter their cell-specific interactions. *Methods*: Anti-EGFR nanobodies, which serve as targeting ligands, were covalently coupled to PEG-phospholipid micelles. Micelles were incubated with EVs derived from EGFR-negative Neuro2A cells or platelets. After purification of EVs, nanobodies present on the EV surface were detected by western blotting and electron microscopy. Cell uptake of retargeted EVs by EGFR-positive tumour cells was assessed using flow cytometry. *Results*: Upon incubation of EVs with phospholipid-nanobody micelles, a temperature-dependent transfer of nanobodies to the EVs was observed. Moreover, transfer efficiency was increased when micelles containing shorter lipid chains were employed, indicating a post-insertion mechanism of transfer. The presence of nanobodies on the EV membrane and sustained EV integrity was confirmed by electron microscopy. Importantly, cell association and uptake of retargeted EVs by EGFR-positive tumour cells were significantly increased when compared to unmodified EVs or EVs modified with control nanobodies. *Summary/conclusion*: For EVs to be deployed as drug delivery systems, it is essential that they can be (re)targeted to specific cell types or tissues. Post-insertion of lipids bearing targeting ligands into the EV membrane is an easy and efficient strategy to modify the tropism of EVs, while maintaining their integrity. This strategy may also be exploited to introduce other functionalities to the EV surface, such as stealth properties or near-infrared probes for imaging purposes.

O2B-074

Milk-derived exosomes as a drug carrier

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Introduction: Exosomes shuttle in and out of cells, and carry a cargo of macromolecules. These natural nanoparticles are being explored as nanodevices for the development of new therapeutic applications. However, no natural source is reported to produce large quantities of exosomes needed for drug delivery exploration. *Methods*: Differential centrifugations were employed to isolate the exosomes from bovine mature milk. The particle size was measured by NanoSight. Exosomal proteins were analysed by western blot, and RT-PCR was performed to identify immune-related miRNA and mRNAs. A variety of lipophilic and hydrophilic compounds, including chemotherapeutic drugs, were incubated with exosomes to achieve drug loading. Antiproliferative and anti-inflammatory activities were assessed by MTT assay and EMSA, respectively. Potential toxicity of the milk exosomes was assessed in S/D rats. *Results*: The isolation method reproducibly resulted in large quantities of exosomes. Exosome size ranged from 30 to 100 nm. The size was confirmed by SEM and AFM. The identity of exosomes was confirmed by surface protein markers (CD63, CD81), buoyant floatation density (1.12 g/ml) and high content of immune-related miRNAs and mRNA. Biologically, significantly higher antiproliferative and anti-inflammatory activities were found with the drug-loaded exosomes versus the free drugs in human lung, breast, ovarian and cervical cancer cells in vitro. Intriguingly, the milk exosomes per se showed significant protective effects in vitro and in vivo, suggesting the presence of cancer cell-killing factors in these particles. Further, no systemic toxicity was found in rats following daily oral dosing with the milk exosomes for 2 weeks. *Summary/conclusion*: This is the first demonstration of identification of a scalable source of exosomes with wide chemopreventive and therapeutic applications for cancer and many other inflammatory diseases.

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O2B-075

Development of exosomes-mediated delivery of therapeutic oligonucleotides

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Introduction: The most serious limitation to realize the full potential of oligonucleotide-based therapies (ONTs) is the highly inefficient transit of oligonucleotides from outside cells to the intracellular compartments where functional activity of oligonucleotides takes place. We hypothesize that exploiting natural, evolutionarily conserved, mechanisms and pathways for trafficking of small RNAs across cellular boundaries is the way to fundamentally improve ONTs efficiency. It has been previously demonstrated that exosomes might efficiently transfer therapeutic oligonucleotides to cells and tissues. However, one of the main technical unresolved issues is the loading of exosomes with oligonucleotides. *Methods*: Here, we have developed a novel approach enabling efficient loading of exosomes with oligonucleotides based on self-delivery properties of hydrophobically modified siRNA (hsiRNA). *Results*: We have demonstrated efficient loading of ~50% of hsiRNAs into U87-derived exosomes. Loaded exosomes were characterized by electron microscopy, Nanosight and Zeta-Sizer (Malvern) and show expected size distribution with a decrease in membrane zeta-potential, indicative of efficient loading (stable interaction). In addition, hsiRNA-loaded exosomes resulted in dose-dependent efficient target silencing in primary neurons and HeLa cells. High-resolution imaging in living cells shows similar kinetic uptake and intracellular distribution of "naked" exosomes and hsiRNA-loaded exosomes. Besides, in vivo data on hsiRNA-loaded exosome silencing efficacy and cellular distribution will be presented. *Summary/conclusion*: This novel approach is being explored for development of novel therapies for Huntington disease. "Nature based" ONTs are expected to have dramatically better potency and will transform the field of therapeutic oligonucleotides expanding their utility to a wide range of clinical applications.

Van Weelde/Mees room
Symposium Session 2C - Hypoxia and EV
Chair: *Matthias Belting and Pia Siljander* 14:00-15:00

O2C-076

Hypoxic induction of carbonic anhydrase IX in glioblastoma exosomes – structural and functional implications

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Introduction: We have studied the role of EVs, i.e. exosomes, as mediators and biomarkers of hypoxia dependent development of glioblastoma (GBM). These highly aggressive brain tumors are characterized by severe hypoxia, endothelial cell hyperplasia and hypercoagulation. Our recent findings reveal that the proteome and mRNA profiles of exosomes closely reflect the oxygenation status of donor glioma cells and patient tumors, and that exosomes derived from GBM cells grown at hypoxic as compared with normoxic conditions are potent inducers of tumor growth through phenotypic modulation of stromal cells (Svensson, et al., PNAS, 2011; Kucharzewska, et al., PNAS, 2013). *Results*: Here, we show that carbonic anhydrase IX (CAIX), i.e. a major regulator of hypoxia-induced acidosis, is highly enriched in exosomes from hypoxic GBM cells. At the structural level, we provide first evidence that CAIX can be conjugated with glycosaminoglycans (GAGs) of both chondroitin sulfate (CS) and heparan sulfate (HS) type. Interestingly, both GAG- and non-GAG conjugated CAIX protein is associated with exosomes. This finding was validated with exosomes isolated from GBM patient serum, showing substantial levels of the two variants of CAIX. GBM exosomes have been shown to utilize cell-surface HS proteoglycans (HSPGs) for their internalization (Christianson, PNAS, 2013). Further, it was shown that exosomes carry HS on their surface. The present study extends these data to show that exosomes specifically derived from hypoxic GBM cells are highly enriched in the CSPG CAIX. *Summary/conclusion*: Given the known role of CAIX in acidosis regulation of the tumor microenvironment as well as in cancer cell migration, our data implicate that the tumor promoting effects of exosomes derived from hypoxic GBM cells may be related to the activity of CAIX and its proper glycosylation. Moreover, the strong enrichment of CAIX in GBM patient derived CAIX may serve as a relevant biomarker of the tumor oxygenation status and aggressiveness.

O2C-077

Leukemic exosomes attenuate bone marrow niche signalling and coordinately suppress haematopoietic stem cell function

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Introduction: The conversion of the low-oxygen bone marrow (BM) environment to a self-reinforcing leukaemia niche that promotes drug resistance and compromises physiologic BM function is of great interest. The impact of leukemic exosomes on the function of normal hematopoietic stem cell (HSC) and BM stroma during leukemic invasion has not been studied. *Methods*: NOD/SCID/IL2rgnull (NSG) mice, AML cells, HSC and stromal cells. CFU, miRNA array, IHC, qRT-PCR and flow cytometry. *Results*: We show in a xenograft model that hypoxia-conditioned AML cells suppressed normal HSC function even at low disease chimerism. In addition, hypoxia-conditioned AML cells downregulated the expression of HSC retention-factors (SCF1 and CXCL12) in recipient BM stromal cells. To understand the respective contribution of AML exosomes (versus AML cells) on suppressing HSC function, we conducted correlative in vitro studies showing a significant decrease in HSC progenitor renewal capacity and systematic downregulation of CXCR4 resulted in a decrease of migration of Lin^- BM cells after AML exosome exposure. Exposure to leukemic exosomes also downregulated the expression of haematopoietic-regulatory factors (HOXA9, E2F3 and SHIP1) in c-kit+ HSC, all targets of miR-210 and miR-155 (highly enriched in hypoxia-conditioned AML exosomes). During in vitro culture of stromal cells, AML exosomes downregulated key hematopoietic supporting factors (SCF1, CXCL12, ANGPT1, TGF-b1, and TGF-b2) that extrinsically regulate HSC retention and survival. Together, the results suggest that AML exosomes participate in altering stromal function and residual haematopoietic activity directly through exosome transfer and miRNA regulation. *Summary/conclusion*: Our results reveal a new mechanistic role of AML exosomes in which exosomes can differentially affect cellular components of the BM niche that together promote the coordinate loss of haematopoietic function during leukemic invasion of the niche.

O2C-078

Hypoxia enhances angiogenesis via exosomal miR-135b derived from multiple myeloma cells

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Introduction: Hypoxia plays an important role in the evolution of cancer cells and their microenvironment. In multiple myeloma (MM), the massive proliferation of malignant plasma cells causes hypoxia. To date, the majority of in vitro hypoxia studies for cancer cells have used acute hypoxic exposure (3–24 h). We therefore aimed to clarify the role of MM-derived exosomes in hypoxic bone marrow using MM cells that grow continuously in vitro under chronic hypoxia (hypoxia-resistant (HR) cells). *Methods*: We established three sublines from the MM cells (RPMI8226, U266, KMS-11) by culturing them under hypoxia (1% O_2) for >6 months until they showed continuous growth. Exosomes were isolated from the culture medium of the HR cells by Exoquick (SBI) and analysed by the nanoparticle tracking assay (NanoSight). Cellular and exosomal miRNAs were profiled by the TaqMan low-density array (ABI). The endothelial tube formation assay was used to evaluate MM cell–endothelial cell communication. The gene silencing effects of exosomal miRNAs were assayed in endothelial cells transfected with a luciferase reporter vector. *Results*: The HR cells produced more exosomes than the parental cells. The HR cells possessed a distinct exosomal miRNA signature compared with the parental cells. Notably, miR-135b was significantly upregulated in exosomes from RPMI8226/HR and KMS-11/HR cells. Exosomal miR-135b directly suppressed its target, factor inhibiting HIF-1 (FIH-1), in endothelial cells. Finally, exosomal miR-135b from RPMI8226/HR cells enhanced endothelial tube formation under hypoxia via the HIF-FIH signalling pathway. *Summary/conclusion*: The ''in vitro HR-myeloma cell model'' will be useful to better understand MM cell–endothelial cell interaction under hypoxic conditions, which may mimic the in vivo bone marrow microenvironment. Although tumour angiogenesis is regulated by various factors, exosomal miR-135b may be a promising target for controlling MM angiogenesis.

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O2C-079

Extracellular membrane vesicles from umbilical cord blood derived MSC protect against ischemic acute kidney injury, a feature that is lost after inflammatory conditioning

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Introduction: Mesenchymal stromal cells (MSC) are shown to have a great therapeutic potential in many immunological disorders. Currently the therapeutic effect of MSCs is considered to be mediated via paracrine interactions with immune cells. Umbilical cord blood is an attractive but still less studied source of MSCs. We investigated the production of extracellular membrane vesicles (MVs) from human umbilical cord blood derived MSCs (hUCBMSC) in the presence (MVstim) or absence (MVctrl) of inflammatory stimulus. *Methods*: hUCBMSCs were cultured in serum free media with or without IFN-g, and MVs were collected from conditioned media by ultracentrifugation. The protein content of MVs was analysed by mass spectrometry. Hypoxia-induced acute kidney injury rat model was used to analyse the in vivo therapeutic potential of MVs, and T-cell proliferation and induction of regulatory T cells were analysed by co-culture assays. *Results*: Both MVstim and MVctrl showed similar T-cell modulation activity in vitro, but only MVctrls were able to protect rat kidneys from reperfusion injury in vivo. To clarify this difference in functionality, we made a comparative mass spectrometric analysis of the MV protein contents. The IFN-g stimulation induced dramatic changes in the protein content of the MVs. Complement factors (C3, C4A, C5) and lipid binding proteins (i.e apolipoproteins) were only found in the MVctrls, whereas the MVstim contained tetraspanins (CD9, CD63, CD81) and more complete proteasome complex accompanied with MHCI. We further discovered that differently produced MV pools contained specific Rab proteins, suggesting that same cells, depending on external signals, produce vesicles originating from different intracellular locations. *Summary/conclusion*: We demonstrate by both in vitro and in vivo models accompanied with a detailed analysis of molecular characteristics that inflammatory conditioning of MSCs influences the protein content and functional properties of MVs revealing the complexity of the MSC paracrine regulation.

Networking coffee — Arcadis Room — 15:00-15:30

Poster Viewing Sessions 1A, 1B, 1C, 3A, 3C, 2B, 10A continued
Posters not-attended by authors — Arcadis Room — 15:00-15:30

Willem Burger room
Symposium Session 3A - EV in the immune system
Chair: *Kok Hian Tan and Graça Raposo* 15:30-17:30

O3A-080

Apoptotic cell-derived extracellular vesicles as attractants for phagocytes

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Introduction: Damaged, aged or unwanted cells are removed from the body by an active process known as 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 and prevention of inflammatory and autoimmune disorders. We have shown previously that dying lymphocytes release apoptotic cell-derived extracellular vesicles (ACDEV) and these are strongly attractive to phagocytes in vitro. These ACDEV carry molecules including ICAM-3 that we have shown plays an important role in promoting phagocyte migration. Here we extend our previous studies to address EV from other apoptotic cells. *Methods*: Supernatants from serum-free cultures, induced to apoptosis by UV, were centrifuged (1,000 × *g*) to remove cell debris and large apoptotic bodies. Dihydroxyvitamin D3-stimulated THP-1 cells, that efficiently remove apoptotic cells, were used as a model monocytic human phagocyte for these studies. Phagocyte migration to EV-replete supernatants was assayed (a) in a horizontal Dunn chemotaxis chamber where cell migration was mapped using Image J after time-lapse microscopy over 4 h or (b) using Cell-IQ live cell imaging to assess cell migration across a barrier of endothelial cells. Involvement of specific molecules was assessed using monoclonal antibodies or ICAM-3-deficient cells *Results*: Our data reveal that ACDEV promote strong directional migration of phagocytes in a manner dependent upon ICAM-3 released in ACDEV from the surface of dying leukocytes. Monoclonal antibody studies demonstrate that blockade of ICAM-3 can reduce phagocyte migration. Using transwell studies, we further demonstrate that THP monocytes are attracted across endothelial cell barriers to dying cells, also in an ICAM-3-dependent manner. Finally, our preliminary data indicate that ICAM-3 may be important in recruiting phagocytes to apoptotic lipid-laden macrophages (foam cells) but not control macrophages. *Summary/conclusion*: Taken together our data suggest ICAM-3 may play an important role in the recruitment of macrophages to sites of cell death. Such sites may include 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.

O3A-081

Extracellular vesicles released from apoptotic cells are a source of autoantigens and act as modulators of immune responses

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Introduction: During apoptosis a multitude of extracellular vesicles (EV) is released from the apoptosing cell (apoptotic blebbing). We studied the molecular content of these EV and investigated their influence on the function of innate immune cells. Further, we analysed the role of apoptotic-cell-derived-EV (apoptotic-EV) in the pathogenesis of human SLE (systemic lupus erythematosus). A disturbed regulation of apoptosis is a known feature of SLE, and we postulated a role of apoptotic-EV in the pathogenesis of this disease. *Methods*: Cells were obtained from healthy donors or SLE patients. Lymphocytes were isolated and apoptosis was induced by UV-B irradiation to harvest apoptotic-EV by filtration and ultracentrifugation. Myeloid dendritic cells (mDC), plasmacytoid dendritic cells (pDC) and monocytes isolated and stimulated by apoptotic-EV to investigate their immunemodulatory function. *Results*: We found that nuclear autoantigens accumulate within apoptotic-EV early after apoptosis induction. Interestingly, most of these antigens are well known players in SLE pathogenesis. When we analysed the effects of apoptotic-EV on the function of pDC, we observed an increase secretion of IFN-α (a ''marker-cytokine'' of SLE) which was triggered via TLR-9 by DNA. Apoptotic-EV induced an alternative maturation of mDC (characterized by a downregulation of MHC class II molecules), which was absent in cells obtained from SLE patients (correlating to disease activity). In monocytes/macrophages, apoptotic EV induced a rather pro-inflammatory phenotype. *Summary/conclusion*: Taken together we provide evidence that apoptotic-EV can act as a source of autoantigens. We showed an immunemodulatory function of apoptotic-EV in various cellular systems. Finally, we could demonstrate that this function is altered in human SLE. We conclude that apoptotic-EV can modify innate immune responses and play a role in the pathogenesis of autoimmune diseases.

O3A-082

Kinetics and analysis of extracellular vesicles released from unstimulated or IgE-activated mast cells

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Introduction: Mast cells, primarily recognized for their role in allergic reactions, are known to release extracellular vesicles (EVs) both under resting conditions and after IgE-mediated activation. Differences in the composition of EVs released during these conditions and the kinetics of EV release are largely unknown. In this study we characterized EVs released from differently activated primary mast cells and compared the kinetics of release of EVs and release of granule-stored soluble mediators after IgE-mediated activation. *Methods*: EVs were isolated from culture supernatants of unstimulated bone marrow-derived mast cells (BMMC) after overnight culture. To obtain EVs from activated BMMC, DNP-IgE primed BMMC were stimulated for 1.5 h with DNP-HSA and EVs were directly isolated from culture supernatants or after overnight culture. EVs were collected by differential centrifugation and subsequently floated into a sucrose gradient and analysed by western blotting. For multiparameter analysis by high-resolution flow cytometry, EVs were stained with specific antibodies and PKH67 prior to floatation. *Results*: Both unstimulated and activated mast cells release a heterogeneous EV population as indicated by differences in buoyant density, light scattering and variation in CD9 and CD63 contents. IgE-mediated mast cell activation resulted in a 10–20-fold increase in EV release compared to unstimulated or LPS stimulated BMMC.

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The vast majority of EVs was released very rapidly, within the first 1.5 h of activation. Comparable antigen dose dependency was observed between EV-release and release of the soluble mast cell mediator beta-hexosaminidase. Although most EVs from IgE-stimulated mast cells had buoyant densities of 1.13–1.19 g/mL, comparable to EVs from unstimulated BMMC, a relatively high increase in EV number was found at densities between 1.21 and 1.23 g/mL. Based on scatter values by high-resolution flow cytometry, these latter EVs are relatively small and this population is enriched for CD63 positive EVs. *Summary/conclusion*: IgE-mediated mast cell activation leads to a rapid and massive increase in EV release. The EV release pattern follows the kinetics of the release of a soluble mast cell mediator stored in mast cell granules.

O3A-083

Extensive phenotyping of extracellular vesicles from mono- and co-cultures of human dendritic cells and allogeneic CD4+ T cells

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Introduction: Extracellular vesicles (EVs) could be important for intercellular communication between immune cells and for the orchestration of immune responses. In this relation, the phenotype of the EVs might provide clues about their functionality. The maturation state of dendritic cells (DCs), either immature (iDCs) or mature (mDCs), may possibly be reflected in both the EV phenotype as well as the ability of the DC EVs to stimulate T cells. An extensive phenotyping of a membrane subproteome of EVs from co-cultures of either iDCs or mDCs and allogeneic CD4 + cells was made, including general exosomal, cancer and immune markers. *Methods*: Human DCs were differentiated from monocytes using IL-4 and GM-CSF. The DCs were matured with lipopolysaccharide (LPS) or left immature and co-cultured with isolated allogeneic CD4+ T cells for 6 days. Monoculture controls were incubated for 48 h. The EVs from the cell culture media were captured on the EV Array containing antibodies against 47 different markers. The EV phenotype was determined with a cocktail of antibodies against CD9, CD63 and CD81. The cellular phenotypes were determined by flow cytometric analyses. *Results*: Of the general exosomal markers, only CD81 was present on EVs from all cell cultures. Some proteins could solely be detected on EVs from the co-cultures of DCs and T cells, such as CTLA-4 and the co-stimulatory molecule CD80. This was also the case for several T-cell-specific markers, including CD4. Other proteins, like ICAM-1, were present in both the co-cultures and the mDC monoculture. *Summary/conclusion*: The EV phenotype from co-cultures of either iDCs or mDCs and CD4+ T cells differ to some extent. The differences are more pronounced for the monocultures. Nonetheless, some observations were as expected and correlate with the cellular phenotypes. Interestingly, the only general exosome marker CD81 seems to be present on EVs from the CD4+ T-cell monoculture.

O3A-084

Intercellular transfer of microRNA between regulatory T cells and dendritic cells: a possible regulation mechanism of dendritic cell function

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Introduction: Regulatory T cells are a subpopulation of $CD4^+$ T cells which function to suppress target immune cells including effector T cells and dendritic cells. Regulatory T cells (Tregs) employ numerous mechanisms to suppress the action of these key target cells including the direct intercellular transfer of suppressive cytoplasmic molecules on immunomodulatory exosomes. Recent research has demonstrated that selected microRNAs (miRNA) are intercellularly transferred via exosomes between human $CD4^+$ T-cell lines and antigen-presenting cells (APCs) leading to gene regulation in the latter cell type. Whether $CD4^+$ Treg-specific microRNAs are intercellularly transferred to APCs via exosomes has yet to be elucidated. *Methods*: A microRNA array was conducted on both a murine Treg line and bone marrow-derived dendritic cells (BM-DCs) to identify miRNAs that were highly expressed in Tregs as compared to DCs. miRNAs were validated in both cells and Treg-derived exosomes, isolated by ultracentrifugation, by qPCR. CFSE labelled exosomes were incubated with BM-DCs to validate the acquisition of these molecules by these cells. *Results*: Several miRNAs, including miR-125b, were identified as being highly expressed in our Treg line as compared to BM-DCs. Upon co-culture of Tregs and BM-DCs increased levels of miR-125b was observed in BM-DCs. One explanation for this may be the intercellular transfer of this miRNA via exosomes. Indeed, we observed that miR-125b was present in Treg-derived exosomes and that these vesicles were acquired by BM-DCs resulting in altered DC phenotype, lower CD80 and CD86 expression and function, reduced IL-12 production following LPS stimulation. *Summary/Conclusion*: Our data suggests that intercellular transfer of microRNAs via exosomes may be a novel mechanism of $CD4^+25^+$ T-cell regulation on DC function.

O3A-085

How can suppressor T-cell exRNA not in exosomes functionally target particular antigen-specific effector T cells to mediate their suppression?

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Introduction: We studied an immunosuppressive extracellular RNA (exRNA) free of exosomes. This exRNA mixture was produced by CD3+ CD8+ suppressor T cells that were induced by antigen-high-dose tolerization to inhibit contact sensitivity (CS) in mice. *Methods*: The activity of the exRNA was identified as due to a small double-stranded miRNA subfraction by its susceptibility to RNase and RNase III treatment and enrichment by progressive phenol chloroform extraction (PCE), Qiagen column separation (QRNA) and electrophoretic sizing gel analysis. Although we failed to find exosomes or immunoglobulins in the exRNA. *Results*: Remarkably, the miRNA fractions suppressed CS-effector cells antigen (Ag)-specifically, and inhibited the IL-2 responsiveness of an HT-2 T-cell line in vitro. The unexpected Ag-specific suppression by the exRNA free of exosomes was discovered to be due to transfection of antibody-coated exosomes released by a B1a B cell subpopulation in the targeted CS-effector cell mixture and mediated by miRNA among the exRNA that finally suppressed the CS-effector T cells. Blockage of the total free QRNA by anti-miRNA-150 and transfection of the Ag-specific B1a cell exosomes with pure miRNA-150 showed that miRNA-150 was responsible for the suppression. *Summary/conclusion*: Thus we propose an alternate mode of exRNA inter-cell exchange. In contrast to the classical transit of miRNA to targeted cells by exosomes released by donor cells from multivesicular bodies (MVB), in the alternate pathway miRNA is released by donor cells among exosome-free exRNA that is protected from plasma RNases by chaperones and transfects exosomes from the targeted cells, possibly the targeted acceptor cells or their companion cells. These exRNA transfected exosomes from the targeted cell population are then taken up by the targeted acceptor cells to mediate subsequent function of their miRNA by inhibiting mRNA translation in the targeted cell. To our knowledge, the alternate pathway is the first demonstration that freely circulating miRNA not in exosomes can inhibit function of acceptor cells by transfecting exosomes produced by the targeted cell population for passing functional information encoded by free

extracellular miRNA between cells. These findings likely have great biologic and immunologic significance, and also relevance to eventual treatment of human diseases.

O3A-086

Exosome-based vaccines: a novel TLR-7 agonist enhances immune stimulatory properties of DC-derived exosomes

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Introduction: Dendritic cell (DC)-derived exosomes are promising vaccine candidates since they carry key molecules from DCs, essential to efficiently activate adaptive immune cells, without the risks associated with cell-based vaccines. The objective of this work was to identify suitable adjuvants that, when paired with a model antigen, stimulate DCs to produce exosomes capable of activating antigen-specific T cells. *Methods*: To initiate exosome production, we cultured DCs in the presence of the antigen ovalbumin (0.25 mg/ml) and one of three adjuvants: LPS (50 ng/ml), CpG-B (8 μg/ml), or a novel TLR-7 agonist, termed poly(mannose methacrylate-co-Imiquimod methacrylate) (p(MMA-IMA)) (40 μg/ml). After 24 h, exosomes were harvested from DC supernatant via sequential (ultra)centrifugation and 20 μg of the isolated exosomes were added to naïve OT-I cells. OT-I cells were cultured with either LPS-, CpG-B-, or p(MMA-IMA)-exosomes for either 24 h or 72 h and analysed for surface markers indicative of activation (CD69), exhaustion (PD-1) and proliferation (CFSE dilution) via flow cytometry. Upon 72 h of stimulation, the concentration of TNFα and IFNγ in the cell supernatant was measured via ELISA. *Results*: Exosomes from DCs matured via LPS, CpG-B or p(MMA-IMA) initiated a time-dependent increase in $CD69^+$ OT-I cells. Interestingly, treating OT-I cells with p(MMA-IMA)-exosomes resulted in significantly more $CD69^+$ OT-I cells as compared to LPS- and CpG-B-exosomes. For example, after 72 h, 83.6% of OT-I cells treated with p(MMA-IMA)-exosomes were $CD69^+$, whereas only 64.3% of OT-I cells treated with LPS-exosomes and 5% of OT-I cells treated with CpG-B-exosomes were $CD69^+$. While there was no detectable proliferation of OT-I cells treated with LPS- and CpG-B-exosomes, 17% of the OT-I cells treated with p(MMA-IMA)-exosomes proliferated after 72 h. None of the exosomes used in this study resulted in OT-I exhaustion as evidenced by a lack of increase in PD-1 surface expression. Finally, OT-I cells treated with p(MMA-IMA)-exosomes released high amounts of TNFα in the supernatant (1011.25 pg/ml). *Summary/conclusion*: In this work, we demonstrate that p(MMA-IMA) is a promising candidate for the production of DC-derived exosome-based vaccines given their ability to activate T cells and initiate T-cell proliferation without exhaustion and the release of immunostimulatory cytokines. These characteristics are essential to initiate and maintain the immune capability of an activated T cell for longer periods of time.

O3A-087

Extracellular vesicles in human semen impair antigen-presenting cell function and decrease antigen-specific T-cell responses

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Introduction: Exposure to semen is the primary route of transmission for many sexually transmitted infections. Mounting evidence suggests that components in semen directly influence leukocytes, which could help to explain the difficulty of inducing vaccination-based protection in the genital mucosa. *Methods*: Extracellular vesicles isolated from semen donated by healthy men, termed SE, were pooled from multiple donors. Dendritic cells (DCs) were derived from blood monocytes, and Langerhans cells (LCs) from CD34+ cells or isolated from vaginal tissue explants. DCs, LCs and PBMC cultures were exposed to labelled SE and assayed for SE uptake by flow cytometry and confocal microscopy. SE-exposed cells were also assayed for immune functions and production of cytokines in response to bacterial lipopolysaccharide and peptide antigens by flow cytometry, Luminex, and quantitative PCR. *Results*: Extracellular vesicles from semen are present at an average concentration of 2.2×10^{13} particles per ejaculate (n = 18 donors). These SE efficiently and rapidly entered peripheral and vaginal DCs, whereas T-cell uptake of SE was poor. SE impaired the immune responses of DCs stimulated by bacterial lipopolysaccharide, where cytokine production was reduced at both the mRNA level in the cells and the protein level in supernatants. Preliminary experiments also indicated that SE downregulate CD80 and CD86 expression on DCs. In PBMC cultures, SE impaired memory T-cell function, reducing the production of TNF-alpha and/or IFN-gamma in response to virus-derived peptides an average of 73% for CD4+ T cells and 55% for CD8 + T cells, in a dose-responsive manner (n = 4 PBMC donors). *Summary/conclusion*: SE likely inhibit antigen-specific T-cell responses by affecting the co-stimulatory capacity of antigen-presenting cells. Understanding how programmed immune responses are altered by the presence of semen is important to developing the next generation of vaccine and preventative treatments against sexually transmitted disease.

Jurriaanse room
Symposium Session 3B1 - Single vesicle analysis
Chair: *Alain Brisson and Rienk Nieuwland* 15:30-17:00

O3B1-088

Refractive index of extracellular vesicles by nanoparticle tracking analysis

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Introduction: Vesicles are often studied by methods that detect light scattering, such as flow cytometry. The amount of light scattered by a vesicle depends on its size and refractive index. Consequently, knowledge of the vesicle refractive index is required to determine the size distribution of vesicles from the flow cytometry scatter signal. The refractive index may also become a new label-free parameter, for example to distinguish vesicles from protein aggregates. At present, however, no method is available to determine the refractive index of single vesicles. *Methods*: We have measured the diameter and light scattering of vesicles and beads by tracking their Brownian motion with nanoparticle tracking analysis (NTA; NS500, Nanosight Ltd). We analytically described the relation between the diameter, refractive index and light scattering of beads using Mie theory to determine the refractive index of vesicles from cell-free human urine. Urine was used because it contains a high concentration of vesicles with low contamination from lipoproteins and protein aggregates compared to blood plasma. *Results*: The median refractive index of urine vesicles was 1.36 with 90% of the vesicles between 1.35 and 1.41. *Summary/conclusion*: The refractive index of single vesicles in suspension can be determined by NTA. Urine vesicles have a median refractive index of 1.36. Taking into account that vesicles have a phospholipid membrane with a refractive index of 1.48, our findings indicate that the lumen of most urine vesicles have a refractive index of 1.34, which is equal to water. Since the cytosol typically has a refractive index of 1.38 ± 0.02 due to the abundant presence of proteins, our findings suggest a relatively low protein concentration in most urine vesicles.

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O3B1-089

Measurement of refractive index by nanoparticle tracking analysis reveals heterogeneity in extracellular vesicles

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Introduction: Light scattering techniques are widely used in the characterization and enumeration of extracellular vesicles (EVs). A lack of suitable calibrators has hindered efforts to standardize EV measurements. Polystyrene reference beads are widely used but many consider polystyrene to be a poor choice owing to its high refractive index (RI) relative to that of EV. It is often assumed that EVs have RI similar to their cell of origin but this is unproven. *Methods*: EVs from a wide range of cells were analysed by nanoparticle tracking analysis (NTA). Light scattering intensity (LSI) and size data were recorded for each vesicle. Silica and polystyrene beads of known RI (1.470 and 1.633, respectively) ranging from 50–500 nm in diameter were also analysed. We developed software, based on Mie scattering code, to enable the calculation of RI. This modelled theoretical LSI for particles of given sizes and RI. The model was verified using LSI from polystyrene and silica beads, then applied to different EV populations. Silica and polystyrene beads were analysed with each batch of tests and EV LSI measurements normalized to these measurements to minimize day-to-day variation. *Results*: The following mean (95% CI) RI measurements were obtained: urinary exosomes 1.419 (1.412–1.431); neuroblastoma exosomes 1.429 (1.427–1.431); blood EV 1.429 (1.426–1.432); EV from activated platelets 1.431 (1.425–1.439); small placental EV 1.381 (1.378–1.384); and 1.50 (1.477–1.514) for large placental EV (>200 nm). The spread of RI values was narrower for small EV than for the more heterogenous large EV. *Summary/conclusion*: Using NTA and Mie scattering theory, we have demonstrated that it is possible to estimate the RI of EV. Exosomes typically had RI of 1.38–1.43, similar to that of intact cells and platelets (1.38 and 1.39, respectively), whereas values of >1.50 were observed for some large microvesicles. We believe that RI may be a useful parameter for characterizing different EV subtypes and for developing calibrators for EV measurement.

O3B1-090

Reproducible minimum detectable size for extracellular vesicle measurement with resistive pulse sensing

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Introduction: The size of extracellular vesicles (EV) can be determined with a tunable resistive pulse sensor (RPS). For each measurement, however, four settings need to be configured: the conductivity of the electrolyte, the pressure across the pore, the voltage across the pore, and the pore stretch. Because the relationship between pore stretch and pore diameter varies from pore to pore, the minimum detectable size will also vary. We investigated the reproducibility of the minimum detectable size by targeting (1) the commonly applied constant stretch, or (2) a constant current through the pore, or (3) a constant blockade height. *Methods*: Experiments were performed with the qNano system (Izon), with NP200 pores. First, experiments were performed with beads and a standard urine vesicle sample using three pores to find measurement conditions that result in the lowest detection limit. Next, we evaluated three methods to configure the instrument. In each method pressure and electrolyte were the same, but for the three methods the voltage and stretch were changed to achieve: (1) constant voltage and stretch, (2) constant voltage and baseline current, (3) constant blockade height. The size distribution of urine vesicles was measured four times with a single pore, and four times with four different pores. *Results*: The lowest detection limit was achieved with the following parameters: 0.5 V, 45 mm stretch, 117 nA baseline current, 15 mbar pressure and 0.28 nA blockade height for 200 nm beads. For constant stretch, constant current and constant blockade height in single pore measurements, the minimum detected vesicle diameter was 96, 110 and 121 nm, with a coefficient of variation (CV) of 3.6, 4.7 and 4.3% respectively. When comparing 4 different pores, however, the CV of the minimum detected vesicle diameter of 118, 124 and 123 nm was 15.4, 11.0 and 4.5% with constant stretch, current or blockade height, respectively. *Summary/conclusion*: Setting a constant blockade height leads to the best reproducibility of the minimum detectable size with tunable resistive pulse sensing. Setting a constant voltage and stretch, or constant voltage and current, leads to lower reproducibility.

O3B1-091

EV enumeration by nanoparticle tracking analysis compared with asymmetric flow-field flow fractionation and cryo-TEM imaging

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Introduction: Small cell-secreted EVs consist primarily of two populations, exosomes and shedding vesicles, that are released from all cell types in response to specific stimuli by entirely different mechanisms. However, individual contributions from the two EV populations in transmitting differentiation-regulating signals to target cells have not been determined. We apply cryo-TEM imaging and asymmetric flow-field flow fractionation coupled with multi-angle light scattering (A4F-MALS) to determine vesicle size distributions and enumerate particle numbers secreted per cell for the exosome population of EVs. The results are used to evaluate nanoparticle tracking analysis (NTA) as an expedient method for EV enumeration. To this end, A4F-MALS and NTA are applied to quantify the number of exosomes secreted by thyroid cancer cell lines in response to treatment and to evaluate a novel approach to non-specific exosome capture by tethered cationic lipoplex nanoparticles (TLNs). *Methods*: Conventional differential centrifugation was used for EV isolation from cell culture. The recovery efficiency of our protocol was determined using A4F-MALS and synthetic liposomes. EV morphologies and size distributions were derived from 500 to 1,500 cryo-TEM images of individual vesicles within each EV population from different cell lines. The size distributions and morphological characteristics such as bilayer thickness were used in the model-dependent analysis of MALS from the eluting fractions of EVs in the A4F. With a reliably accurate model for light scattering established, we calculated EV number density as a function of vesicle diameter. Parallel measurements of size distributions and number densities by NTA allowed us to evaluate and optimize NTA. *Results*: We find exosome size distributions derived from A4F-MALS and cryo-TEM imaging to be in good agreement for vesicle diameters less than 200 nm, with a slight bias towards larger diameters seen in the A4F-MALS distributions. Exosome size distributions obtained by NTA are shifted to larger diameters. The number of exosomes secreted per cell derived from A4F-MALS is also greater than that obtained by NTA. We apply these methods to quantify the number of exosomes secreted by different thyroid cancer cell lines as a measure of stress response to treatments, and find that exosome size distributions are independent of cell type and insensitive to the treatments. However, the number of exosomes secreted per cell increases dramatically in response to treatment in a pathway-dependent manner. NTA quantitation of exosome capture by TLNs establishes the feasibility of this non-specific isolation method. *Summary/conclusion*: We demonstrate the utility and limitations of EV enumeration by A4F-MALS and NTA. A4F-MALS circumvents the limitations of light scattering from larger particles in polydisperse samples by first fractionating the sample based on particle size. NTA has advantages of convenience and simplicity.

This work is supported by NIH (P01 CA124570 and 1UH2TR000914-01).

O3B1-0910

Comparative analysis of extracellular vesicles and reference particles using high-resolution flow cytometry, tunable resistive pulse sensing and nanoparticle tracking analysis

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Introduction: In recent years novel instruments have become commercially available for quantification and characterization of individual extracellular vesicles (EVs). The underlying technology and measuring principles of these instruments are fundamentally different, and it is not known how this affects the comparability in measurements of diverse nanosized particles on these instruments. We sought to investigate the comparability, strengths and limitations of nanoparticle tracking analysis (NTA), tunable resistive pulse sensing (tRPS) and high-resolution flow cytometry (hFC). *Methods*: Dilution ranges and mixes of 100 and 200 nm polystyrene beads were analyzed for quantification accuracy. In addition, fluorescent liposomes were prepared as reference particles and quantification was compared on the three instruments. Comparative analysis of EVs was performed using PKH67-labelled, density-separated EVs from two different tumour cell lines. For each instrument, specific settings influencing the detection accuracy were studied. *Results*: Each instrument allowed accurate quantification of a dilution range of polystyrene beads at constant settings. The size and absolute values of the optimal particle concentration range differed between the platforms, with hFC being accurate over the largest concentration range (500-fold vs. 64-fold dilution range for NTA and tRPS). Mixes of 100 and 200 nm beads could be analyzed with similar accuracy using tRPS and hFC, whereas adaptations of settings were necessary for accurate identification of the two populations using NTA. Quantification of a homogeneous population of liposomes was comparable and accurate on all three instruments. However, due to heterogeneity in size, analysis of the EV preparations resulted in differences in exact quantifications and size distributions. In contrast to NTA and tRPS, analysis of heterogeneous EV populations with hFC was achieved with constant settings. However, measurements on this instrument required fluorescent labelling and did not yield absolute values for size. *Summary/conclusion*: Quantification of homogeneous particles was similar on all three instruments, but larger variation was observed in quantification of heterogeneous EV populations. For both NTA and tRPS, the EV size profiles were influenced by the instrument settings.

O3B1-0911

Luminal labelling of extracellular vesicles: advantages and pitfalls

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Introduction: Labelling of extracellular vesicles (EVs) with (fluorescent) probes is required for analyzing the interaction of EVs with cells using techniques such as fluorescence microscopy and flow cytometry. At present, mainly lipid labelling is used, in which hydrophobic labels are incorporated into the vesicle membranes. As lipid labels are exposed on the outside, spontaneous transfer of the label might occur and vesicle behaviour might be influenced. An advantage of the luminal labelling method used in this research is the entrapment of the probe inside the vesicle. *Methods*: EVs were labelled with modified probes, after which labelling stability was assessed and the purification method was optimized. Uptake of labelled EVs by recipient cells was examined using flow cytometry and fluorescence microscopy. *Results*: We were able to stain EVs using luminal probes. Labelling was stable and EVs could be used for uptake studies using flow cytometry and microscopy. However, optimization of purification methods for the labelled EVs appeared essential, as in some purification protocols EV-associated probes could spontaneously diffuse into cells, resulting in non-EV related fluorescent signal. *Summary/conclusion*: Luminal labelling is a promising novel method to label EVs and has advantages over generally used lipid membrane dyes. Nevertheless, one should remain alert on the presence of impurities in the EV sample as they could have a major effect on the outcome in EVcell interaction studies.

Jurriaanse room
Symposium Session 3B2 - Novel developments in the EV field: sponsored session
Chair: *Alain Brisson and Rienk Nieuwland* 17:00-17:30

O3B2-092

Tuneable resistive pulse sensors for high-resolution characterization of extracellular vesicles

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Introduction: Accurate high-resolution characterization of extracellular vesicles is critical to understanding their properties, function and potential utilization as predictive markers or therapeutic agents. Tuneable resistive pulse sensors (TRPS), such as the Izon qNano, have generated considerable interest to accurately characterize the size, charge and concentration of nano- to micro-scale particulate suspensions. Measuring the properties of each particle (e.g. exosome, microvesicle or cell) as is passes through the sensor provides analysis capabilities beyond that of other measurement techniques. Furthermore, the capacity to measure the size and charge on a particle-by-particle basis provides a unique insight into the role that these properties play. Herein we describe the principles and demonstrate how TRPS has been used to improve the characterization of extracellular vesicles. *Methods*: Measurements were made using an Izon qNano (NZ) unless otherwise stated. Polystyrene particles were purchased from Bangs Laboratories (USA), extracellular vesicles were isolated from urine, plasma and cell culture media by ultracentrifugation and ExoQuick from System Biosiences. Samples were diluted in either 100 mM KCl with 0.01% Tween 20 or PBS for analysis. Particle concentration, size and charge (zeta-potential) were calculated using Izon Control Suite Software. *Results*: The properties of synthetic particles and extracellular vesicles isolated from a range of media were measured using an Izon qNano. Fundamental modelling of the TRPS signal enables an accurate measure of vesicle concentration as well as simultaneous measurement of individual particle size and charge (zeta-potential). This holds significant promise for studying extracellular vesicles where differences in the expression levels and surface properties of vesicles may elucidate the cause of their formation or biological function. The analysis capabilities and reproducibility of TRPS were assessed via measuring a series of model complex samples of varying size and concentrations in addition to extracellular vesicles isolated from a range of media. TRPS was found to be one of the most sensitive and accurate techniques for measuring complex particle systems. *Summary/conclusion*: High-resolution particle-by-particle analysis techniques, such as TRPS, can provide much needed analysis detail for researchers and industry to better understand and utilize extracellular vesicles.

O3B2-093

The practical use of nanoparticle tracking analysis to characterise extracellular vesicles in general and when using fluorescence markers

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Introduction: Extracellular vesicles (30–100 nm) are released from endosomes in a wide variety of cells. It is increasingly accepted that exosomes are implicated and appear in a multitude of pathological conditions and show much promise as diagnostics for many different diseases such as cancer, heart disease, diabetes, Alzheimer's, pre-eclampsia, etc. However, developments in this area are constrained by limitations in the technology available for their measurement. *Methods*: Nanoparticle tracking analysis (NTA) (NanoSight Ltd, Amesbury) offers the potential to both enumerate and speciate (through fluorescent markers) these microparticles in a rapid manner. In this method the particles in suspension scatter laser light which is collected by a CCD (or sCMOS) camera via a microscope-type configuration. Particles between 10 and 2,000 nm are tracked individually and their diffusion coefficient, and therefore size, calculated directly from their speed. This characterization gives a direct measurement of the concentration and size distribution of the particles in the field of view. *Results*: If the particles are fluorescently labelled for expressed markers, then the concentration and size distribution are those of the labelled particles only, allowing a proportion of labelled particles to be calculated. However, this requires a combination of aspects to be identified and optimized during the process. *Summary/conclusion*: This talk will look at some of the challenges and considerations that need to be taken into account during the protocol development and measurement. We will discuss examples of good methodologies and appropriate controls, sharing data for fluorescence labels such as QDots and Alexa Fluor®488.

Van Weelde/Mees room
Symposium Session 3C - EV in the nervous system
Chair: *Eva-Maria Krämer-Albers and Lawrence Rajendran* 15:30-17:30

O3C-096

Neurotransmitter signalling triggers exosome transfer from oligodendrocytes to neurons

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Introduction: In the central nervous system, the generation of the axon-myelin unit is controlled by bidirectional interactions between oligodendrocytes and neurons, which continue throughout life. Lack of glial support occurs in mice deficient for the myelin proteins PLP and CNP and eventually results in degeneration of axons. Oligodendrocytes release extracellular vesicles (EVs) with the properties of exosomes. These exosomes contain PLP as well as CNP. *Methods*: We utilize differential centrifugation for exosome purification and characterize exosomes using nanoparticle tracking, western blot and quantitative proteomics. Moreover, we employ the LoxP-Cre-system to validate exosome transfer in co-culture assays. *Results*: Exosome secretion from oligodendrocytes is controlled by neuronal electrical activity via the neurotransmitter glutamate, which stimulates Ca^{2+} entry through oligodendroglial NMDA and AMPA receptors. In turn, neurons internalize oligodendroglial exosomes and functionally recover the exosomal cargo, which improves their metabolic activity under cell stress. Importantly, we demonstrate that PLP as well as CNP-deficient oligodendrocytes release reduced amounts of exosomes, which display specific abnormalities. *Summary/conclusion*: Oligodendrocytes influence the neuronal metabolism by an exosome-dependent transfer of biomolecules to neurons. Thus, disturbed intercellular transfer of substances by exosomes may contribute to the degeneration of axons in PLP and CNP null mice.

O3C-097

Ectosomes: a new mechanism for non-exosomal secretion of Tau protein

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Introduction: Tau is a microtubule-associated protein that aggregates in neurodegenerative disorders known as tauopathies. Recently, studies have suggested that Tau may be secreted and play a role in neural network signalling. However, once deregulated, secreted Tau may also participate in the spreading of Tau pathology in hierarchical pathways of neurodegeneration. The mechanisms underlying neuron-to-neuron Tau transfer are still unknown; given the known role of extra-cellular vesicles in cell-to-cell communication, we wondered whether these vesicles could carry secreted Tau. In this context, we investigated in cell media and body fluids if Tau might be present into (a) plasma membrane originating vesicles, the ectosomes, and/or (b) in multivesicular bodies deriving vesicles, the exosomes *Methods*: Ectosomes and exosomes were purified by differential centrifugations from culture media of neuroblastoma cell lines, primary cultures of rat embryonic cortical neurons and brain interstitial fluid coming from a rat model of Tauopathie (1). The presence of Tau was analysed using electron microscopy and biochemical assays. *Results*: Under basal conditions, Tau is present in ectosomes, which supports the concept of a new physiological function for Tau. We provide evidence that an ectosomal pathway at least partly mediates Tau secretion. These specific vesicles enabled cytosolic Tau to be shuttled to the extracellular media. The presence of Tau in vesicles coming from our rat model of sporadic tauopathy also suggests that the over-accumulation of intracellular Tau results in targeting to MVBs, leading to release in exosomes. *Summary/conclusion*: This study brings new direct evidences that Tau is cell-to-cell transferred at least thanks to vesicular systems. Deregulation of Tau secretion secreted Tau may also participate to the spreading.

Reference
1. Caillierez R, Begard S, Lecolle K, Deramecourt V, Zommer N, Dujardin S, et al. Lentiviral delivery of the human wild-type tau protein mediates a slow and progressive neurodegenerative tau pathology in the rat brain. Mol Ther. 2013;21:1358–68.

O3C-098

Virally activated monocyte-derived exosomes transfer miR-223 to neural cells: implication for neurocognitive impairment

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Introduction: Neurocognitive disturbances in HIV/HCV co-infection continue to persist in $>50\%$ of infected subjects despite successful treatment for HIV. We recently showed that peripheral monocytes from co-infected subjects with undetectable HIV (<50 copies/ml) express type-I interferon (IFN) gene activation profile that correlates with neurocognitive impairment. Given that HCV is not neurotropic, it suggests that indirect viral mechanisms may be responsible for neural cell dysfunction. Several reports indicate that exosomes facilitate intercellular communication wherein host RNA, including miRNAs, can be transferred to recipient cells. We hypothesized that in co-infection a viral-activated monocyte IFN response mediates neurodysfunction through the exosomal transfer of monocyte miRNAs to neural cells. *Methods*: Primary human monocytes from five co-infected subjects and three healthy subjects treated in vitro with type-I IFN were cultured overnight. Exosomes were harvested from conditioned medium with Exoquick and characterized by electron microscopy and protein markers. Identification and relative levels of exosomal miRNAs were determined using a hybridization array with 381 probes of well-characterized human miRNAs. Internalization of DiI-labelled exosomes by astrocytes was visualized by confocal microscopy. *Results*: Monocyte-derived exosomes harvested from co-infected subjects as well as exosomes from IFN-activated monocytes were highly enriched in miR-223. Exosomes from IFNα-treated primary monocytes were internalized by human astrocytes and significantly elevated intracellular levels of miR-223. A 3′UTR luciferase assay confirmed that exosomal miR-223 was functionally transferred. *Summary/conclusion*: Intracellular levels of miR-223 were significantly elevated in astrocytes following incubation with IFNα-activated monocyte-derived exosomes. Transfer of biologically functional miR-223 from activated monocytes to astrocytes via exosomes may explain, in part, the neurological abnormalities associated with co-infection.

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O3C-099

Interaction with hsc70 is essential for alpha-synuclein loading into exosomes: implication in Parkinson's disease pathology

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Introduction: Alpha-synuclein aggregation plays a central role in Parkinson's disease pathology and there is increasing evidence that the cellular release of alpha-synuclein in the brain may underpin the progression of this pathology. It is important to understand how cells process alpha-synuclein and in particular what regulates its release from cells. Alpha-synuclein contains a generic pentapeptide motif ($_{95}$VKKDQ$_{99}$) which interacts with hsc70 and is important for its degradation by chaperone mediated autophagy (CMA). However, when lysosomal function is compromised alpha-synuclein release from cells in exosomes is dramatically increased. Recently hsc70 has also been shown to direct proteins containing the generic KFERQ pentapeptide motif (hsc70 interaction) to late endosomes/multivesicular bodies (LE/MVB) and exosomes. We propose that the interaction of hsc70 with alpha-synuclein is important for the targeting of alpha-synuclein to exosomes which is increased when lysosomal function is compromised. *Methods*: Using stable cell lines over-expressing WT or $_{98}$AA$_{99}$ alpha-synuclein we have analysed; alpha-synuclein hsc70 interactions by co-precipitation; alpha-synuclein turnover, total and exosomal release by ELISA and WB. Exosomes were isolated by ultracentrifugation or immunoprecipitation. *Results*: Mutant $_{98}$AA$_{99}$ alpha-synuclein did not interact with hsc70 and was not degraded by CMA. While WT alpha-synuclein was detected in released exosomes, $_{98}$AA$_{99}$ alpha-synuclein was barely detectable. Inhibition of lysosomal function by bafilomycin increased exosomal WT alpha-synuclein release from the cells, but $_{98}$AA$_{99}$ alpha-synuclein release was unchanged. *Summary/conclusion*: These results imply that the interaction of alpha-synuclein with hsc70 is necessary for the transfer of alpha-synuclein to either lysosomes for degradation or LE/MVB/exosomes. The increase in release of alpha-synuclein in exosomes involves a hsc70 dependent mechanism.

O3C-100

Brain-derived microvesicles confer sickness behaviours by switching on the acute phase response in the liver

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Introduction: The hepatic response to brain injury, which includes the acute-phase response, is an essential part of the process that leads to immune cell recruitment to the brain and the exacerbation of disease. Local injury to the brain or other sites induces NFκB activity in the liver in a highly specific manner, but the process by which injury signals reach, and selectively activate, the liver to produce acute-phase response (APR) proteins is unknown. Endogenous microvesicles (MVs) have the potential to carry signals from the CNS to the liver. *Methods*: Here we examined potential of brain-derived MVs from animals receiving a CNS injection of IL-1β to activate a systemic APR in a naive animal. Labelled MVs were examined using intercalating membrane dye PKH-67. Bloods were removed and MV fraction was isolated for nanoparticle tracking analysis (NTA) and for transfer into naive animals. Behavioural experiments were performed on animals receiving both platelet-free plasma and MV fractions. The APR was examined using qPCR. *Results*: NTA shows that the inflammatory response after IL-1 into the striatum is a significant enough injury to induce increased circulating MVs. Plasma fractionation and transfer experiments in rats showed that the MV fraction contributes to the induction of the APR in the liver and to sickness behaviour after CNS injury. By labelling membranes in the CNS during the central injury using membrane dye PKH-67 we were able to observe CNS-derived MVs in the livers of IL-1β-injected animals. *Summary/conclusion*: These experiments reveal that increased circulating MVs, which are associated with a number of CNS disorders, are able to elicit a systemic APR to brain injury and this can result in sickness behaviours.

O3C-101

Study of exosomes shed new light on physiological amyloidogenesis

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Introduction: Amyloids are insoluble proteins or peptide aggregates with a cross beta-sheet structure and are often associated with pathological conditions including Alzheimer's disease and prion diseases. Amyloids also have a normal biological function as demonstrated by the functional amyloids derived from the protein PMEL that act as structural scaffolds for melanin deposit in melanosomes of pigment cells. PMEL amyloidogenesis occurs in specific intracellular compartments, i.e. multivesicular bodies (MVB), where PMEL is sorted and processed on intraluminal vesicles (ILVs). Our previous electron tomography reconstruction of melanosomes supports the hypothesis that ILVs are the site of nucleation of amyloid fibrils. To investigate the formation of amyloids at the surface of ILVs, we have based our analysis on ILVs secreted as exosomes. *Methods*: Exosomes, ILVs and cells have been analysed by cryo-electron microscopy and conventional electron microscopy coupled to mass spectrometry, cell imaging and biochemistry. *Results*: Our observations at a close to native state by cryo-EM revealed unreported multilayer structures on the surface of exosomes. Our work has identified these structures and revealed their role in PMEL amyloidogenesis within MVBs. *Summary/conclusion*: These data provide further insight into the role of MVBs and exosomes in functional amyloidogenesis and opens new avenues for the understanding of some pathological amyloidogenesis.

O3C-102

Exosomal miRNA as biomarkers for diagnosing neurodegenerative diseases

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Introduction: Alzheimer's, Parkinson's, and prion diseases are neurodegenerative disorders of humans associated with the spread and deposition of misfolded proteins. Exosomes are known to contain a number of these proteins (e.g. prion protein, Aβ, tau, and α-synuclein), suggesting these vesicles may play a role in the pathogenesis of these diseases. *Methods*: Using deep sequencing we have analysed the RNA content of exosomes from a cohort of healthy aged and Alzheimer's patients using samples from the Australian Imaging, Biomarkers and Lifestyle Flagship Study (AIBL). miRNAs associating with disease were validated using qPCR and additional risk factors collected during the 4.5 year AIBL Study were included, which consisted of baseline and 54 month clinical, medical and cognitive assessment including Amyloid-PET imaging. *Results*: We developed a workflow for the analysis of exosomal miRNA from

human blood serum that has diagnostic potential for AD. We have identified an AD specific, 16 miRNA signature, derived from the deep sequencing data and validated in an additional set of samples. Adding established risk factors including age, sex, and APOE allele status to the panel of deregulated miRNA resulted in a sensitivity and specificity of 87% and 77%, respectively, for predicting AD. Furthermore, Amyloid-PET information for those HC participants incorrectly classified with AD suggested progression towards AD. *Summary/conclusion*: These data suggest that an exosomal miRNA signature may be a suitable peripheral screening tool for AD and other neurodegenerative disorders such as Prion and Parkinson's diseases.

O3C-103

Intracerebral administration of glycosphingolipid-enriched exosomes reduces Alzheimer's pathologies in mouse brain

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Introduction: Increased levels of amyloid-β peptide (Aβ) in human brain are linked to the pathogenesis of Alzheimer's disease (AD). We have previously demonstrated using cell cultures that extracellular Aβ can bind to neuronal exosomes in a glycosphingolipid-dependent manner, and the Aβ associated with the exosome was internalized into microglia to degrade. The exosomes may work for Aβ clearance. In this study, we performed continuous intracerebral administration of the exosomes into the brains of AD model mice to evaluate the effects of the exosomes on AD pathologies in vivo. *Methods*: The exosomes were isolated from culture supernatants of neuro2a cells (N2a) or murine primary cortical neurons by sequential ultracentrifugation. Heterozygotic transgenic mice that express the human APP (amyloid precursor protein) bearing the Swedish and Indiana (KM670/671NL, V717F) mutations (J20 strain) were used as AD model mice. We continuously infused the exosomes into lateral ventricle or hippocampus of wild type or the APP transgenic mice (4- or 12-month-old) for 2 weeks using osmotic mini pumps. After the treatment, we measured Aβ levels with ELISA, and evaluated amyloid depositions and Aβ-mediated synaptic toxicity with immunohistochemistry. *Results*: Continuous intracerebral administration of the exosomes into the APP transgenic mice resulted in marked reductions in Aβ levels, amyloid depositions and Aβ-mediated synaptotoxicity in the hippocampus. In addition, we determined that glycosphingolipids, a group of membrane glycolipids, are highly abundant in the exosomes, and the enriched glycans of the GSLs were essential for Aβ binding and assembly on the exosomes, both in vitro and in vivo. *Summary/conclusion*: Our data demonstrate that exosomes in the brain act as potent scavengers for Aβ by carrying it on exosome surface glycosphingolipids. Improving of Aβ clearance by exosome administration would provide a novel therapeutic intervention for AD.

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Willem Burger room
Oral with Poster 1
Chair: *Michiel Pegtel* 17:45-18:20

OP1-104

Human mast cell exosomes regulate lung cancer cell cycle: possible role of c-kit transfer

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Introduction: Human mast cell release exosomes containing mRNA, miRNA and proteins. However, their potential effects on tumour cell are still unknown. *Methods*: The total RNA of exosomes was analysed by Bioanalyzer, and c-kit mRNA was identified by RT-PCR. The uptake of PKH67-labelled exosomes by lung cancer cells was examined by measuring fluorescence using flow cytometry and fluorescent microscope. The percentage of cells in different phases of the cell cycle was analysed by FACS and cell cycle associated protein detected by western blot. *Results*: In this study, we observed that the purified exosomes from mast cells can be taken up by lung cancer cells. Our results also show that exosomes express and transfer protein to lung cancer cells and subsequently activate signal transduction. *Summary/conclusion*: Our results indicate that a novel mechanism transfers protein between cells via exosomes and affects a recipient cell signalling events through receptor–ligand interactions.

OP1-105

Characterization of circulating microparticle origin in patients with myeloproliferative neoplasms (MPN) by flow cytometry

Carmen Julia Tartari[1], Marina Marchetti[1], Romaric Lacroix[2], Laura Russo[1], Francesca Piras[1], Guido Finazzi[3], Alessandro Rambaldi[3] and Anna Falanga[1]
[1]Division of Immunohematology and Transfusion Medicine, Hospital Papa Giovanni XXIII, Bergamo, Italy; [2]Department of Hematology and Vascular Biology, Hospital La Conception, Marseille, France; [3]Division of Hematology, Hospital Papa Giovanni XXIII, Bergamo, Italy

Introduction: Essential thrombocythemia (ET) is an MPN characterized by thrombocytosis, high rate of thrombotic complications and increased plasma concentration of procoagulant microparticles (MPs) (Marchetti et al. A.J.H. 2013). The present study was designed to characterize the cellular origin of MPs in ET patients and extend the analysis to polycythemia vera (PV), another MPN at high thrombotic risk. The influence of JAK2V617F mutation and therapies was also evaluated. *Methods*: A total of 36 ET patients (24F/12M, 18 JAK2V617F carriers), 36 PV patients (18F/18M, 29 JAK2V617F carriers) and 36 control subjects (13F/23M) were included in the study after informed consent. MPs were analysed in platelet free plasma by flow cytometry (Navios cytometer; Beckman Coulter). To define MP cellular origin anti-CD31-PE (endothelial cells), anti-CD41-PC7 (platelets), anti-CD11b-APC (leukocytes) anti CD235-APCAlexa750 (erythrocytes) monoclonal antibodies were used. Annexin V (AnV)-FITC staining was used to evaluate the expression of phosphatidylserine (PS) on MPs. Results are expressed as MP count/μL or%. *Results*: Significantly ($p < 0.05$) higher MP levels were found in ET (4,199 ± 495/μL) and PV (3,719 ± 388/μL) patients compared to controls (2,201 ± 272/μL). More than 90% of circulating MPs were AnV positive, indicating the expression of PS on their surface. In controls, 71% of MPs was positive for platelet (P-MP), 24% for erythrocyte (E-MP), 4% for endothelial cell (EC-MP) and 1% for leukocytes (L-MP) markers. In ET and PV subjects, the percentage of P-MP was significantly higher (80%; $p < 0.05$) and E-MP levels significantly lower (15%; $p < 0.05$) than controls, while L-MP and EC-MP were similar between patients and controls. The absolute counts of P-MP and L-MP were higher ($p < 0.05$) than controls in both ET and PV. Furthermore, MPN patients displayed significantly elevated number of P-MP co-expressing the endothelial marker CD31. No significant correlations were found between the platelet, leukocyte and erythrocyte counts with MP (both as count/μL or as%) in study subjects. No influence of JAK2V617F mutation or therapy on MP levels was observed. *Summary/conclusion*: Our data confirm in a larger population of ET patients increased concentrations of plasma MP, supporting their role in the previously reported procoagulant activity. In addition, the study demonstrates increased levels of MP in PV patients. A similar profile of MP cellular origin appears from the analysis of MP from ET and PV. The lack of correlation found between the total and subtype MP counts with the corresponding cell of origin counts suggests that MP formation is not constitutive, but may result from an active stimulation.

OP1-106

Exosomes: future diagnostic tool in prostate cancer

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Introduction: Prostate cancer (PCa) is the leading diagnosed and third most lethal malignancy in men. If diagnosed early, PCa is curable at early stage with surgery or radiation treatment however, with time, which varies from months to years; many PCas metastasize and, even with aggressive hormone therapy treatment approaches, progress to castration-resistant prostate cancer (CRPC) and lethality with no effective long-term treatment for CRPC. Serum PSA measurement is the main screening test for PCa detection. While PSA screening is very controversial, prostate biopsy, albeit an invasive procedure, remains the only definitive diagnostic test for PCa. Therefore, there is an urgent current need, for the discovery of relevant biomarkers to replace the existing diagnostic tests for better and earlier detection of PCa. One possible source of biomarkers which could be used as part of diagnostic tests is exosomes. Exosomes are nanovesicles secreted from normal and cancer cells and are present in different biological fluids such as blood and urine. Exosomes secreted from cancer cells carry different cell components compared to normal cells and hence could be used as a diagnostic tool in different pathological conditions. *Methods*: Exosomes were purified from the conditioned media of different PCa cell lines as well as PCa patient's biological fluids. Further analysis using NanoSight, western blot (WB) and transmission electron microscopy (TEM) validated the size, purity and integrity of isolated exosomes. Proteomic analysis was performed using a Waters LC-QTOF/MS in conjunction with ProteinLynx software and the searchable MASCOT peptide-sequence database for validation of potential PCa biomarkers using our published list of PCa derived exosomes from prostate cell lines as a guide. Furthermore, the presence of identified PCa biomarkers (e.g. FOLH1, CLU, etc) was validated in biological sample using LCMS and WB. *Results*: Our EV characterization data confirmed a pure enrichment of exosomes in the isolated exosome. Our proteomic data show that exosomes derived from specific PCa cells or patient's biological fluids

present several biomarkers with the potential to depict PCa progression. *Summary/conclusion*: This study highlights a potential for differential protein composition of exosomes as a source of diagnostic biomarkers in addition to therapeutic targets for PCa via non-invasive testing.

OP1-108

Exosomes mediate drug resistance in melanoma

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Introduction: Melanoma cells can develop BRAF inhibitor resistance by acquiring de novo mutations or by activation of cell signalling in the absence of genetic mutation. Recent findings demonstrate that exogenous growth factors can rescue cells from drug inhibition, suggesting the extracellular environment can mediate resistance. We hypothesized that exosomes released from BRAF inhibitor drug-resistant melanoma cells traffic to neighbouring and distant drug-sensitive melanoma cells, transferring functional characteristics and mediating drug resistance. *Methods*: Exosomes were isolated from the conditioned media of melanoma cell lines and characterized by protein and miRNA composition, density and morphology. Exosomes isolated from BRAF inhibitor-sensitive or -resistant cells were incubated with recipient cells in the presence/absence of inhibitor, and then functional studies including motility, migration, invasion and proliferation assays performed. Exosomes also underwent comparative proteomic and miRNA profiling. *Results*: Melanoma-derived exosomes were found to mediate a phenotypic switch in recipient cells, thereby contributing to drug resistance. Specifically, exosomes isolated from BRAF inhibitor-resistant cells transferred resistance to sensitive cells. The proteomic and genomic composition of exosomes derived from drug resistance cells is distinct to the composition of exosomes from sensitive cells providing insight into both biomarkers and therapeutic targets for the treatment of BRAF inhibitor-resistant melanoma. *Summary/conclusion*: If cancer therapies are to become more effective, the study of treatment failure and its mechanisms is a priority. We have identified a mechanism which appears to mediate the transfer of functional characteristics including drug resistance between heterogeneous melanoma cell populations.

OP1-109

Extracellular vesicles isolated from equine synovial fluid bind to and are internalized by chondrocytes and synoviocytes in vitro

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Introduction: In both the healthy and diseased joint, extracellular vesicles (EVs) can possibly play an important role in the communication between chondrocytes and cells of the surrounding articular tissues. Gaining insight into this form of communication between cells in the joint may be of great benefit for the progress of cartilage regenerative medicine and treatment of articular diseases. The aim of this study is to verify the presence of EVs in equine synovial fluid and analyse their potential role in communication between articular cells in an *in vitro* model system. Current experiments examine binding and internalization of EVs by chondrocytes and synoviocytes *in vitro*. *Methods*: EVs were isolated from equine synovial fluid of healthy horses, labelled with PKH-67 and separated according to buoyant density by iodixanol gradient ultracentrifugation. PKH-67 labelled EVs were analysed by high-resolution flow cytometry (BD Influx). The EVs containing gradient fractions were added to a monolayer culture of C20A4 chondrocytes or HFLS-*408-05a* fibroblast-like synoviocytes. After 2 or 5 h incubation, the cells were processed for regular flow cytometry to measure binding/uptake of EVs by cells. In addition, intracellular localization of the PKH-67 signal was visualized by confocal microscopy. The lipid dye LD-540 was used to stain lipid droplets. *Results*: EVs could be successfully isolated from synovial fluid and subsequently labelled with PKH-67. High-resolution flow cytometry data verified the presence of EVs in iodixanol gradient fractions with densities ranging from 1.04 to 1.18 g/ml. Interaction of labelled EVs with both chondrocytes and synoviocytes, as measured by regular flow cytometry, was most pronounced after 5 h. Confocal microscopy showed high amounts of internalized EV-derived PKH-67 (compared to the PKH-67 control condition without EVs). Interestingly, diffuse staining surrounding the nucleus and specific co-localization with lipid droplets was seen in both cell lines. *Summary/conclusion*: The results of the present study indicate that synovial fluid derived EVs can bind to and be internalized by articular cells. Co-localization of the PKH-67 dye (derived from labelled EVs) with lipid droplets suggests intracellular trafficking of EVs into specific cell compartments. Live imaging will be performed to study this process in more detail. Furthermore, activation of downstream pathways in these cells will be studied. This work was supported by a grant from the Dutch government to the Netherlands Institute for Regenerative Medicine (NIRM).

OP1-110

Extracellular proteasomes are generated by blebbing of microvesicles

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Introduction: The 20S proteasome, a barrel-shaped protease composed of four stacked, seven-membered rings, is responsible for the degradation of a multitude of intracellular proteins. Extracellular proteasomes are present in cell culture supernatants of different cell types and in diverse body fluids of healthy individuals. Under pathological conditions like inflammatory or autoimmune disorders and various cancers, the levels of extracellular proteasomes can be dramatically increased. In previous experiments, we have shown that 20S proteasomes can be released into the extracellular environment by microparticle blebbing and that subsequent breakdown of these microvesicles by sphingomyelinase leads to free proteasomes. The aim of this study was to further characterize extracellular proteasomes, compare different cells for proteasome release and evaluate stimuli for package of proteasomes into extracellular vesicles. *Methods*: We cultivated primary T cells, HeLa and Jurkat cells, and isolated the vesicle fraction after stimulation of the cells by 2 μM Calonophore A23187 and 1 mM CaCl2 in MP-free medium for 45 min. The cell culture supernatant was transferred for differential centrifugation, and finally the microvesicles (MVs) were isolated by ultracentrifugation for 20 min at 100,000 *g*. For native gel electrophoresis, MV samples were mixed with TSDG buffer (10 mM Tris/HCl, 25 mM KCl, 10 mM NaCl, 1.1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 2 mM ATP, 10% glycerol, pH7) and native gel loading buffer. Native gel electrophoresis was accomplished on 3–12% BisTris gels, the loaded proteins were separated in 1 M BisTris/1 M tricine at 4°C overnight and in-gel tryptic activity was determined by incubation for 15 min at 37°C with 200 μM Bz-VGR-AMC substrate. Cytokine stimulations were done for 24 h with 200 U/ml interferon-γ (IFNγ), 10 μg/ml tumour necrosis factor α (TNFα) or 50 U/ml interleukin-2 (IL-2), and SDS-PAGE was performed by mixing MVs with RIPA and loading buffer. *Results*: (1) Comparison of different cell types revealed that proteolytically active 20S as well as 26S proteasomes were released by microvesicles. (2) Induction of microvesicle shedding by A23187 increased ubiquitinylated proteins in T cells and in T-cell-derived microvesicles. (3) Stimulation of T cells by different cytokines revealed that upon interferon-γ stimulation for 24 h, but not TNFα and IL-2 stimulation, the microvesicles were enriched with proteasomes. *Summary/conclusion*: Proteasomes in extracellular vesicles

are active proteases. Ca/A23187 stimulation increased the amount of ubiquitinated proteins in cells and microparticles. Until now, is not clarified, which functions are performed by vesicular proteasomes. The cytokine INFγ induces the production of immunoproteasomes in cells, also resulting in increased proteasome content of microvesicles. Therefore cytokine production might influence elevated plasma proteasome levels in pathological immune responses.

OP1-111

The role of extracellular vesicle-mediated transfer of donor genomic DNA to recipient cells in genetic influence between cells

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Introduction: Extracellular vesicles (EVs) carry signals within or at their limiting membranes, providing a mechanism by which cells can exchange more complex information than what was previously thought. Recent studies have shown that both mitochondrial DNA and chromosomal DNA were found in EVs. However, whether the transferred EV DNAs are functional or not is not clear. *Methods*: We first examined the existence of genomic DNA (gDNA) in EVs derived from human plasma and cell supernatants in culture by Solexa sequencing and PCR. Then, we studied the transport of gDNAs (AT_1 receptor DNA) in EVs from AT_1 receptor-transfected HEK293 cells or VSMCs to non-transfected HEK293 cells. Moreover, we investigated the effect of endogenous NF-κB, a promoter of the AT_1 receptor gene, on transferred DNAs including AT_1 receptor DNA. Finally, we examined the transfer of BCR/ABL hybrid gene in EVs from K562 cells to normal neutrophils by both cell and animal experiments. *Results*: Solexa sequencing indicated the presence of at least 16,434 genomic DNA (gDNA) fragments in the EVs from human plasma. Immunofluorescence study showed direct evidence that acridine orange-stained EV DNAs could be transferred into the cells and localized to and inside the nuclear membrane. We found that EV gDNAs could be homologously or heterologously transferred from donor cells to recipient cells and increase gDNA-coding mRNA, protein expression, and function (e.g. AT_1 receptor). An endogenous promoter of the AT_1 receptor, NF-κB, could be recruited to the transferred DNAs in the nucleus, and increase the transcription of AT_1 receptor in the recipient cells. Moreover, BCR/ABL hybrid gene, involved in the pathogenesis of chronic myeloid leukaemia, could be transferred from K562 EVs to HEK293 cells or neutrophils. Furtherly, two months after injection of K562 EVs into the tail vein of Sprague-Dawley (SD) rats, they showed some characteristics of CML, for example, feeble, febrile, and thin, with splenomegaly and leukocytosis but with reduced neutrophil phagocytic activity. These findings were also observed in immunodeficient NOD/SCID mice treated with K562 EVs; BCR/ABL mRNA and protein were found in their neutrophils. The administration of actinomycin D (an inhibitor of *de novo* mRNA synthesis) and imatinib (a tyrosine kinase inhibitor) prevented the abnormalities caused by K562 EVs in NOD/SCID mice related to CML. *Summary/conclusion*: Our present study shows that the gDNAs transferred from EVs to cells have physiological significance, not only to increase the gDNA-coding mRNA and protein levels, but also influence function in recipient cells both in vitro and in vivo.

Jurriaanse room
Oral with Poster 2
Chair: *Chris Gardiner* 17:45-18:20

OP2-116

Characterization of neutrophilic granulocytes derived apoptotic bodies

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Introduction: Neutrophils (PMN) produce antibacterial microvesicles after complex biological stimulation. The antibacterial capacity is reduced if the activation is partial. In order to assess the importance of cell activation in production of antibacterial microvesicles, in this study we characterize the extracellular vesicles (EVs) produced during spontaneous death of the neutrophils and test their antibacterial effect. *Methods*: PMNs were prepared from the blood of healthy volunteers. After storing the cells in dulbecco's modified eagle's medium in cell culture incubator for 3 days, EVs were separated by two step centrifugation and filtration. The separated vesicles were counted and analysed with EV-optimized flow cytometry. The structure of EVs was analysed with fluorescent and electron microscopy. The antibacterial effect of the EVs was tested in bacterium survival assay. *Results*: During the 3 days storing the number of apoptotic EVs was increased parallel with PMN count decrease. The flow cytometric appearance (forward and side scatter) of apoptotic EV was similar to antibacterial microvesicles, and both populations were positive for annexin V and CD11b, a neutrophil cell surface marker. However unlike microvesicles produced after PMN activation, the apoptotic EVs were ineffective in bacterium survival assay. *Summary/conclusion*: The apoptotic EVs of neutrophils have a right side out vesicular nature, which is similar to the antibacterial microvesicles, but apoptotic vesicles do not have antibacterial effect. These results highlight the need for biological tests in EV research to distinguish different types of EVs produced from the same cell.

OP2-117

Nanovesicles engineered from cells

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Introduction: Exosomes draw attentions due to their roles in intercellular communications. Especially, exosomes secreted from stem cells have potential applications in regenerative medicine. Nevertheless, the researches of exosomes have been hindered due to the small quantity of exosomes; exosomes of 100 ng are collected from 10^6 embryonic stem cells in 24 hr by using density gradient ultracentrifugation. To overcome this limitation, artificial exosome-like nanovesicles derived from cells are practical alternative to exosomes secreted from cells. To generate more exosome-like nanovesicles with homogeneity in both quality and quantity, an extrusion system with centrifugal force was developed and contains RNAs and more cytosolic proteins than exosomes. *Methods*: Nanovesicles generation device has several components: a pair of syringes, pistons and caps, a filter holder and a track-etched polycarbonate filter. Centrifugal force is applied by piston and common centrifuge. All components except the piston were assembled by a screw thread. The murine ES D3 cell line was used as source for the nanovesicles. This cell was crushed and self-assembled to nanovesicles when they pass through the polycarbonate filter repeatedly. The nanovesicles were treated to mouse embryonic fibroblast (MEF) GFP isolated from embryo of C57BL/6-Tg(ACTB-EGFP) mouse, and RNAs and proteins deliveries were evaluated. *Results*: The nanovesicles that had a spherical shape enclosed by lipid bilayer were found by TEM. Their size was similar to exosomes (50 ~ 150 nm). Amount of generated nanovesicles was about 100 times more than exosomes from same number of ES cells. Also, these nanovesicles contained about two times more RNAs and cytosolic proteins than exosomes. We verified that intracellular contents were from ES cells using RT-PCR and western blotting. Also we found that the nanovesicles induced phosphorylation of MAPK regulating proliferation and cell survivals. *Summary/conclusion*: The nanovesicles generated by the extrusion system using centrifugal force contain more intracellular contents than cell-secreted exosomes, and are able to deliver RNAs and proteins to the target cells. Thus, the nanovesicles engineered from living cells are expected to contribute to exosome research fields or vesicle-based therapy.

OP2-118

Characterization of *Leishmania* extracellular vesicles: insights into their immunoregulatory role in the establishment of the infection

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Introduction: The use of secretion pathways for effector molecule delivery by microorganisms is a trademark in pathogenesis. Recent emphasis has been given to the release of extracellular vesicles (EVs) by the parasite *Leishmania*, the etiological agent of leishmaniasis. The absence of vaccines and satisfactory treatments associated to this disease demand a better understanding of the infectious process. The evasion of the immune response is critical for infection establishment. Our hypothesis is that EVs could be implicated in these early evasion mechanisms by acting on immune cells. In this sense, the immunological potential of EVs and vesicle-depleted exoproteome (VDE) has been tested, in vitro and in vivo, on mouse models. *Methods*: EVs and VDE were prepared from stationary promastigotes as recently described by us and further characterized. Subsequently, an air pouch model was used to resemble the biological environment of the first stages of infection, allowing the study of local cell recruitment. Mouse's backs were inflated and subsequently injected with EVs or VDE. At 6 h after inoculation, animals were euthanized, the pouch exudate collected, and the presence of different cell populations studied by flow cytometry. For in vitro studies bone marrow-derived dendritic cells (DCs) and macrophages were used. The expression of several activation markers and cytokines was studied, by flow cytometry/ELISA, after 24 h of incubation with EVs or VDE. Then, the state of responsiveness of the cells was also evaluated by re-stimulation with TLR-ligands. *Results*: The promastigote-secreted EVs are associated with protein markers traditionally found in exosomes, containing also genetic material. Nonetheless, evidences of a heterogeneous vesicle population hints at distinct origins. At the evaluated time point, EVs consistently recruited higher numbers of DCs and eosinophils when compared with VDE or an inflammatory stimulus as LPS. In vitro, both EVs and VDE are able to diminish the response capacity of DCs and macrophages to exogenous stimulus. *Summary/conclusion*: *Leishmania*-released EVs might promote a specific immune cell

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recruitment and subsequent anergy, contributing to the survival of the parasite in the early stages of the infection.

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OP2-119

Human tears and sweat trigger clotting of blood: the role of tissue factor-exposing vesicles

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Introduction: The epithelium provides a barrier to microorganisms and produces fluids as saliva, tears (lacrima) and sweat to trap, kill and remove microorganisms. Tears contain a thus far unidentified coagulant activity. Recently, we observed high levels of coagulant tissue factor (TF)-exposing vesicles in human saliva and urine (*Blood* 2011;117:3172–80), and we hypothesized that such vesicles are involved in host defence by supporting clot formation and thus maintenance of the barrier integrity. In this study, we investigated whether the procoagulant activity of tear fluid and sweat is also associated with TF-exposing vesicles. *Methods*: We collected tear fluid and sweat from healthy subjects and studied the presence of vesicles (transmission electron microscopy), vesicle-associated TF antigen (flow cytometry and western blot) and TF coagulant activity (fibrin generation assay). *Results*: Tear fluid and sweat contained vesicles of 40–140 nm. Tear fluid from all subjects (n = 13) shortened the clotting time of normal pool plasma from 3,600 s to 1332 s ± 502 ($p < 0.0001$). This shortening was completely TF-mediated ($p < 0.0001$). TF in tear fluid was exclusively associated with cell-derived vesicles because centrifugation completely abolished the coagulant activity of tear fluid. Also sweat contained detectable levels of TF-exposing vesicles (n = 8), albeit on average 3–4 fold lower than in tear fluid. Sweat from two subjects triggered TF-mediated clotting of plasma. *Summary/conclusion*: This study demonstrates that tear fluid contains coagulant TF-exposing vesicles, and sweat of some subjects contains detectable levels of vesicle-associated coagulant TF. These findings further support our hypothesis that epithelial fluids contribute to protection and maintenance of our ''milieu-interieur'' by promoting clot formation.

OP2-120

The effect of rosuvastatin on circulating microparticles in treatment-naïve HIV-patients

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Introduction: Various studies suggest that ongoing activation of coagulation and of the immune system may play a role in excess non-AIDS-related diseases in HIV patients. Coagulation and inflammatory events have been shown to involve microparticles (MPs), which may be modulated by statins. In this study we explored the impact of HIV and the effect of rosuvastatin on the number of MPs and their marker expression. *Methods*: Using flow cytometry, levels of circulating MPs and their marker expression were measured in plasma samples of healthy controls (n = 10) and treatment-naïve HIV-patients (n = 20), in the OLVG Hospital in Amsterdam. We determined markers of T cells (CD3, CD4 and CD8), monocytes (CD14), platelets (CD41), endothelium (CD144), leukocytes (CD45), granulocytes (CD66acde), erythrocytes (CD235a), and activation (PSGL1, HLA-DR, CD38, P-selectin, E-selectin and CD44) and tissue factor (CD142). A randomized double-blind, placebo-controlled cross-over study explored the effect of rosuvastatin 20 mg qd for 8 weeks on MPs and their marker expression in treatment-naïve HIV-patients. *Results*: The total numbers of circulating MPs in HIV-infected patients and controls were similar (median 11822 vs. 14561/μl, $p = 0.48$). However, HIV-patients had higher levels of erythrocyte-derived MPs (346 and 2.8 vs. 111/μl and 0.60%, $p < 0.01$/ < 0.01) and leukocyte-derived MPs (20 and 0.17 vs. 12/μl and 0.1%, $p = 0.04$/0.02) and lower levels of granulocyte-derived MPs (10 and 0.08 vs. 20/μl and 0.18%, $p = 0.04$/0.05). There was a correlation between leukocyte-derived MPs and CD8 count ($R = 0.52$/$p < 0.01$) and T-cell activation levels (e.g. CD38 + HLA-DR+ of CD8+ T cells $R = 0.20$/$p = 0.03$). Rosuvastatin treatment did not affect the concentration of circulating MPs. *Summary/conclusion*: HIV infection is associated with an increased number of erythrocyte-derived and leukocyte-derived MPs that are most likely the result of destabilization of erythrocytes and immune activation, respectively. Rosuvastatin treatment did not influence the total number of MPs nor influence their marker expression.

OP2-121

Unfiltered beer – a rich source of yeast extracellular vesicles

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Introduction: Yeast cells secrete extracellular vesicles (EVs) by molecular traffic across the cell wall. In yeast these EVs have been implicated in the transport of proteins, lipids, polysaccharide and pigments to the extracellular space. Unfiltered beer is made from grains, water and yeast allowing preservation of the B-complex vitamins, trace minerals and metals like chromium and selenium, since they are not filtered out at the end of the process. Hitherto, the presence of yeast EVs in beer as food source remains largely unreported and characterized. *Methods*: Unfiltered beer was acquired from multiple sources (IPA, Weissbier, Ale and pilsner). Purification of yeast EVs was optimized based on methodology previously described (Oliveira, 2010). Purified MVs were validated by nanoparticle tracking analysis (NTA), electron microscopy (EM) and atomic force microscope (AFM). The EV sub proteome was characterized and differentiated to yeast cells using quantitative mass spectrometry followed by functional annotation of the identified proteins. *Results*: Bilayered vesicles with diameters at the 55–300 nm range were found in extracellular fractions from EV particles recovered by ultracentrifugation using NTA and EM. Label-free quantitative proteomic profiling of vesicular fractions to yeast cells revealed more putative EV proteins than previously reported including more than 55% of previously associated proteins including proteins having catalytic activity in metabolic processes and a wide range of membrane proteins. Further analysis of putative EV proteins by GO highly supports an extracellular role of the proteins. Current, efforts serve to validate the extracellular nature of these proteins within the EV subproteome. *Summary/conclusion*: Unfiltered beer is a rich source of EVs, and our findings indicate a far higher number of EV-associated proteins than previously reported for yeast EVs.

OP2-122

Towards traceable size determination of extracellular vesicles

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Introduction: Characterization of extracellular vesicles (EVs) is challenging because of their small size (30 nm–1 μm), low refractive index

and heterogeneity. Since vesicles are below the detection range of common methods, many new techniques have not been validated yet. Consequently, there is a need for traceable size determination of EVs, meaning that the measurement result can be related to the SI unit ''meter'' through an unbroken chain of comparisons with known uncertainties. *Methods*: Erythrocyte-derived EVs were isolated from erythrocyte concentrates by centrifugation at 18,890 $\times$ g for 0.5 h. The size distribution of the sample was determined using small-angle X-ray scattering (SAXS), size exclusion chromatography coupled with dynamic light scattering, nanoparticle tracking analysis, resistive pulse sensing and electron microscopy. We selected vesicles isolated from erythrocyte concentrates as those offer an excellent opportunity to purify high concentrations of vesicles from a single cellular origin. *Results*: The mode diameters of the size distributions of the studied erythrocyte EVs reported by the different methods are around 130 nm, but the full widths at half maximum of the distributions ranges from 50 to 200 nm. Interestingly, most of the methods indicate vesicles with a diameter below 100 nm, despite the fact that the applied g-force during the isolation is thought to isolate primarily vesicles $>$100 nm. SAXS reveals the presence of plasma proteins that are not associated to the vesicles, and can also characterize the inner structure of EVs. *Summary/conclusion*: The commonly used techniques result slightly different size distributions that also depend on the settings of the given technique. SAXS is a promising technique in respect to traceability. To reach the traceable measurement of EVs, monodisperse and highly concentrated samples are required. This work was funded by EMRP under the MetVes project. The EMRP is jointly funded by the EMRP participating countries within EURAMET and the European Union.

Van Weelde/Mees room
Oral with Poster 3
Chair: *Rienk Nieuwland* 17:45-18:20

OP3-128

Release of microvesicles during haemapheresis correlates with surface roughness of adsorbent polymers

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Introduction: Blood contact with adsorbent polymers requires biocompatible surfaces to minimize activation of coagulation, platelet adhesion and release of thrombogenic microvesicles (MVs). Since data on MV generation in blood purification are scarce, we assessed the influence of adsorbent morphology on MV generation from thrombocyte concentrates. *Methods*: DALI and Liposorber D, which are applied in lipoprotein apheresis, and a non-commercial polymer (DALI-N) were characterized by scanning electron microscopy and atomic force microscopy. To determine MV generation, freshly isolated thrombocytes (3×10^5/μl; total volume 50 mL) were circulated over columns (6 × 1.8 cm; downscaled equivalent to clinical use) packed with the adsorbents at a flow rate of 1.2 ml per min for 2 h. Thrombocytes were quantified in the flow-through every 30 min using a blood cell counter (Sysmex). Flow cytometric analysis was performed with a Gallios Flow Cytometer (Beckman Coulter) after staining of thrombocytes with a CD61 antibody and calibration with fluorescent beads to cover the MV (0.5 and 0.9 μm) and the thrombocyte size ranges (0.9 and 3 μm). *Results*: Thrombocyte passage over DALI-N resulted in significantly higher levels of MVs as compared to DALI (5.7% vs. 4.3% vs. 4% for the control after 120 min, n = 5), while Liposorber and DALI showed comparable MV release (4.4% vs. 4.1%. vs. 3.8% for the untreated control). Thrombocyte adhesion was significantly higher for DALI-N as compared to DALI and Liposorber. The increased MV generation and thrombocyte adhesion correlated with higher surface roughness of DALI-N vs. DALI and Liposorber. In all experiments, the percentage of $CD61^-$ MVs and thrombocytes increased over time as compared to the control, indicating that activated ($CD61^+$) thrombocytes are preferentially bound by the adsorbent. *Summary/conclusion*: MV release correlates with surface roughness and can serve as marker of blood compatibility of adsorbent polymers.

OP3-129

ACE2 mRNA expression in urinary exosome from patients with kidney disease

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Introduction: Angiotensin-converting enzyme-2 (ACE2) plays a critical role in regulation of renin-angiotensin system (RAS) and is important for the development of chronic kidney disease (CKD). ACE2 is highly expressed in tubular epithelial and glomerular cells. Urinary exosomes are microvesicles released by tubular epithelial cells and podocytes containing information of their originated cells. This study aimed to investigate expression of ACE2 mRNA in urinary exosome in different stages of CKD and to explore its correlation with renal function and histological changes. *Methods*: Urine samples were collected from 31 patients with chronic kidney disease (CKD) who underwent kidney biopsy and 7 controls. Urinary exosome was isolated with differential ultracentrifugation, and ACE2 mRNA was detected by real-time RT-PCR on RNA isolated from urinary exosome. *Results*: The pellet microvesicles were positively stained with exosome marker, AQP2. The mRNA level of ACE2 showed no difference in patients with kidney disease compared with controls. However, ACE2 mRNA was differently expressed in different stages of kidney disease ($p = 0.009$ by Kruskal-Wallis test). In CKD patients with eGFR < 90 ml/min/1.73 m2 (n = 18), ACE2 mRNA correlated with eGFR ($r = -0.480$, $p = 0.044$), BUN ($r = 0.527$, $p = 0.025$) and score of glomerulosclerosis ($r = -0.521$, $p = 0.027$). No correlation was found between ACE2 and score of tubulointerstitial fibrosis (TIF). Interestingly, in group of TIF < 30%, ACE2 correlated positively with levels of glomerulosclerosis ($r = 0.668$, $p = 0.007$) while in group of TIF < 30%, ACE2 correlated negatively with glomerulosclerosis ($r = -0.663$, $p = 0.007$). In both groups, no correlation was found between eGFR and glomerulosclerosis. *Summary/conclusion*: ACE2 mRNA could be readily detected in urinary exosome with different levels in different stages of CKD. It might be a novel biomarker for kidney disease reflecting the severity of glomerulosclerosis and renal function.

OP3-130

Uptake of macrophage exosomes by the human placenta

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Introduction: During pregnancy, the placenta acts as the interface between the maternal and foetal circulations. It is well established that the human placenta sheds high levels of extracellular vesicles into the maternal circulation. Both microvesicles and exosomes shed by the placenta can interact with maternal immune cells to alter their activation status. Whether this trafficking is bi-directional has not been established. We propose that exosomes shed by maternal immune cells can also interact with placental tissue. This could have significance for the foeto-placental response to maternal inflammation. *Methods*: The human THP-1 monocytic cell line was transduced with CD63-GFP lentiviral particles to create a stable tracer cell line producing GFP-labelled exosomes. Alternatively, isolated exosomes were stained with PKH-26 fluorescent tracker. Methods were optimized for isolation of exosomes from culture supernatants using a commercial Total Exosome Isolation Reagent (TE reagent; Invitrogen), compared to differential centrifugation. Exosome phenotype was determined using Nanosight Tracking Analysis (NTA) and western blotting for CD63 and calnexin. Uptake of THP-1-derived exosomes by the BeWo trophoblast cell line was measured by flow cytometry and confocal microscopy. *Results*: Isolation using TE reagent resulted in soluble, high-purity, high-yield exosome preparations, positive for CD63 protein and negative for calnexin. NTA demonstrated a size range comparable to exosomes isolated by differential ultracentrifugation, and at a higher yield. Culture supernatants of as low as 1 ml were used in subsequent isolations. Dose-dependent binding and uptake of fluorescent THP-1-derived exosomes by BeWo cells were observed by both confocal microscopy and flow cytometry. *Summary/conclusion*: This study demonstrates for the first time that exosomes derived from immune cells can

interact/enter human placental cells. Ongoing studies are examining the potential impact of this interaction on placental cell function, but this finding reveals a potential novel mechanism by which the foeto-placental unit could respond to maternal inflammation and infection.

OP3-131

Utilize extracellular vesicles as biomarkers of vitreal diseases

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Introduction: We address a potential novel diagnostic for inflammatory uveitides and B-cell lymphoma of the vitreous which often present diagnostic dilemmas. Uveitis ''masquerade syndromes'' include non-malignant diseases and malignancies such as B-cell lymphoma. The yield for diagnostic vitrectomy in masquerade syndrome has ranged from 14.3 to 61.5%. *Methods*: Vitreous specimens and patient records of seven patients were studied under IRB approval. Vitreous biopsies obtained during primary diagnostic vitrectomy were processed with standardized techniques and centrifuged. MCV were pelleted and fixed for analysis. Identification of MCV was performed nanoparticle tracking analysis and demonstration of bi-layered membranous structures by TEM, followed by PCR for detection of disease-specific markers such as VEGF. *Results*: By NTA, all seven specimens contained MCV, with concentrations from $1.27 \times 1{,}010$ to $4.44 \times 1{,}010$ particles/ml. By TEM, MCV primarily of two sizes (50–100 and 100–500 nm) were noted. All seven specimens contained MCV of 50–400 nm of varied shapes (round, oval, cigar-shaped), most with double-membrane surrounding a central or eccentric electron-dense core, with some cases of single membrane thin-walled MCV. Using PCR we show presence of VEGF in vitreal fluid. MCV were found in greatest concentration in the birdshot retinochoroidopathy, multifocal choroiditis-panuveitis and idiopathic intermediate uveitis patients. The lymphoma specimen showed a lower concentration of MCV. *Summary/conclusion*: Analysis of MCV in other biofluids has provided diagnostic or prognostic biomarkers. ''Masquerade syndromes'' are intraocular processes which cause secondary inflammation and often present diagnostic dilemmas. We present a total of seven out of seven uveitis and B-cell lymphoma cases in which NTA and TEM analyses of the vitreous specimens revealed the presence of MCV. MCV analyses of vitreous aspirates offer potential for the detection of diagnostic biomarkers of inflammatory and neoplastic diseases in pauci-cellular specimens such as VEGF mRNA. Further studies are indicated to explore a role for MCV analysis in the diagnosis and/or monitoring of ocular diseases and response to treatment.

OP3-132

Urine microvesicle isolation: effect of storage and THP on RNA yield

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Introduction: Urine-derived microvesicles (MVs) are a potential source of information to determine changes in kidney, bladder and prostate physiological conditions. These changes can affect secretion and cargo composition of MVs. Thus MVs may be a good source of RNA and protein candidate biomarkers for non-invasive urine-based diagnosis. However, urine microvesicles isolation methods vary between different laboratories. To obtain reproducible results, standardization of the isolation method is essential. Here we compare different isolation methods, including pre-handling of urine before isolation. In addition, since the presence of the Tamm-Horsfall protein (THP) in urine results in the formation of large polymeric network that traps vesicles, we determined whether reducing this entrapment increased the yield of MVs. *Methods*: Urine was collected after signed informed consent, and was approved by the local medical ethical committee of the VU University Medical Center. Samples were examined in following conditions: (1) 3 days storage at 4°C, (2) storage of full urine at −80°C and (3) pre-centrifugation before −80°C storage. In addition, for all these conditions the effect of dithiothreitol (DTT) was evaluated. Microvesicles were isolated by differential ultracentrifugation, currently the most widely used method, and were treated with RNaseA. Efficiency of microvesicle isolation was based on expression level of three different miRNAs as measured by RT-qPCR. Isolation by ultracentrifuge was also compared with commercially available Norgen Biotek Urine Exosome RNA Isolation Kit. *Results*: After MV isolation from urine kept for 3 days at 4°C, lower RNA yields were obtained compared to purified MVs after −80°C storage or pre-centrifugation, which were comparable. When DTT was added, the levels of detected miRNAs were increased significantly for each storage method, which may relate to an increase in microvesicles yield due to release from THP. Importantly, the ratios between the 3 miRNAs were comparable between the different urine storage methods, including after addition of DTT. As an easier and faster method of isolating exosomes from urine, we examined commercially available Norgen Biotek Urine Exosome RNA Isolation Kit, which requires low volumes of urine compared to ultracentrifugation. However the efficiency of MVs isolation seemed very low, with nearly undetectable presence of the miRNAs. *Summary/conclusion*: Here we show that storage and removal of THP from urine sample before microvesicles isolation are important for providing high RNA yield for evaluation with RT-PCR. Although the ratios between the miRNAs were comparable, standardization of urine microvesicle isolation may be important in order to obtain inter-laboratory reproducible results.

OP3-133

Regulation of exosome RNA cargo

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Introduction: While tissue specificity of gene expression and regulation has been extensively studied, the effects of genetic differences on intercellular communication are less well characterized. One way cells may communicate is through small extracellular vesicles such as exosomes. We characterized the transcriptomes (miRNA and mRNA) of exosomes in a large family and studied how genetic differences affect exosomal contents, which may in turn alter intercellular communication. *Methods*: Messenger and small RNA were isolated from exosomes and cells of lymphoblastoid cell lines derived from 17 family members across three generations. Exosomes were obtained by ultracentrifugation and 200 nm filtration, and total RNA was isolated using Trizol with Bioanalyzer RIN quality scores of 9–10 subsequently used for cDNA library construction. Transcriptomics data were combined with publicly available genomic information on the 17 individuals to determine whether *cis*-regulated genetic associations with expression levels were replicated between the cell and exosome. We also assessed differential expression of transcripts between cells and exosomes to determine, genome-wide, which mRNA and small RNA species are selectively exported in exosomes. Finally, targeted cells were transfected with exosomes to measure the response of select genes by qPCR. *Results*: We outline the genes

that are differentially expressed between cells and exosomes as well as the functions they are enriched for. We also find that several *cis* associations with gene expression are detected in both cells and exosomes. Finally, we examined the response of certain genes in target cells transfected with exosomes containing miRNAs targeting those genes. *Summary/conclusion*: Learning about the RNA species packaged into exosomes and how their expression levels are influenced by genetic variation will allow us to better understand how exosomes communicate with neighbouring cells. Our study has examined which genes are selectively included or excluded from exosomes, how genetic variation affects exosomal transcript levels, and how transcripts packaged in exosomes exert epigenetic changes in target cells. This work offers a way of considering exosome contents in the context of genetic variability.

OP3-134

Proteomic profiling of outer membrane vesicles derived from uropathogenic *Escherichia coli* strain 536

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Introduction: In humans the urinary tract is the one of the most common sites for bacterial infection, where uropathogenic *Escherichia coli* (UPEC) are responsible for 90% of cases. Outer membrane vesicles (OMVs) that are released from bacteria into the extracellular milieu have important roles in bacterial survival and pathogenesis. Environmental conditions are known to affect the release of OMVs, but the mechanisms involved and pathological relevance are unclear. *Methods*: UPEC strain 536 was cultured overnight at 37°C in RPMI 1640 ± 10 mM $FeCl_3$ to mimic the environment during initiation and progression of an infection. OMVs were isolated from culture supernatants by ultracentrifugation. The effect of iron restriction on the protein composition of UPEC bacteria and their OMVs was evaluated by SDS-PAGE and liquid chromatography-tandem mass spectrometry. *Results*: The UPEC OMVs were enriched for virulence factors, for example, haemolysin, and outer membrane proteins. OMVs from UPEC grown in iron-restricted conditions showed more individual protein species and were rich in iron repressible outer membrane proteins, for example, several siderophore receptors (including FepA, FhuA and FyuA) and the hemin receptor. The comparison of the protein profiles from OMVs and whole bacterial cells now provides evidence to identify those proteins selectively packaged into OMVS. *Summary/conclusion*: This present study provided a global proteome profile of UPEC and their OMVs, and compared the effect of iron restriction on UPEC OMVs. The identification of selectively packaged (or excluded) proteins in OMVs now provides a basis to investigate the mechanisms controlled selective packaging and the roles of OMVs in the progression of bacterial infection.

JEV Editorial Meeting — Schadee Room — 18:30-19:30

Poster Presentations

Arcadis Room
Poster Session 1A - EV in the tumor micro-environment
Chair: *Clark C. Chen and Janusz Rak* 13:00-14:00

P1A-018

Role of tumour-derived exosomes in growth and metastasis of cancers

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Introduction: Cancer burdens and death are largely due to metastasis in several cancer types, including lung and breast. The pervasive mortality from metastasis highlights the shortcomings of traditionally accepted hypotheses on the metastatic process. Exosomes are biological nanovesicles (30–100 nm) of endocytic origin that mediate local and horizontal transfer of information. Studies indicate tumour-derived exosomes (TDEs) as critical mediators of tumourigenesis and metastasis. Here, we examine the role of TDEs in lung and breast cancer metastasis. *Methods*: Non-metastatic lung (H522) and breast cancer (T47D) cells were treated with fluorescent dye (PKH67) labelled exosomes from metastatic lung (H1299 and A549) and breast (MDA-MB-231) cancer cells, respectively. Changes in growth rate, migratory and invasive behaviour were assessed. EMT-associated proteins were analysed by western blot. Metastasis-associated miRNAs were analysed by RT-PCR. *Results*: Our findings suggested: (a) TDEs had a mean size of 92 nm and carried exosomal marker proteins CD63 and CD81, (b) uptake of exosomes from highly metastatic cancer cells by non-metastatic recipient cells, (c) faster wound healing rate, increased migratory behaviour, and invasiveness in recipient cells, (d) increased endothelial cell tube formation, reflective of angiogenesis ability of TDEs, (e) modulation in expression levels of key EMT-associated in non-metastatic cells treated with TDEs favouring EMT shift, (f) difference in metastasis-associated miRNAs levels in exosomes from invasive and non-invasive cancer cells. *Summary/conclusion*: Exosomes from metastatic cancer cells carry the cargo essential for EMT and have the ability to change phenotypic traits of the recipient cells. Understanding the role of TDEs in cellular process would enable better management of cancer metastasis and will have great potential to uncover new targets and lead to new therapies for metastatic disease.

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P1A-019

Role of miR-126 shuttled by exosomes in the crosstalk between chronic myelogenus leukaemia and endothelial cells

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Introduction: Exosomes are nanovesicles that mediate intercellular communications and modulate tumour microenvironment. Our previous work showed that exosomes released by myeloid chronic leukaemia cells, LAMA84, stimulate angiogenesis, in vitro and in vivo. Because exosomes are involved in horizontal transfer of information through the export of miRNAs, we focused on the role of miRNAs on crosstalk between CML cells and endothelium. *Methods*: Exosomes were collected by LAMA84-conditioned medium by ultracentrifugation. miRNAs profiling was performed by miRNA-array and RT-PCR. HUVECs, transfected or not with miR-126 mimic or inhibitor, were treated with exosomes. CXCL12 and VCAM1 mRNA were assessed by RT-PCR. CXCL12 secretion was evaluated by ELISA. Biological effects of miR-126 shuttled by exosomes in HUVECs were evaluated by motility and adhesion assays. *Results*: Exosomes released by LAMA84 transport microRNAs. Among of 124 miRNAs identified in LAMA84-Exo, we focused our attention on miR-126. This miRNA was upregulated in exosomes respect to cells and targets CXCL12 and VCAM1. We transfected LAMA84 with labelled miR-126-Cy3 and leukaemia cells were co-cultured with HUVECs. miR-126-Cy3 was shuttled to endothelial cells. The treatment of LAMA84 cells with GW4869 blocked this transport. As the treatment of HUVEC with LAMA84-exo for 24 h reduced CXCL-12 and VCAM-1 expression, we ascribed these effects to exosomal miR-126 internalized by endothelial cells. By luciferase activity assay, we confirmed that exosomal miR-126 targets CXCL-12 and VCAM-1 3'UTR mRNA in HUVECs. Moreover, we observed that reduced levels of CXCL-12 and VCAM1 affect negatively LAMA84 motility and cells adhesion. MiR-126 inhibitor reverted the decrease of CXCL12 and restored the motility and adhesion of LAMA84. Over-expression of miR-126, with miR-126 mimic, showed opposite effects. *Summary/conclusion*: Our data suggest that CML-exosomes facilitate mobilization of leukemic blast from bone marrow and their diffusion in the bloodstream.

P1A-020

Lung cancer-derived extracellular vesicles promote cancer progression by triggering oncogenic signals and increasing vascular permeability in an autocrine/paracrine fashion

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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 largely unknown. In this study, we tried to find out the biological functions of EVs in lung cancer and evaluate the clinical applications. *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, Nanosight, and western blotting. 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 both these two methods with different capacity revealed by EM and Nanosight. Specific EV markers including Alix, CD63 and, Tsg101 were detected

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in the isolated EVs. The EVs carried various RNA species that small RNAs seemed to be enriched. Furthermore, the EVs could be uptaken by lung cancer cells and trigger oncogenic signals such as Stat3 and Akt. Previously, we have shown that IL-6/Stat3/VEGF pathway plays an important role in lung cancer angiogenesis and metastasis. Here, we showed that EVs from lung cancer samples carried high level of VEGF and triggered vascular permeability changes in mice. *Summary/conclusion*: Using these methods, we isolated EVs not only from culture supernatants but also various lung cancer-associated clinical samples. Furthermore, the EVs could promote cancer progression by triggering oncogenic signals and increasing 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.

P1A-021

Crosstalk between chronic myelogenous leukaemia and bone marrow stromal cells: role of exosomes in the il8-dependent signalling mediated by egfr activation

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Introduction: Chronic myelogenous leukaemia (CML) is a myeloproliferative disorder characterized by Bcr-Abl oncoprotein with a constitutive tyr-kinase activity. Exosomes (exo) shed by cancer cells potentially affect tumour–stroma interaction through the establishment of a bi-directional crosstalk. Interleukin-8 (IL8) is a pro-inflammatory chemokine that regulate proliferation and survival of cancer cells. We previously demonstrated that CML-derived exo modulate bone marrow microenvironment through the IL8 secretion from stromal cells. EGFR, as well as IL8, regulate cell proliferation and survival; it has been recently demonstrated that EGFR ligands can signal via exosomes shed by cancer cells. We hypothesized that the effects induced by IL8 are EGFR mediated and exosomes are involved in this pathway. *Methods*: Human cell lines used are LAMA84 (CML cells) and HS5 (stromal cells); gene expression analysis was performed by RT-PCR and western blot with antibodies for EGFR, pEGFR and AREG. For in vivo experiments, LAMA84 cells were inoculated in NOD/SCID mice and treated with IL8, vehicle (PBS), SB (IL8 receptors inhibitor) or IL8 + SB. After 50 days, tumours were removed to calculate their weights and RNA was extracted from biopsies. *Results*: On the basis of the in vitro effects of IL8 on LAMA84 cells, we studied the in vivo role of this cytokine. Mice treated with IL8 develop larger tumours than control groups; co-treatment with SB resulted in a slower tumour growth compared with mice treated with IL8 alone. The expression of pro- and anti-apoptotic genes confirmed a pro-survival role of IL8 in vivo. LAMA84 cells and their exo showed different EGFR ligands. Exo treatment of stromal cells increases EGFR expression possibly inducing activation of survival pathways mediated by IL8. *Summary/conclusion*: Our data show that IL8 promotes tumour growth and survival in vivo. Exo, carrying EGFR ligands, modulates bone marrow microenvironment through activation of EGFR signalling on stromal cells, demonstrating a new extracrine signalling mediated by EGFR ligands.

P1A-022

Role of exosomes released by colon cancer stem cells in the modulation of tumour microenvironment

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Introduction: Colon carcinoma is characterized by a heterogenic pool of cells with distinct differentiation patterns. It was reported that a population of undifferentiated cells from the primary tumour, called cancer stem cells, can reconstitute the original tumour on xenotransplantation. Colon cancer stem cells (CCSCs) are able to initiate and sustain tumour growth. Recently it was reported that renal cancer stem cells released exosomes that stimulate angiogenesis and pre-metastatic niche formation. Exosomes are nanosize vesicles derived from endosomal compartment released in extracellular space. Exosomes contain proteins, mRNA, and microRNAs and function as mediators in cell-to-cell communication. We characterized vesicles released by CCSCs and investigated on their involvement in angiogenesis and in pre-metastatic niche formation. *Methods*: Exosomes were collected from condition medium of CCSCs grown as sphere (Sphere) and SDAC (sphere-derived adherent cells). Vesicles were characterized by nanoparticles tracking analyses (NTA), western blotting and enzymatic assay. HUVECs were treated for 6 h with exosomes, gene expression was analysed with RT-PCR and protein secretion was evaluate by ELISA. *Results*: CCSC released vesicles that enriched in Alix and acetylcholinesterase. NTA showed that the peaks of particle size were approximately 100 nm for Sphere and 60 nm for SDAC, according with the expected size of exosomes. CCSCs–exosomes were internalized by endothelial cells, and they modulated IL8, IL6, VCAM1 and CXCL12 mRNA expression. Exosomes induced CCSC adhesion to endothelial cells pre-treated with CCSCs–Exo. In vitro and in vivo angiogenesis assay showed that exosomes induced neovascularization. *Summary/conclusion*: CCSCs–Exo are involved in modulation of tumour microenvironment, in particular they induced an angiogenic phenotype in endothelial cells. Work is in progress to investigate if CCSCs–Exo are able to modulate the pre-metastatic niche formation.

P1A-023

Modification of protein and microRNA profile in extracellular microvesicles by UPR in CML cells

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Introduction: There is a growing need to determine the role of the microenvironment in regulation of growth, self-renewal and drug resistance of leukaemic cells in the bone marrow, as it seems that the communication of leukaemic progenitor and stroma cells might play an important role in the cancer progression. Secretome, which bypass the information and regulatory signals by the usage of proteins and microRNA, plays a crucial role in this crosstalk. Oncogene activity leads to deregulation of various signalling pathways. Our recent data showed that BCR-ABL1 activity in CML cells leads to activation of the unfolded protein response (UPR) (Kusio-Kobialka *Cell Cycle* 2012). This results in rearrangement of mRNA translation, thus allowing for adjustment of cellular proteome and adaptation of cancer cells to stress conditions. The aim of this study was to verify if composition of the microvesicles secreted by CML cells might be modified respectively to the activation of UPR and how it influences MVs properties. *Methods*: Using mass spectrometry analysis, we compared which proteins are present in mouse 32D cells-parental and expressing BCR-ABL1 as well as in human K562 CML cell line. Profile of microRNAs secreted in microvesicles by K562 cells was determined using microarrays and confirmed by real-time PCR analysis. The influence of isolated microvesicles either from bone marrow stroma fibroblasts or CML cells conditioned medium on proliferation and apoptosis of targeted cells was assayed using multiparameter flow cytometry. *Results*: Bioinformatic analysis indicated that most of the proteins identified in the CML microvesicles are known to play role in the cell movement, modification of extracellular matrix and intercellular signal transduction. We experimentally confirmed that presence of CML microvesicles facilitates cells motility and invasiveness in matrigel invasion assay. Furthermore,

we found modified microRNA profile in MVs and decreased invasive potential driven by the exosomes. *Summary/conclusion*: Altogether our results indicate that secreted microvesicles may play a crucial role in the stroma-leukemic cells crosstalk and could have significant impact on the cancer progression.

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P1A-024=OP1-104

Human mast cell exosomes regulate lung cancer cell cycle: possible role of c-kit transfer

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P1A-025

Free light chain mediated extracellular vesicles processing in multiple myeloma

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Introduction: Multiple myeloma (MM) is a haematological malignancy caused by a microenviromentally aided persistence of plasma cells in the bone marrow. Monoclonal plasma cells often secrete high amounts of immunoglobulin free light chains (FLCs) that could induce tissue damage. Recently we showed that FLCs are internalized in endothelial (Hvec) and myocardial (H9C2) cell lines and secreted in extracellular vesicles (EVs). In MM patients we observed an increase of EV production and the EVs were strongly positive for FLCs, annexin V and c-src compared with MGUS and control patients. Our data suggest that c-src positive EVs are involved in MM pathogenesis. Therefore we wanted to investigate the effect of MM serum EVs on proliferation and survival of Hvec and H9C2 cells. Furthermore we characterized the role of FLCs in EV generation and uptake. *Methods*: Serum samples from MM, monoclonal gammopathy of undetermined significance (MGUS) and healthy patients were collected and stored at -80°C. Afterward we isolated and characterized serum EVs with ultracentrifugation and sucrose density gradient protocols. The EV population was analysed with protein quantification, western blot, acetylcholinesterase assay, SEM and atomic force microscopy (AFM). To evaluate their effect on endothelial and myocardial cells, we labelled EVs with PKH67 (Green Fluorescent Cell Linker Kit, Sigma). After labelling, EVs were incubated or not with a polyclonal anti-FLCs antibody (the binding site) and subsequently added in the serum-free culture medium. To stop the lysosomal processing, cells were previously treated or not with NH_4Cl. *Results*: Our results clearly show that only MM-derived EVs are rapidly internalized in endothelial and myocardial cells. Indeed MM EVs induce an increase of the cellular proliferation rate. Intriguingly Hvec and H9C2 cells incubation with anti-FLCs antibody pre-treated EVs reduced their uptake and release ($p < 0.001$). *Summary/conclusion*: C-src-enriched MM EV uptake is FLCs mediated in a lysosomal-independent pathway pointing out a new potential therapeutic target.

P1A-026

Exosomal transfer of microRNAs and their variants from ovarian cancer-associated omental adipose tissues to ovarian cancer cells

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Introduction: Most ovarian cancers are diagnosed at an advanced stage when the tumour is widely metastatic. The 5-year survival drops to 50% for the cancer cases that spread beyond the pelvis to the omentum. However, the mechanisms underlying the effect of omental adipose tissue (OMT) on ovarian cancer progression are poorly understood. Recent studies showed that exosomes also contain non-coding RNAs such as microRNAs (miRNAs). Thus, we hypothesize that the transfer of miRNAs and their variants from ovarian cancer-associated OMTs to ovarian cancer cells via exosomes may contribute to the nearby microenvironment for ovarian cancer metastasis and cancer progression. *Methods*: Ion Torrent next generation sequencing was performed on miRNAs isolated and enriched from exosomes and cell lysates of ovarian cancer cell lines (OVCA), the epithelial component of microdissected omental ovarian cancer tissues (CT), normal omental adipose tissues (OMN) and ovarian cancer-associated omental adipose tissues (OMT). Besides, exosomes isolated from OMTs were incubated with ovarian cancer cells to examine the transfer of exosomal miRNAs from OMTs to ovarian cancer cells. *Results*: By integrating the miRNA expression profiles, 65 miRNAs were expressed at significant higher levels in OMT-derived exosomes compared with those in OMN-derived exosomes and OVCA-derived exosomes. A set of miRNAs (miR-32a, miR-221, miR320a and miR-421), which had been implicated in controlling cell growth and chemoresistance, was identified. Also, the Ion Torrent results were validated and exosomal transfer of OMT-derived miRNAs was confirmed in vitro. *Summary/conclusion*: The exosomal communication between adipose tissues and ovarian cancer cells in the omental tumour microenvironment is verified. The transferable miRNAs and their variants may remain functional in the recipient ovarian cancer cells and confer more aggressive phenotypes in these cells.

P1A-027

Comparison of two populations of extracellular vesicles in glioma and benign human endothelial cells

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Introduction: Tumour cells shed different populations of extracellular vesicles (EVs) that vary in biogenesis, size and content. They range from 30–100 nm (exosomes) to 1–10 um (large oncosomes). Large oncosomes have been reported as products of fast migrating amoeboid prostate cancer cells and other types of tumour cell with varying aggressiveness (Di Vizio et al., *Cancer Res* 2009, Di Vizio et al., *Am J Pathol* 2012). Given their atypical size, large oncosomes can accommodate greater amounts of tumour-derived macromolecules than smaller populations of EVs (Morello et al., *Cell Cycle* 2013), and we sought to quantify levels of the oncoprotein EGFRvIII in exosomes and large oncosomes released by U87 glioblastoma cells in an attempt to improve the signal-to-noise ratio of circulating biomarkers of glioma (Skog, *Nat Cell Biol*, 2008; Al-Nedawi, *Nat Cell Biol* 2008). *Methods*: The two EV populations were purified from glioma U87 cells and HUVECs and were compared using differential centrifugation, nanoparticle tracking analysis (NTA), cryoEM, immunofluorescence (IF), qRT-PCR and μNMR (nuclear magnetic resonance) (Shao et al. *Nat Med*, 2012). We also implanted subcutaneous tumours expressing the EGFRvIII oncogene into nude mice and purified small EVs and large oncosomes from the serum of these mice. *Results*: Both cell lines released more exosomes than large oncosomes, and the number of large oncosomes recovered was significantly higher in U87 cells than HUVECs, in line with previous results in different organ systems in which the detection of large oncosomes shed from benign cells was negligible. A quantitative comparison of the content of EGFRvIII between the two EV populations demonstrated a higher number of copies of EGFRvIII in large oncosomes than in small EVs. This result was reproduced in vivo following subcutaneous injection of U87-EGFRvIII cells in nude mice. Strikingly, EGFRvIII RNA and protein load, quantified by qRT-PCR and μNMR, respectively, was significantly higher in

circulating large oncosomes than in smaller EVs. *Summary/conclusion*: Here we report for the first time that large oncosome shedding is significantly increased in glioma cells, while a population of smaller EVs containing exosomes is released at similar rates from glioma and benign human endothelial cells. Large oncosomes are enriched in tumour-derived molecules, and our data provide a strong rationale for developing clinically applicable platforms that allow their analysis along with other classes of EVs. Large oncosome isolation does not require ultracentrifugation and thus may represent an advantage over exosomes as a clinical indicator of disease status. Furthermore, because of their atypically large size, they may allow single particle profiling in the circulation of patients with solid tumours.

P1A-028

Intercellular transfer of miR-122 and a reciprocal anti-miR-122 signal control human hepatic cell growth

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Introduction: miRNAs are 20–22 nt long post-transcriptional regulators of gene expression in metazoan cells. miRNAs follow a tissue- and cell- specific expression pattern, and aberrations in this pattern are associated with various diseases. It is not clear how the various cells in a tissue communicate with each other to maintain homeostasis of miRNA expression. *Methods*: miR-122 is a liver-expressed miRNA known to be downregulated in hepatocellular carcinoma (HCC) cells. To determine the importance of crosstalk between liver cells in the homeostasis of miRNA expression, we used human hepatoma cells HepG2 and Huh7 for our study. Despite its hepatic origin, HepG2 does not express miR-122, whereas Huh7 expresses this miRNA. *Results*: We found that miR-122 is transferred by extracellular vesicles between the human hepatic cells Huh7 and HepG2. Exosomal miR-122 from Huh7 cells causes repression of various target genes, and reduces cellular proliferation in miR-122-depleted recipient HepG2 cells. Interestingly, in a reciprocal process, HepG2 cells secrete Insulin-like growth factor-I (IGF1) which causes increased mTOR phosphorylation in co-cultured Huh7 cells and increases the cell growth and the proliferation rate of the cells. The affected Huh7 cells also show decreased miR-122 expression and reduced levels of endogenous miR-122. *Summary/conclusion*: In this study we have established a model in which cells having different miRNA profiles when co-cultured together can reciprocally regulate each others' miRNA levels using an exosomal way of intercellular communication and thereby affects proliferation and senescence status of both cells.

Arcadis Room
Poster Session 1B - Novel developments in characterization of EV
Chair: *Jennifer Jones and Yuana Yuana* 13:00-14:00

P1B-029

Measurement of unstained microvesicles by flow cytometry

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Introduction: Measurement of cell-derived microvesicles (MVs) by flow cytometry can provide information on the number and size of MVs, as well as the amount of a specific antigen on individual vesicles. However, the laser wavelength, which is in the size range of MVs, challenges the sensitivity limits of flow cytometry. In order to overcome the problem of a dim signal and to increase the specificity of MV measurement, the staining with annexin V or antibodies are often used. Our aim was to achieve quick, easy and cost-efficient MV measurement; we therefore decided to measure unstained MVs by flow cytometry using a high-power laser. *Methods*: MVs from peripheral blood were isolated by differential centrifugation and washing, and counted by flow cytometry. Unstained polystyrene microspheres with known size were used as size references. Flow cytometric data acquisition and analysis were performed by the Altra flow cytometer (Beckman Coulter) with a high-power 488 nm laser (200 mW, water-cooled). Forward and side scatter (FS/SS) parameters were set at logarithmic gain; FS Discriminator was set to minimum. *Results*: Our results indicate that particles larger than 500 nm are clearly detectable, while particles between 300 and 500 nm can only be partially detectable by our equipment. Although we can detect only a part of the entire MV population (larger MVs), this part of population is also clinically relevant, as we have recently shown by measuring the amount of MVs in blood samples from patients with gastrointestinal stromal tumour. *Summary/conclusion*: Measurement of unstained MVs by flow cytometry, although this is subject to limitations, can provide clinically relevant information. Because it is fast, easy and cheap, it is therefore the ideal technique for high-throughput purposes in a clinical setting.

P1B-030

Large inter-individual variation in the fraction of small-size vs. large-size platelet-derived microparticles measured by flow cytometry

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Introduction: Flow cytometry is a commonly used method for detection of cell-derived microparticles (MPs). A frequent limitation of this method is that many flow cytometers are unable to detect particles below the size of 0.5 μm. The aim was to establish a method to detect platelet-derived MPs (PMPs) below the size of 0.5 μm using a sensitive new generation digital flow cytometer, and to compare the fraction of PMP below 0.5 μm (small-size particles) and above 0.5 μm (large-size particles) in an in vitro experimental set-up on plasma from healthy donors. *Methods*: Blood samples were obtained from 11 healthy donors after obtaining informed consent and after approval of the study protocol from the local ethics committee. Platelet-rich plasma (PRP) and platelet-free plasma (PFP) were subjected to 30 min incubations without or with oxidized low-density lipoprotein (oxLDL) or native LDL (nLDL). PFP isolated from this set-up was analysed by flow cytometry after labelling of PMPs with FITC-conjugated lactadherin and APC-conjugated anti-CD41 antibody, and a MP-gate was established using a blend of fluorescent calibrated beads (0.2, 0.5, 0.9 and 3.0 μm). Total PMP releases during incubations were enumerated. Small-size particles were separated from large-size particles using the size distribution of fluorescent beads in a forward (FSC-H) and side scatter (SSC-H) setting. The lower limit of the small-size MP gate was set to include the 0.2 μm beads, and the upper limit of the large-size MP gate was established just above the size distribution of the 0.9 μm beads. The 0.5 μm bead size distribution defined the upper and lower limits, respectively, of the two MP gates. *Results*: Total PMP number was increased by fourfold after 30 min incubation of PRP at room temperature ($p < 0.05$), whereas the fraction of small-size vs. large-size PMPs was unaffected ($p = 0.2$). Addition of oxLDL in increasing concentrations to PRP induced no significant release of PMPs ($p = 0.958$), whereas a trend was seen towards increasing PMP number with increasing concentrations of nLDL. Small-size vs. large-size PMP fractions were unaffected by oxLDL or nLDL. *Summary/conclusion*: The fraction of small-size vs. large-size PMPs seems to be stable within an individual, whereas there is a large variation between individuals. Addition of oxLDL to PRP does not seem to induce release of PMPs.

P1B-031

Characterization and expression of markers on erythrocyte-derived microvesicles, either naturally released or stimulated with normal human serum

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Introduction: The detection and profiling of markers on erythrocyte-derived microvesicles (eMVs) is important in the context of developing a potential tool for early diagnosis and monitoring of haematological disease progression. For eMVs to be a useful tool in clinical investigation, the expression of markers must be elucidated. *Methods*: Erythrocytes isolated from fresh venous blood by ficoll-Paque™ and centrifugation; eMVs were formed in two ways: (1) stimulated − erythrocyte pellet was incubated for 45 min at 37°C with RPMI 40, and $CaCl_2$ and (2) as (1) plus normal human serum. eMVs were isolated −25,000 ×g for 90 min, then labelled with a panel of markers, including CD235ab, annexin-V and CD41(-ve). eMVs were confirmed and quantified using guava easyCyte™ flow cytometry, and SDS-PAGE with SYPRO®Ruby protein gel stain. *Results*: Fluorescence-based methodology allows an estimation of eMVs numbers/levels and sample purity (>93% for (+ve) control and <6% for (−ve) control in both populations of eMVs). In addition, both populations of eMVs expressed 50 kDA band (glycoprotein A/integrin-associated protein) via SDS-PAGE. *Summary/conclusion*: Sample purity and fluorescence-based detection and characterization of eMVs is a step closer towards profiling eMVs as a diagnostic tool. This work forms part of ongoing research into the characterization and expression of different markers by eMVs to develop reference profiles.

P1B-032

Biophysical characterization of extracellular vesicles with fluorescence fluctuation spectroscopy

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Introduction: Cells secrete extracellular vesicles (EVs) into their microenvironment and ultimately into biological fluids. A major difficulty in the field is the development, improvement and standardization of technics allowing their characterization. Electron microscopy, flow cytometry, nanoparticle tracking analysis and dynamic light scattering represent most of the actual tools available. Here we used fluorescence fluctuation spectroscopy (FFS) to characterize the physical and biological properties of EVs secreted by platelets (PLT) and erythrocytes (ERY). *Methods*: This technique analyses the fluctuations of fluorescence signal originated from the translocation of fluorescently labelled EVs through an open diffraction-limited observation volume. Fluctuation analysis was here performed both in time and amplitude, giving access to diffusion times, mean particle occupancy and brightness which are parameters linked to size, concentration and labelling density, respectively. Labelling was achieved through the use of amino-reactive dyes, which are non-specific, and fluorescent antibodies against CD63 membrane proteins (anti-CD63), which are recognized as markers of exosomes. *Results*: Using this procedure, we have observed that ERY secreted a homogeneous population of vesicles, whereas PLT EVs were heterogeneous, corroborating electron microscopy data. In addition, the population of PLT EVs positively stained with anti-CD63 exhibited faster diffusion times compared to unspecifically labelled ones. This subset of EVs was estimated to have a diameter smaller than 100 nm, confirming previous findings that CD63 positive vesicles are mainly exosomes. *Summary/conclusion*: FFS is an elegant method for EVs characterization as it requires small sample volumes and enables fast data acquisition. More importantly, this method can analyse EVs with single-molecule sensitivity. Consequently, size and protein expression level of EVs are not critical parameters for this technique, overcoming the limitations of classical techniques. Finally, this method enables to discriminate heterogeneities within a single EVs population.

P1B-033

Grating-coupled interferometry as a novel tool to characterize the surface adhesion of extracellular vesicles

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Introduction: The aim of the study was to demonstrate the applicability of a novel label-free evanescent-field based optical technique to investigate the adhesion of extracellular vesicles (EVs) onto different surfaces. In this study 100–1,000 nm EVs (microvesicles, MVs) were examined. *Methods*: MVs secreted by the CCRF-CEM T-cell line (ECACC) were isolated using differential centrifugation and gravity-driven filtration. The quality of the MV samples was controlled by transmission electron microscopy, and the MV size distribution was determined by scanning ion occlusion sensing (qNano). Adhesion of the isolated MVs was monitored using grating-coupled interferometry (GCI). The strength and the kinetics of the binding of the MVs onto fibronectin-, poly-L-lysine- and collagen-coated surfaces were determined. *Results*: In this work we demonstrated the applicability of the GCI technique in the field of EV research. Our results indicate that MVs can irreversibly bind to poly-L-lysine and fibronectin. In contrast, MVs did not adsorb onto type-I collagen-coated surface irreversibly. The adsorption curves showed that the saturation level was reached in less than 5 min, suggesting strong interactions between the MVs and extracellular matrix proteins. *Summary/conclusion*: Our results demonstrate for the first time the suitability of the label-free optical biosensor GCI for the assessment of surface molecular interaction of EVs. Due to a lack of moving parts and assembly of simple and inexpensive components, the GCI technology could provide new opportunities to monitor EVs as diagnostic biomarkers in various diseases.

P1B-034

Real-time analysis of microvesiculation using a Quartz Crystal Microbalance

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Introduction: Characterization of microvesicles (MVs) is essential for understanding their mechanisms of action and biological importance. Stimulated MVs (sMVs) are released through the activation of cells by a multitude of factors. We aimed to use a quartz crystal microbalance (QCM) or piezoelectric quartz resonator able to determine small mass changes, to monitor MV release and to determine MV mass. *Methods*: A QCM (Q-Sence E1) was used to analyse MV release from THP-1 leukaemic promonocytes. The cells in RPMI and 2 mM Ca^{2+} were applied to the QCM to establish a steady baseline. The sample on the sensor was stimulated to microvesiculate with 10% exosome- and MV-free normal human serum. The QCM was then able to monitor sample density and fluid rigidity. Over the same time frame, the level of apoptosis of cells releasing MVs was assessed by staining with annexin V and 7-aminoactinomycin D (Guava Nexin Reagent). Using the QCM we were also able to measure MV mass directly by measuring their ability to quench the oscillating momentum of the QCM. *Results*: Using the QCM, we were able to monitor deposition of cells on the crystal and then sMV release from cells, in the absence of any labelling or fluorescent probe, by measuring cell mass change. Cells (10^5) were deposited onto the QCM electrodes, and the frequency decreases over the first 1000s indicating attachment. The cells were then stimulated with 10% EV-free NHS in RPMI and Ca^{2+} (2 mM) or, as a control, with heat inactivated NHS. During the ensuing 6.5 min, the resonate frequency remained stable. Then, over the following 10 min there was a 30 Hz increase indicating a loss in mass, consistent with the high rate of sMV observed. Given the crystal constant, C as 17.7, $\triangle$f as 19 Hz and v (the third overtone) as 3, and with the crystal area at 0.2 cm^2, using the Sauerbrey equation we calculated the mass loss to be 23 ng which corresponded to 0.25 pg per MV given that 0.92×10^5 MVs were released. The 16 min period over which MVs continue to be released as determined on the QCM coincides with the MV increase measured by FACS and with an increase in early apoptosis from 4% plateauing at 10%, levels of late apoptosis remaining at 1–3%. We also looked at deposition of sMV on the sensor. Given a Δf of 271 ± 7 Hz for the deposition of 1.3×10^6 sMVs, we estimate the mass of an sMV by this approach as 0.241 ± 0.006 pg. *Summary/conclusion*: Using the QCM we were able to measure a significant change in cellular mass, beginning at 6.5 min post-stimulus and peaking at 1000 s post-stimulus. The QCM also detected a decrease in media fluidity, attributed to the process of membrane blebbing on THP-1 and MV release. The QCM was able to provide an accurate measurement of sMV mass (0.25 pg) by calculating the loss in mass of the stimulated cells. By measuring the quenching of the oscillating momentum on the QCM as sMVs are deposited on the sensor, we were also able to calculate the mass of an sMV as 0.24 pg.

P1B-0360

Proteomics driven design of EV Array

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Introduction: Functional analysis of biological classes of extracellular microvesicles (EVs) necessitates efficient means for specific detection and quantification. The quantity and molecular composition of

exosomes shed from different cell types differ considerably. The ''barcode'' of protein profiles, lipid composition and post-translational modifications (PTMs) from circulating classes of microvesicles are important features for surface recognition of circulating microparticles to target tissues. The EV Array is a well-functioning high-throughput method for exosome characterization and phenotyping using only minute amounts of starting material such as blood, plasma and conditioned cell culturing media. *Methods*: Crude MC and exosomes of different biological origins including plasma from human donors, stable-isotope labelled cancer cell cultures and synovial fluid from rheumatoid arthritis patients. Purified MVs were validated by nanoparticle tracking analysis (NTA), electron microscopy (EM) and atomic force microscope (AFM). The EV subproteome were characterized and differentiated using quantitative mass spectrometry followed by functional annotation of the identified proteins and PTMs. *Results*: Analysis of crude MVs and enriched exosomes for design of multiplexed platform of protein microarray were carried out. For validation of the surface-exposed subproteome, we have optimized a dual label strategy based on chemical stable-isotope labelling combined with data-independent tandem mass spectrometry for characterization. Proteome profiles and PTMs specific for subgroups of biospecimens were target for selection of markers for the EV array in combination with literature-based markers. *Summary/conclusion*: An increased panel of putative markers are being investigated for optimization of the antibody-based EV array.

P1B-0361

A new analysis tool for profiling proteins of individual extracellular vesicles

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Introduction: Extracellular vesicles (EVs) including exosomes have drawn considerable attention in the field of cell biology and medicine. Due to the heterogeneity of EVs, surface protein profiling using antibodies is the most desirable approach for characterizing EVs. However, it is difficult to obtain profile of EVs using conventional flow cytometry because the number of fluorescence-labelled antibodies attached on the surface of exosomes of several tens of nanometre in diameter is so small that low S/N ratio and photobleaching are serious problems. Here, we will present the development of a new analysis tool for profiling surface proteins of individual EVs employing the principle of on-chip particle immunoelectrophoresis. *Methods*: Particle immunoelectrophoresis is an immunoassay method that can provide both qualitative and quantitative information on surface proteins/oligoproteins of EVs based on particle electrophoresis using a microcapillary chip and immunoreaction. We have demonstrated usefulness of this new method by some feasibility studies. For example, EV samples isolated from culture medium of human breast cancer Sk-Br-3 cell line (high Her2 gene expression) by differential centrifugation were incubated with either anti-human Her2 antibody or IgG (control experiment) for 30 min at 37°C. In electrophoresis experiments, the velocity of migrating exosomes was automatically analysed using a custom-developed particle tracking velocity (PTV) software. Zeta potentials of exosomes were deduced from the measured electrophoretic mobility (EPM) using the Henry equation. *Results*: It was revealed that zeta-potential distribution of EVs after reaction with anti-Her2 antibody showed positive shifts compared to that of with IgG. The degree of the shift is related to the electrical charge and number of antibody molecules. Thus, the particle immunoelectrophoresis enables researchers to analyse surface protein profile of individual exosomes. *Summary/conclusion*: To make our immuno-analysis technique available to many users, we are developing a prototype of the ''exosome immuno-profiler''. It comprises a microcapillary electrophoresis chip with multiple microchannels, a dark-field microscope imaging system and an automated analysis software. The exosome immuno-profiler is expected to contribute the ever-growing exosome research.

P1B-0362

Size and charge fingerprints of exosomes by particle tracking analysis

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Introduction: Particle tracking analysis (PTA) provides the scientist with information about size, concentration and zeta potential of low concentrated exosome samples. Even more physical/chemical information about the sample can be gathered by the variation of pH and ionic strength resulting in discrete charge fingerprints. *Methods*: PTA is highly favourable when analysing low concentrated samples where single particles of the sample are visualized and traced. By analysis of several thousands of single particle traces, the particle size distribution is calculated from Brownian motion using the Einstein-Smoluchowski equation. The zeta potential of single particles is determined by microelectrophoresis. The physical–chemical parameter zeta potential has long been known as measure for stability and particle–particle interactions. *Results*: In general, the zeta potential of blood cell-derived exosomes and HEK cell line exosomes is affected by pH and ionic strength of the buffer, depending on sample composition. By variation of buffer composition, even more information about the sample can be gathered: Distributions of particle size and zeta potential are visualized as function of buffer concentration (i.e. conductivity) resulting in discrete charge fingerprints of the sample. *Summary/conclusion*: We utilize the advantages of PTA with emphasis on practical aspects relevant for exosome characterization. Potential applications, such as monitoring of interactions sensitive to ionic strength or pH in combination with multi-parameter characterization will be discussed on selected examples.

P1B-0363

SEM-BSE direct immunoassay for profiling protein expression on the surface of exosomes

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Introduction: Protein characterization of exosomal targets using standard immunoassays (ELISA, Luminex) is not very useful in understanding the efficiency of the vesicle capture or the specificity of the antibodies to exosomal targets. We developed a method of exosomal protein characterization which enables direct visualization of exosomes in SEM by combining secondary electron (SE) imaging with simultaneous assessment of exosomal protein profiles by gold labelling detected in back scattered electron (BSE) mode. *Methods*: (a) Exosomes input: 1–5 ug/25 K beads; (b) detector biotinylated Ab input: ~10 ug/ml; (c) one-step labelling: mix Ab with streptavidin-gold (SA-G); 50 ng/ul SA-G stock ($2.1 \times 10^{12} - 3.5 \times 10^{12}$ nanoparticles/ml); (d) unreacted Ab removal: treat the Ab-SA-G mix with SA-DynaBeads: 40 ug SA beads per 1 ug Ab; (e) beads bound to poly-L-lysine cover slips and underwent standard SEM preparation with carbon coating. *Results*: The method was developed using Vcap cell line exosomes captured on α-CD9 Ab conjugated to MicroPlex beads (Luminex) and detected with CD9/gold complex. Validation of the method on human plasma exosomes, isolated via ultracentrifugation, showed the need to increase exosome input 3–5 times. Next, same beads with CD9-captured exosomes were assessed with both Luminex immunoassay and SEM-BSE. SEM shows the presence of captured exosome-like shapes carrying CD9-activated gold nanoparticles on their surface. The number of gold particles per bead

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correlated with the fluorescent signal in Luminex and titered with the exosomal input. Individual gold particles could be discerned suggesting that this method may provide absolute quantitation of co-expression of proteins expressed on the surface of microvesicles. We further evaluated the method using other antibodies (CD63, CD81, EpCam) and differently shaped gold particles to expand the multiplexing capabilities of this method. *Summary/conclusion*: This approach offers a clear and direct visualization method to assess co-expression of proteins on the surface of extracellular vesicles and could be used as an alternative to more common indirect immunoassays such as Luminex and ELISA.

Arcadis Room
Poster Session 1C - Pathogen-derived EV
Chair: *Paul Wilmes and Meta Kuehn* 13:00-14:00

P1C-037

Secreted exosomes from *Heligmosomoides polygyrus* modulate cellular responses of the murine host

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Introduction: Exosomes are nanovesicles providing a mode of communication amongst eukaryotic cells through transfer of proteins and RNAs. Recent indications suggest that pathogen-derived exosomes, such as those discovered in *Leishmania donovani,* are able to modulate the onset of a host inflammatory immune response, thus promoting parasite survival. Here, we examine secreted vesicles from the murine gastrointestinal nematode *Heligmosomoides polygyrus,* and their potential role in host-helminth interactions. *Methods*: Transmission electron microscopy of cross-sections from *Heligmosomoides Polygyrus* adult worms. Ultracentrifugation of *H.polygyrus* excretory/secretory product to isolate exosomes. Protein mass spectrometry of *H.polygyrus* exosomes. Microarray analysis of exosome–small intestinal cell culture experiments. Real-time quantitative reverse transcription PCR (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA) to measure RNA and protein production of cell lines. Flow cytometry to study cellular character of lung inflammation following co-treatment with *H.polygyrus* exosomes. *Results*: Transmission electron microscopy reveals vesicle-like structures of 50–100 nM in the secretory product recovered by ultracentrifugation, and potential evidence of multivesicular bodies in the worm intestine. An intestinal origin is supported by proteomic data which show enrichment of worm intestinal proteins in the exosomes. Microarray analysis of exosome-treated small epithelial cells reveals significantly reduced expression of a number of genes, including those involved in the regulation of signalling and the immune response, such as dual specificity phosphatase 1 (DUSP1). Furthermore, we found that exosomes significantly reduce expression of classical activation markers, as well as inflammatory cytokine production in the macrophage cell line RAW 264.7. Finally, preliminary in vivo studies using a model of lung inflammation indicate that exosomes may modulate some cellular components of this response, shown by a reduction in type-2 innate lymphoid cells from lung tissue and bronchoalveolar lavage (BAL) fluid eosinophils. *Summary/conclusion*: This work suggests that exosomes secreted by parasitic nematodes could mediate cross-phylum communication and may help to suppress the host inflammatory response.

P1C-038

Induction of peritoneal and sepsis-like systemic inflammation by bacteria-free extracellular vesicles from faeces

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Introduction: Mammalian cells and bacteria shed nano-sized proteolipids, known as extracellular vesicles (EVs). EVs derived from Gram-negative and Gram-positive bacteria have multiple roles in inter-bacteria and inter-kingdom communication, especially, multiple pathophysiological roles in bacterial infection-related human diseases. This study aims to determine whether faeces contain bacterial EVs, and whether these faeces EVs have any local and systemic pro-inflammatory effects in murine model. *Methods*: EVs were isolated from faeces from mice by ultracentrifugation. The characteristics of faeces EVs were determined by TEM and the presence of lipid A, lipoteichoic acid, and actin was measured by ELISA. These isolated faeces EVs were introduced to mice, and local and systemic inflammatory responses were examined. Macrophages were stimulated with faeces EVs in vitro and several signalling molecules were studied. Finally, the role of TLR2 and TLR4 in faeces EV-induced inflammatory responses was studied in knockout mice. *Results*: faeces EVs originated from Gram-negative and Gram-positive bacteria, as well as from the mouse itself. Peritoneally injected faeces EVs induced a local inflammation and increased pro-inflammatory cytokines and cells in blood and lung. In addition, macrophages have the capacity to take up faeces EVs, and respond with a significant release of TNF-α and IL-6. The release of these cytokines was differentially regulated by intracellular signalling molecules most likely downstream signals of TLR2 and TLR4, as TLR2 or TLR4 knockout mice had a reduced inflammatory response to faeces EVs. *Summary/conclusion*: This study shows that faeces EVs cause a dose-dependent peritoneal and systemic inflammation when introduced intraperitoneally, confirmed by the increase in both pro-inflammatory cytokines and inflammatory cells in the blood and lung. The role of faeces EVs in clinical diseases warrant further studies, as faeces EVs may contribute to the pathology observed in bacterial sepsis.

P1C-039=OP2-118

Characterization of *Leishmania* extracellular vesicles: insights into their immunoregulatory role in the establishment of the infection

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P1C-040

RNA transcriptome of fungal extracellular vesicles from Saccharomyces cerevisiae, Cryptococcus neoformans, Paracoccidioides brasiliensis, and Candida albicans

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Introduction: Extracellular vesicles (EVs) have been characterized in pathogenic fungi and *Saccharomyces cerevisiae*. They can transpose the fungal cell wall and deliver to the extracellular milieu their

contents that potentially have a role in nutrition, intercellular communication, biofilm formation, induction of immune response and also damage to host cells. A variety of proteins, neutral lipids, glycans, and pigments have been identified in fungal EVs. We presently characterized EV small RNA enriched fractions (EVRNA) isolated from *S. cerevisiae* and the human pathogens *Cryptococcus neoformans, Paracoccidioides brasiliensis*, and *Candida albicans*. *Methods*: EVs were obtained from cell-free yeast culture supernatants after a final ultracentrifugation step for 1 h at 100,000 *g*. RNA was isolated using miRNeasy and RNeasy MinElute Cleanup (Qiagen). RNA sequencing was performed by next generation technology (SOLiD) and data were analysed by CLC Genomics Workbench software. *Results*: EVRNA was composed mainly by molecules smaller than 250 nt that aligned either at intergenic and intronic regions or at specific positions of the mRNA, mostly in the reverse orientation characteristic of small interference RNA. In *C. neoformans*, 90% of the reads mapped in intronic regions and 10% in exons. In *P. brasiliensis*, 17% of the reads mapped in intronic regions, 82% in exons and 21% in exon–intron regions. In *S. cerevisiae* and *C. albicans,* generally lacking introns, 90% of the reads mapped in exons. Among species, there were 32 common small nucleolar (snoRNA), 3 small nuclear (snRNA), 7 transfer (tRNA), 3 ribosomal (rRNA), and 3 non-coding (ncRNA) RNA, besides 20 sequences compatible with miRNA. We also observed a small proportion of co-purified mRNA suggested by reads along entire mRNA sequences, including those related to vesicle-mediated transport and metabolic pathways. *Summary/conclusion*: Our results show that fungal EV can export different types of RNA. Financial support: FAPESP, CNPq, Capes.

P1C-041

The role of *Staphylococcus aureus* extracellular vesicles in blood coagulation

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Introduction: Coagulopathy, including intravascular thrombosis and disseminated intravascular coagulation, occurs often in patients with severe sepsis or septic shock. However, the underlying mechanisms of coagulopathy are not fully understood. Recently, it has been reported that a wide variety of Gram-positive bacteria produced extracellular vesicles (EVs) and these vesicles have been proposed to play diverse roles. *Staphylococcus aureus*, the most virulent of the many staphylococcal species, is a leading cause of both nosocomial and community-based infections. Here, we studied the role of *S. aureus* EVs in blood coagulation. *Methods*: EVs were purified from the *S. aureus* culture supernatant by ultracentrifugation. *S. aureus* EV-induced thrombosis was assessed by in vitro and ex vivo measurement of clot turbidity and lung pathology in mouse model. After *S. aureus* EVs exposure to platelet rich plasma, platelet activation and aggregation were examined using the light microscopy. The levels of soluble tissue factors released from human umbilical vein endothelial cells were assayed by ELISA with anti-tissue factor antibody. *Results*: Histological analyses of mouse lung tissues, after a single-dose injection of *S. aureus* EVs, showed thrombus formation and peri-bronchial inflammation. In addition, *S. aureus* EVs significantly induced not only the fibrin formation *in vitro*, but also platelet activation and aggregation of platelet-rich plasma. When human umbilical vein endothelial cells were treated with *S. aureus* EVs, the levels of soluble tissue factors were increased in a dose-dependent manner. *Summary/conclusion*: This study suggests that *S. aureus* EVs are able to develop intravascular thrombosis through platelet activation and aggregation as well as the induction of tissue factor expression in human endothelial cells. Although the detailed mechanisms remain to be elucidated, *S. aureus* EVs could be the potential target of therapeutic agents to prevent or treat sepsis-induced coagulopathy.

P1C-042

Microvesicles released from *Giardia intestinalis* modulate the parasite–host cell interaction

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Introduction: *Giardia intestinalis* (GI) is an anaerobic protozoa and aetiological agent of giardiasis, an infection that induces a loss of epithelial barrier function and functional injuries of the enterocyte, producing diarrhoea and other symptoms. GI has a simple life cycle alternating between cyst and trophozoite. Cyst passes by oral route to the stomach and becomes trophozoite at the intestine in a multifactorial process. Recently, microvesicles (MVs) have been widely detected in various biological fluids and eukaryotic cells. In GI, we have observed a release of MVs during the interaction with mammalian hosts. This work aims to investigate the role of MVs from GI in the pathogenesis of giardiasis. *Methods*: Trophozoites of GI are maintained in culture at 37°C, in modified TI-S base medium. To obtain MVs, a log phase culture of GI trophozoites was incubated during 1 h in a TI-S base medium without adult bovine serum with addition of Ca^{++} 1 mM. After the induction, MVs were purified via ultracentrifugation (100,000 g for 1.5 h), quantified by flow cytometry analysis and used to different essays. Caco cells were used as a model of cell interaction, and proteomic analysis of MVs was performed by mass spectrometry. *Results*: GI responds to different environment conditions (pHs from 3–8, normal human serum and bili) with a high release of MVs derived from the plasma membrane. MVs from log phase, and not from cyst-induced culture of GI trophozoites, were able to increase the attachment of GI trophozoites to Caco cells. We observed that lipid rafts are involved in GI biogenesis and attachment to Caco cells and that the MVs increase the proliferation of Caco cells. Proteomic analysis showed variants surface proteins (VSPs) abundance and some putative markers, which are under investigation. *Summary/conclusion*: We have seen a high production of MVs from GI trophozoites in response to different environmental conditions during the course of infection. MVs from log phase trophozoites increase two times the attachment of the parasites to Caco cells. In contrast, MVs from cyst-differentiated trophozoites had no effect. MVs from GI alter the proliferation and integrity of Caco cells releasing some virulent factors. Proteomic analysis of MVs is under investigation.

P1C-043

Peroxiredoxins of *Trypanosoma cruzi*: beyond the peroxidase activity

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Introduction: The protozoan flagellate *Trypanosoma cruzi*, the causative agent of Chagas disease, has the ability to invade almost any kind of human cells. During invasion process, both surface and secreted proteins from *T. cruzi* play an essential role in host–parasite interaction. In order to identify relevant proteins in infection process, we analysed the proteome of shed vesicles produced by the parasite. *Methods*: Extracelullar vesicles shed by *Trypanosoma cruzi* were isolated and purified by ultracentrifugation. The proteome of purified shed vesicles was analysed by mass spectrometry. Among the identified proteins, cytosolic tryparedoxin peroxidase (*Tc*c-TXNPx) was found. Immunofluorescence analysis was done to study the interaction of extracellular vesicles and *Tc*c-TXNPx with human cells. Invasion and proliferation assays were performed in order to determine the role of secreted *Tc*c-TXNPx in the infection context. Finally, transcriptomic analysis of cells incubated with *Tc*c-TXNPx was also carried out using next generation sequencing. *Results*: In this

work, we analysed the proteome of shed vesicles from *Trypanosoma cruzi* identifying almost 30 proteins. We also describe that *Tcc*-TXNPX is secreted in different parasite stages both in extracellular vesicles and also in a vesicle-independent way. Parasitic peroxiredoxin is able to enter host cells through vesicles and also on its soluble form through an endocytic process, and this interaction enhances parasite infectivity. Besides, the interaction of *Tcc*-TXNPx with human epithelial cells promotes proliferation as well as the induction of pro-inflammatory cytokines in a dose- and time-dependent manner, and this interaction is independent of its peroxidase activity. Moreover, interaction of *Tcc*-TXNPX with cells triggers an unfolded protein response (UPR) leading to endoplasmic reticulum stress. *Summary/conclusion*: Taken together, these results suggest that secreted tryparedoxin peroxidase plays an important role in infection and immunomodulation.

P1C-044

Identification of miRNAs in exosomes from the parasitic trematode *Dicrocoelium dendriticum*

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Introduction: *Dicrocoelium dendriticum* is the most widespread parasite among the ruminants, causing the parasite infection called Dicrocoeliosis, a fluke-like disease of relevance in livestock. Extracellular vesicles constitute a newly discovered form of communication among different organisms, including parasites, and emerge as an important mechanism for protein export in trematodes. In this context, with the aim of characterizing the molecules involved in the interaction of *D. dendriticum* adults and the host, we have identified and characterized the components of exosomes in the excretory/secretory products (ESP) of *D. dendriticum*. *Methods*: Adult parasites obtained from the infected livers of cattle were incubated for 5 h in RPMI-1640 (25 worms/mL) to obtain ESP. Exosomes were purified from ESP supernatants using a protocol based on ultracentrifugation coupled to membrane filtration. Exosomes were washed with PBS and analysed using proteomic approaches (LC-MS/MS). Proteins were characterized using currently available datasets including the transcriptome of the related species *Echinostoma caproni*. We have used a homology-based approach because this parasitic trematode lacks an assembled genome sequence. The miRCURY™ RNA Isolation Kit (Exiqon) was used to isolate short RNA molecules. miRNAs were analysed by Bioanalyzer, subjected to high-throughput sequencing, and characterized by computational analysis using different algorithms and available datasets (miRbase v20). *Results*: We have identified 65 distinct proteins in *D. dendriticum* exosomes, exhibiting a distinct proteomic profile from the described for the related parasitic species *E. caproni* and *F. hepatica*, indicating specificity in their cargo. In addition, we have revealed the presence of miRNAs in *D. dendriticum* exosomes. We have identified 30 miRNAs from the parasite by homology to other parasitic helminthes, as well as 113 miRNAs corresponding to the host. *Summary/conclusion*: Our work represents the first report of miRNAs in parasitic helminth exosomes.

P1C-045

The characterization biochemical and proteome of different strains of *Trypanosoma cruzi* vesicles

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Introduction: Infective trypomastigote forms of *Trypanosoma cruzi* release membrane vesicles rich in α-galactosyl epitopes (alpha-GAL). These parasite vesicles induce a potent host pro-inflammatory innate immune response through the activation of TLR-2. In this study, we evaluated the biochemical characterization (from Y strain) and proteomic analysis of vesicles from Y, Y (from blood), Yu-Yu and CL-14 strains. *Methods*: The vesicles from different strains were obtained from the conditioned medium of cell-derived trypomastigotes and fractionated by gel-filtration. The vesicles released by strains of *T. cruzi* with different degrees of virulence were characterized. We performed ELISA assays, SEM, and LightScattering in solution and characterized the vesicles by molecular mass spectrometry (ESI-MS). *Results*: Trypomastigotes from the YuYu strain released more protein in the supernatant of the parasites than Y, CL-14 and Colombiana strains. The purified vesicles from *T. cruzi* Y, YuYu and CL-14 strains have differences in expression of alpha-GAL epitope among the parasites. The release of vesicles by trypomastigotes of the Y strain is not decreased at 4°C. The addition of glucose in serum-free medium increases shed vesicles in the supernatant, and variation in pH of the culture medium alters the release of vesicles. The vesicles had a diameter of 20–30 nm. We have identified specific proteins of *T. cruzi*, including several members of the *trans*-sialidase/gp85 (TS-gp85) and gp63 super-families. We have also found several polypeptides related to mammalian exosomes mucin (MASP). In addition, several peptides of cytoskeletal proteins are present in vesicles from different strains. We found enolase, Rab GTPases, S-receptor kinase and elongation factor as components of the vesicles from different strains of *T. cruzi*. There are differences in the distribution of protein classes on different strains (functions of the genes found). An overlap of sequences found shows that only the TS-gp85 is common in all strains. *Summary/conclusion*: Our data clearly show that vesicles secreted by *T. cruzi* contain the major virulence factors responsible for promoting the parasite entry into the host cells and for its subversion from the host immune response.

P1C-046

Capture of membrane-derived microvesicles from *Giardia intestinalis* induces activation of human monocyte-derived dendritic cells

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Introduction: Pathogens may use plasma membrane-derived vesicles (PMVs) in order to alter the immune response aiming immune evasion. *Giardia intestinalis* is an extracellular parasite, causative agent of giardiasis. The parasite is known to shed encystations-specific vesicles (ESVs) when undergoes encystation, and we recently observed PMV secretion by *G. intestinalis*. As dendritic cells are critical against infection, we studied these cells' interaction with PMVs secreted by *G. intestinalis*. *Methods*: Purified PMVs were obtained from axenically cultured *G. intestinalis'* trophozoite and from the THP-1 cell line by calcium stimulation. They were quantified by protein content correlation and characterized by transmission electron microscopy. Human monocytes isolated from peripheral blood of healthy donors were differentiated into dendritic cells (MDDCs). MDDCs were then pulsed with PMVs from either *G. intestinalis* or THP-1 cells stained with PKH-67. Capture of PMVs was assessed by flow cytometry, and the endocytic pathway was studied by confocal microscopy. MDDCs' phenotype after PMVs pulse was determined by activation markers (CD25, CD83, HLA-DR) and confirmed by alloproliferative assays. *Results*: PMVs from *G. intestinalis* and from the THP1 cell line were similarly internalized by human immature dendritic cells (iDCs). Endocytosed PMVs are sorted into the endocytic compartment of iDCs and processed, leading to iDCs activation. Functionally, capture of PMVs from both cell origins induced enhanced T-cell alloproliferation. Intriguingly,

even under limiting conditions for capture (4°C), MDDCs were still positively conditioned for allostimulation. *Summary/conclusion*: *G. intestinalis* and THP-1 PMVs secreted upon high calcium concentrations are captured by iDCs and trigger their activation. PMVs from both cell origins captured by dendritic cells enhance the allostimulation abilities of iDCs towards T cells. Thus, dendritic cells are able to respond to different types of PMVs.

P1C-048=OP2-121

Unfiltered beer – a rich source of yeast extracellular vesicles

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Arcadis Room
Poster Session 2B - EV therapeutics 1
Chair: *Pieter Vader and Sai Kiang Lim* 13.00-14.00

P2B-049

Exosomes as carriers of miRNA for treatment of glioma

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Introduction: Exosomes contain miRNAs which can be transferred between cells, resulting in cross-cellular gene regulation. Bone marrow stromal cells (MSCs) produce exosomes abundant in mature miRNAs. We tested if MSC exosomes could be used as a delivery vehicle for anti-tumour miRNA therapy of malignant glioma. To this end, we administered exosomes from MSCs transfected with miR-146b or miR-145 by intratumour injection to rats bearing glioma. We have demonstrated that mir-146b and miR-145 reduce glioma cell motility and invasion and that EGFR mRNA is a binding target for miR-146b and miR-145 translational inhibition. As miR-146b and miR-145 inhibit EGFR expression and suppress the malignancy in glioma cells, we hypothesized that MSC exosomes containing miR-146b or miR-145 would have a therapeutic anti-tumour effect in rats bearing brain tumour. *Methods*: Expression plasmids were employed to express miR-146b or miR-145 in primary rat MSCs. Exosomes were harvested using ExoQuick-TC precipitation solution. Real-time PCR was employed to detect miRNA in exosomes or 9 L gliosarcoma cells. The Izon qNano system was used to analyse and quantify harvested exosomes. We employed a Fischer rat model of primary brain tumour. Exosomes or delivery vehicle was used to treat tumour-bearing rats by intratumoural injection 5 days (miR-146b) or 7 days (miR-145) after tumour implant. *Results*: MSC cells transfected with miRNA expression plasmids efficiently packaged miR-146b and miR-145 into released exosomes. Treatment with exosomes from miR-146b- or miR-145-transfected MSCs reduced 9 L rat gliosarcoma cell growth, and expression of EGFR protein in 9 L cells in vitro. Intratumour injection of miR-146b or miR-145 containing MSC exosomes significantly reduced glioma xenograft growth in a rat model of primary brain tumour. *Summary/conclusion*: Our findings indicate that MSCs exosomes may be used as biological delivery vehicles to deliver anti-tumour miRNAs to glioma.

P2B-050

Prevention of exosome uptake by synthetic, biomimetic high-density lipoprotein nanoparticles

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Introduction: Exosomes are endogenous nanostructures, which are crucial mediators of several biological processes, and play an important role in the progression of several diseases, including cancer. Intercellular communication between the tumour and its microenvironment is mediated by exosomes and is a vital step in tumour progression and metastasis. Therefore, a means to disrupt exosome signalling in a targeted manner is an intriguing possibility and a potential novel new therapy against cancer, among other diseases. Our group has developed a synthetic route to biomimetic, high-density lipoprotein nanoparticles (HDL-NPs), which specifically target scavenger receptor type B1 (SR-B1) and efflux cholesterol, and modulate cellular cholesterol metabolism. Here we present evidence that HDL-NPs prevent the uptake of exosomes in SR-B1 positive cell lines by targeted disruption of cholesterol homeostasis in lipid raft membrane domains. *Methods*: Exosomes from A375 melanoma cells were isolated using ultracentrifugation and labelled with fluorescent lipophilic dyes. The uptake of exosomes by A375 and human microvascular endothelial cells (HMVECs) was measured by fluorescence microscopy and flow cytometry after treatment with HDL-NPs. Lipid rafts were labelled using cholera toxin subunit B conjugated to Alexa 488 and imaged after HDL-NP treatment. *Results*: HDL-NPs target SR-B1 and decrease the uptake of exosomes in both A375 and HMVEC cell lines. A375 cell lines were further studied and a disruption of lipid rafts was observed. This disruption was attributed to a modulation of cholesterol homeostasis caused by the HDL-NP and was reversed by natural human HDL. *Summary/conclusion*: HDL-NPs block the uptake of exosomes in melanoma and endothelial cell lines due to the targeting of SR-B1 and disruption of cholesterol homeostasis within lipid rafts. Due to the importance of cholesterol metabolism and intercellular signalling in cancer, and many other disease processes, HDL-NPs may be potent next-generation therapies for cancer and other diseases where exosomes play a role.

P2B-051

Mesenchymal stem cell-derived exosomes mediate angiogenesis

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Introduction: Elucidating the mechanisms of new blood vessel formation (angiogenesis) has important implications for numerous diseases, including cardiovascular disease and cancer. Bone marrow-derived mesenchymal stem cells (MSCs) have been well characterized for their immunomodulatory, tissue healing and pro-angiogenic capabilities. Studies have shown that MSCs mediate angiogenesis through the secretion of pro-angiogenic factors. Studies to date have focused on canonical secretory proteins such as VEGF as the mediators of MSC's ability to induce angiogenesis. However, recent studies have shown that MSC also secrete significant amounts of secreted vesicles called exosomes, which can transport biologically active non-secretory proteins and miRNA from their cell of origin to target cells. We aimed to investigate the potential role of MSC exosomes in MSC-induced angiogenesis. *Methods*: Exosomes were isolated from MSC-conditioned media. MSC exosomes were used to stimulate endothelial cells (HUVEC) in vitro. miRNA expression in MSC-exosomes was quantified via qPCR. *Results*: We show that MSC exosomes induce angiogenesis-like tubule formation in endothelial (HUVEC) cells in vitro. We show that MSC-derived exosomes contain pro-angiogenic miRNAs. *Summary/conclusion*: This finding indicates that MSC exosomes have the potential to deliver miRNA payloads and suggests that this capability may be further enhanced through genetic engineering, perhaps leading to new therapeutic avenues involving the delivery of extracellular RNAs.

P2B-052

The potential of adipose-derived stem cell exosomes as vehicles of drug delivery

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Introduction: Glioblastoma multiforme (GBM) is a devastating malignant disease characterized by aggressive tumours in the central nervous system, which have a dysregulated microRNA expression profile favouring growth, invasion and angiogenesis. Exosomes have been shown to possess the ability to deliver gene therapy and to be abundant in culture supernatants from mesenchymal stem cells. This study seeks to investigate whether adipose-derived stem cell (ASC) exosomes are appropriate to use as drug delivery vehicles. *Methods*: Characterization of proteins in the ASC exosomes were analysed by EV array and mass spectrometry. The size distribution of ASC exosomes was determined by nanoparticle tracking analysis (NTA), whereas atomic force microscopy was performed to visualize the shape of normal ASC exosomes versus electroporated ASC exosomes. Transfection efficiency of the ASCs was evaluated by quantification with pEGFP-C1. Finally, the functional effects of ASC exosomes on the proliferation potential of 2 commercially available GBM cell lines (U87 and U251) and an in-house-generated GBM stem cell line (C16) were assessed by flow cytometry. *Results*: Exosomes from ASCs were shown to express relevant exosome-associated proteins, while the size distribution was comparable to those reported for other types of cells. Atomic force microscopy showed that exosomes had a round shape and that electroporation induced the formation of large aggregates in the preparations. The ASCs were transfected with pEGFP-C1 with an efficiency of 12–15%. In addition, ASC exosomes had no apparent proliferation-inducing effect on GBM cell lines. *Summary/conclusion*: The results of this study suggest that ASCs are a relevant choice of donor cell type for the production of targeted exosomes. The lack of any apparent proliferation-inducing effect on GBM cell lines suggests an application of ASC exosomes for delivering therapeutics to GBM tumours. However, care should be taken with regards to electroporation settings and, furthermore, stable transfection of ASCs may be necessary to obtain the desired amount of targeted exosomes.

P2B-053

Exosomes influence proliferation of androgen-sensitive prostate cancer cells

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Introduction: Androgen receptor (AR) stimulation activates androgen response genes to upregulate hormone-dependent prostate cancer cell proliferation. As androgen deprivation therapy will upregulate androgen downregulated genes, characterizing exosomes from advanced prostate cancer patients on systemic therapy may provide indicators of treatment response. We report here the effects of androgen deprivation, dihydrotestosterone (DHT) and androgen antagonist MDV3100 on exosomes from AR+ LNCaP and DuCaP cells. *Methods*: Cells in 5% charcoal stripped serum were treated with ethanol (vehicle) or 10 nM DHT, with or without 10 mM MDV3100. Exosomes isolated from conditioned media by stepwise ultracentrifugation[1] were confirmed by transmission electron microscopy before downstream proteomics and miRNA analysis. Exosomal protein was analysed by LCMS/MS, raw data were generated by Agilent's MassHunter and processed by Agilent's Spectrum Mill, searched against the human database. Quantitative analysis was performed using Scaffold4 v4.2.1, normalized using emPAI.[2] Mass spec data was analysed for activated downstream pathways using Ingenuity Pathway Analysis (IPA). The size of exosomes was measured by qNANO (IZON). *Results*: DHT and MDV3100 treatments changed the composition of LNCaP exosomal proteins. By mass spec, 161/338 total proteins were seen in all samples, including exosome markers CD9, CD81, Alix, TSG101, and a prostate-specific biomarker, Prostate-Specific Membrane Antigen (PSMA/FOLH1). DHT upregulated CD9, Alix and TSG101 in DuCaP, while androgen deprivation affected their expression in LNCaP cells. DHT significantly altered the size of exosomes secreted by LNCaP, but not DuCaP cells. IPA on LNCaP exosomes showed that DHT upregulated cell proliferative and survival pathways. Functional assays showed that treating androgen-deprived LNCaP cells with exosomes from DHT-treated cells increased the proliferative rate of LNCaP cells. SiRNA of Alix-inhibited LNCaP cell proliferation confirming that altering exosome biogenesis plays a role in cellular proliferation. Investigation of miRNA content and pathways involved in this process are currently on going. *Summary/conclusion*: Our data support the idea that androgens, through the AR, regulate exosome secretion in AR+ prostate cancer LNCaP and DuCaP cells. Androgens not only mediate prostate cancer progression by directly regulating AR-related gene transcription but also alter cell–cell communications between prostate cancer cells via secreted nano-sized vesicles, such as exosomes. Funding: USA Department of Defense – Postdoctoral Training Award [W81XWH-12-1-0047], Movember Global Action Plan for Exosome Biomarkers, and Australian Government Department of Health and Ageing.

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P2B-0540=OP2-117

Nanovesicles engineered from cells

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P2B-0541

A new registry and award for EV therapies and vaccines

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Introduction: Therapies and vaccines are topics at ISEV meetings, but the greater ISEV focus is diagnostics and bench research. At the 2012 and 2013 ISEV annual meetings and the education session of the 2013 ISEV, remarkable successes using EV since the 1980s were described by Mulhall and Grandi. So far, despite ISEV's success in getting organizations like the NIH to support basic research on, for example, mRNA, and in EV-related therapeutic R&D, immediate needs of patients still go unmet. Also, it is apparent from the near-exponential increase in EV publications that duplication of earlier research continues because the data on successes in one field is not in a form where it is easily acquired in searches by researchers in other fields. Potential solutions to the dilemma include a registry and award for EV therapies and vaccines. *Methods*: It was proposed at ISEV 2012 and 2013 plenary meetings and education session by Mulhall to develop a database of EV therapeutic and vaccine applications. The database is an important tool for inderdisciplinary learning about successful EV-based therapies as well as successful therapies not yet evaluated for impacts on EV. It is further proposed here that the ISEV sponsors and pursuers support for a registry for EV-related therapies and vaccines with these aims: comparing approaches across disciplines on how successes with anti-bacterial

vaccines support acceleration of cancer vaccines and cataloguing work across multiple disciplines, that is, cancer, coronary disease, renal disease, blood disorders, immunotherapy, regenerative therapies, etc. It is further suggested that the ISEV start a non-monetary recognition award for researches with the most promising practical therapeutic potential. The aim of such an award is to draw the attention of the international funding community, especially drug development agencies for the outstanding achievements in this field. *Results*: The intended results of a registry are to speed up the path from research to clinical applications with a mechanism to adapt data from successful applications in one domain to other domains. The intended results of the award are to accelerate support for outstanding EV therapeutic and vaccine research and broaden awareness of EV potential in the healthcare community. *Summary/conclusion*: For such a registry and award to get started, it is important to gauge ISEV members' support level other than in SurveyMonkey. The ISEV annual meeting is one such venue. Students are encouraged to join the session as a way of familiarizing themselves with the state-of-the-art therapies and participating in the development of the registry and award.

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Arcadis Room
Poster Session 3A - EV in the immune system
Chair: *Maria Mittelbrun and Erzsebet Ligeti* 13:00-14:00

P3A-055

Systemic delivery of bovine milk-derived microvesicles aggravates collagen-induced arthritis in mice

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Introduction: Bovine milk contains microvesicles (MVs), and they may possess immunoregulatory properties. The objective of this study is to determine the effect of commercial milk-derived MVs on macrophage function and on collagen-induced arthritis (CIA), a mouse model of rheumatoid arthritis. *Methods*: Milk MVs were isolated by a serial (ultra)centrifugation method followed by ExoQuick isolation. MVs were labelled with PKH-67 and in vitro incubated with murine RAW264.7 macrophages or isolated adherent splenocytes (APCs). As a control, unlabelled MVs were taken and nonspecific uptake was determined at 4°C. Uptake was measured with FACS and confocal microscopy. miRNAs from MVs were detected by RT-qPCR. RAW 264.7 macrophages and splenic APCs pre-incubated with milk MVs for 24 h were stimulated with LPS for 24 h and release of MCP-1 and IL-6 was measured by Luminex bead assay. In CIA, milk MVs were given twice intravenously, 1 day before immunization and booster (day 21). Arthritis was scored macroscopically and joint pathology by histology. Splenic APCs were stimulated with LPS to measure cytokine production. *Results*: Confocal microscopy and FACS showed uptake of bovine MVs by macrophages and splenocytes within 1 h of incubation. This resulted in significantly increased (65%) production of the pro-inflammatory cytokine IL-6 in LPS-stimulated macrophages, while MCP-1 was significantly reduced (11%). The MVs contained miRNAs miR-Let7a, 223, and 124a, the latter is known to target the 3'UTR of the *MCP-1* gene. Systemic delivery of MVs slightly reduced onset of CIA but at later time points markedly aggravated arthritis. This effect coincided with an increased production of KC (68%), IL-6 (35%) and TNFα (36%) of LPS-stimulated splenic APCs from CIA mice. *Summary/conclusion*: Two injections of bovine milk MVs into mice leads to aggravation of arthritis possibly by modulating the APC response. This raises the question whether bovine milk MVs are involved in human rheumatoid arthritis. Funding: Dutch Arthritis Association RF 12-2-201.

P3A-056

Apoptotic-cell-derived-membrane vesicles transport microRNA from apoptotic cells to phagocytes

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Introduction: Apoptosis leads to the release of extracellular vesicles (apoptotic-EV), which have been identified as mediators of cell-to-cell communication. An impaired clearance of apoptotic debris has been observed in SLE patients. We analysed the microRNA content of lymphocytes and their corresponding apoptotic-EV from both normal healthy donors (NHD) and SLE patients. *Methods*: MicroRNA content of activated and apoptotic lymphocytes and corresponding EV were compared in an Agilent microRNA array and validated by qPCR. Apoptosis was induced by UVB-irradiation. EVs were isolated by filtration and ultracentrifugation from cell supernatants. MiR-155 expression in monocytes after UV-MCVs engulfment was determined by qPCR. Expression of miR-155 target protein Tab-2 was analysed by western blot. *Results*: MiR-155* levels were decreased after apoptosis induction in lymphocytes and apoptotic-EV compared to viable correlates. MiR-155, miR-99a and miR-34b were decreased in apoptotic lymphocytes but increased or not significantly changed in apoptotic-EV compared to EV released from activated cells. This indicates a directional transport of microRNA into EV. MiR-34a was expressed at higher levels in SLE compared to NHD, while MiR-34b expression was decreased in apoptotic cells and EV from SLE patients. Functional assays confirmed higher miR-155 levels and consecutively decreased target protein levels in monocytes after engulfment of apoptotic-EV. *Summary/conclusion*: We showed an unequal distribution of distinct microRNA into EV released by activated or apoptotic lymphocytes suggesting a directional transport rather than a random distribution. Further, we showed a microRNA and protein expression change in phagocytes after EV engulfment. Hence, apoptotic-EV could serve as a transport vehicle for microRNA and influence intracellular processes in phagocytes. Disturbances of this system might contribute to the pathogenesis of SLE.

P3A-057

Comparison of efficacies of different extracellular vesicles to induce immune responses

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Introduction: Extracellular vesicles (EVs) can be classified based on their size and their intracellular origin. Differential ultracentrifugation is the classical method described for EVs purification. In each step of centrifugation, different extracellular components are pelleted. In particular, apoptotic bodies or cell debris are large vesicles described to pellet at 2,000 × g, while vesicles of various sizes (>100 nm diameter) are secreted by budding of the plasma membrane and are isolated after 10,000 × g centrifugation. Finally, exosomes are 50–100 nm vesicles thought to originate from endosomes and are purified by differential ultracentrifugation at 100,000 × g. Several works have described the efficient induction of $CD4^+$ and $CD8^+$ T-cell responses by dendritic cell (DCs)-derived exosomes, but without comparing exosomes with other EVs. Here, we tried to determine the effect of different EVs on $CD4^+$ T cells. *Methods*: EVs released by human monocyte-derived DCs were isolated at all steps of the differential centrifugation purification protocol (2,000 × g, 10,000 × g and 100,000 × g pellets). All EVs expressed MHC class II, as observed by WB and flow cytometry. Activation of $CD4^+$ T lymphocytes by EV-borne MHC class II was measured either by mixing allogeneic EV and T cells or PBMCs and following proliferation by flow cytometry, or by incubating an antigen-specific $CD4^+$ T-cell clone with peptide-loaded EVs and measuring IFN-γ secretion. *Results*: All the pellets from the different purification steps were able to induce, to a similar extent, primary $CD4^+$ T-cell proliferation and IFN-γ secretion by the $CD4^+$ T-cell clone. Limited expansion of $CD25^+FOXP3^+$ $CD4^+$ T cells was also observed in the presence of anti-CD3 and anti-CD28. *Summary/conclusion*: We observed that not only exosomes but also other EVs are able to induce $CD4^+$ T-cell responses. These results highlight the need for a more comprehensive study on the specificity of EVs activity.

P3A-058

TGF-β1 is involved in the suppression of NK cells by PMN-ectosomes

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Introduction: Previous work has shown that ectosomes (Ecto) derived from polymorphonuclear neutrophils (PMN) and platelets (PLT) are immunosuppressive. Recently we showed that PLT-Ecto suppressed natural killer cell (NK cell) function, which was directly related to the expression of TGF-β1 on PLT-Ecto. PMN-Ecto do not express TGF-β1, but they suppress NK cell function. It is known that PMN-Ecto has the potential to induce TGF-β1 release by human monocyte/macrophages, a possible mechanism for an indirect suppression of NK cell function. *Methods*: Therefore, we investigated the direct and indirect interactions of PMN-Ecto with NK cells and PBMC, respectively. We labelled the surface proteins of PMN-Ecto and NK cells with DyLight Amine-Reactive Dye 633 and 488, respectively, and followed their interaction for 20 h by live-cell confocal microscopy. After 20 h exposure to PMN-Ecto, PBMC or NK cells were stimulated by co-incubation with the HLA class-I-deficient target cell line 721.221 for further 6 h. Thereafter, degranulation (CD107) and cytokine production (IFNγ) were assessed by FACS analysis. The results are expressed as the mean percentage of IFNγ and CD107-positive NK cells. In parallel, TGF-β1 was determined by ELISA in the supernatants of PBMC and NK cells exposed for 20 h to PMN-Ecto. When stated, the experiment was performed in the presence of anti-TGF-β1 Abs. *Results*: PMN-Ecto bound to NK cells very rapidly after exposure. With time, the period of interactions increased and could last up to several hours. No uptake was detected even after 20 h. The response of NK cells or PBMC to target cells after pre-incubation with PMN-Ecto reduced their function in terms of IFNγ production (from 26.8% to 13.5%; $p < 0.001$, and from 26.6 to 14.6; $p < 0.001$, respectively) and CD107 expression (from 29.7. to 17.1%; $p < 0.001$, and from 25.6. to 14.7%; $p < 0.005$, respectively). PMN-Ecto induced a significant increase in the release of TGF-β1 during 20 h co-incubation with NK cells (31.5 ± 1.5 to 44.0 ± 1.0 pg/ml; $p < 0.025$), as well as with PBMC (110.0 ± 5.8 to 166.7 ± 27.3 pg/ml). The function of NK cells was partially restored by anti-TGF-β1 Abs (IFNγ from 10.3 to 15.5%; CD107 expression from 10.0 to 15.0%; $p < 0.02$). *Summary/conclusion*: TGF-β1 release by NK cells and PBMC is increased in the presence of PMN-Ecto and is involved in the suppression of NK cells function.

P3A-059

Phenotypic characterization of exosomes present in blood by extracellular vesicle array – is the exosome profile a reflection of the distribution of the leukocyte subpopulations in blood?

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Introduction: It is generally accepted that exosomes play an important role in the communication between cells. Studies have shown that the quantity and molecular composition of exosomes shed from various cell types differs considerably. It is therefore expected that blood contains a wide range of exosomes with different phenotypes, reflecting the phenotype of the cells that produced them. Consequently, it was investigated whether exosomes present in blood reflect the specific subtype composition of the leukocytes in blood from healthy individuals. *Methods*: Blood samples from 10 healthy individuals were analysed by flow cytometry for the subtype composition, using antibodies specific for T cells, B cells, NK cells, monocytes and granulocytes. The expression of the tetraspanin markers CD9, CD63 and CD81 was also determined for each subtype. From the same blood samples, plasma was extracted and used for the phenotypic investigation of the exosomes present in whole blood using an EV Array (Jørgensen *et al*., 2013, JEV). The EV Array is based on the antibody capture of exosomes by a panel of surface markers with subsequent detection of the captured exosomes by biotin-labelled anti-tetraspanin antibodies. The panel included the subtype-specific surface markers and more than 30 other selected markers. *Results*: The EV Array demonstrated only low intensities for the majority of subtype-specific markers, while few surface markers, for example, CD3 and CD14, appeared at higher intensities. In addition, the results indicated that the 3 general exosomal markers, CD9, CD63 and CD81, were differentially expressed on the different leukocyte subpopulations. *Summary/conclusion*: Based on a panel of 12 different immune markers, the results indicate that there is no direct correlation between the amount and phenotype of the exosomes and the corresponding subtype composition of leukocytes in blood.

P3A-060

Tumour-derived microvesicles affect differentiation of human monocytes to macrophages

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Introduction: Blood monocytes are a heterogeneous population of cells playing an important role in inflammatory and anti-tumour responses. Monocytes infiltrating the tumour site are precursors of tumour-associated macrophages (TAM) that are involved in the response to the growing tumour. Monocytes are affected by tumour cells *via* a direct contact, soluble mediators and microvesicles. Tumour-derived microvesicles (TMVs) may modulate biological activity of monocytes by inducing production of reactive oxygen intermediates, cytokines and chemokines. *Methods*: Monocytes were separated from peripheral blood of healthy donors by counter-current centrifugal elutriation. TMVs were isolated from culture supernatants of colon cancer cell lines (CaCO, SW480, SW620 and LoVo) by centrifugation at $50{,}000 \times g$ for 1 h at 4°C. Monocytes were cultured alone (control) or with TMVs applied at different times (first or last day of the culture) and dosage (3 or 30 μg/ml). After 7-day culture, the cells were evaluated in terms of their morphology (by light microscopy), phenotype, phagocytic potential, ROI production (all by flow cytometry) and cytokine secretion (by ELISA). *Results*: Monocytes differentiated in the presence of TMVs have macrophage-like morphology (MDM, or monocyte-derived macrophages). They express CD11b, CD14, CD33, CD36, CD68, CD86, CD163, CD206 and HLA-DR; however, the expression profile depends on regimen of contact with TMVs. MDM differentiated in the presence of TMVs secreted more TGFβ, VEGF, IL-10 and TNF than control MDM. Production of ROI (after PMA stimulation) was significantly elevated in MDM, which encountered TMVs at the final stage of differentiation. These cells have a lower phagocytic potential in comparison to control MDM. *Summary/conclusion*: TMVs may affect the process of monocyte differentiation and may modulate their phenotype and functions. It was presented before that TMVs/exosomes may direct differentiation of circulating monocytes towards MDSC (myeloid-derived suppressor cells). Now, we present the evidence that TMVs derived from colon cancer cell lines are able to ''push'' differentiation of monocytes into MDM. Moreover, our results indicate that the regimen of monocyte contact with TMV may be crucial to the type of MDM polarization.

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Arcadis Room
Poster Session 3C - EV in the nervous system
Chair: *Malene Jørgensen and Marike Broekman* 13:00-14:00

P3C-061=OP3-131

Utilize extracellular vesicles as biomarkers of vitreal diseases

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P3C-062

Plasma microvesicles as a marker for Multiple Sclerosis?

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Introduction: Little is known with respect to the involvement of microvesicles/exosomes in neurological disorders such as multiple sclerosis (MS). It has been suggested that exosomes are involved in the transfer of myelin to neuronal myelin sheaths. Central nervous system-derived exosomes have been reported to be able to cross the blood–brain barrier and to be present in the circulation. *Methods*: We determined exosome markers in plasma lipoprotein profiles prepared by separating lipoproteins using density gradient ultracentrifugation of MS and control patients. *Results*: Exosome markers were present in the HDL subfractions (density range 1.063–1.21 g/ml). In comparison with controls, MS patients showed differences specifically in this density range, in particular when measuring sphingosine-1-phosphate (S1P). S1P has been linked to MS and has been related to maturation and release of exosomes, for example, for the secretion of neurotransmitters. *Summary/conclusion*: Therefore, our data suggest that MS patients present with strongly increased plasma microvesicle/exosome levels. Presently, we are analysing exosome proteins and lipids in these fractions. Data will be presented.

P3C-063

miRNAs associated with neuropathological features in patients with Alzheimer's and Parkinson's diseases

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Introduction: miRNAs are important regulators of cellular processes involved in both homeostasis and disease. miRNAs detectable in peripheral circulation may provide relevant information about human health and disease. To determine what miRNA signals are present in cell-free peripheral biofluids of patients with neurodegenerative disease, we evaluated the miRNA content in 2 biofluids across 2 diseases. We profiled the miRNA content of both cerebrospinal fluid (CSF) and blood serum (SER) from 70 Alzheimer's disease patients, 67 Parkinson's disease patients and 76 neurologically normal controls using next-generation sequencing. *Methods*: We isolated total RNA from 1 ml of cell-free CSF or serum. We used the Illumina TruSeq small RNA library preparation kit to prepare the samples for sequencing. Samples were loaded onto the sequencer and the data analysed. *Results*: For each miRNA, we report the average abundance detected in CSF and in SER. We also describe novel miRNAs discovered in CSF and SER. All subjects were clinically evaluated and had post-mortem neuropathological verification of disease diagnosis. The information provided in the neuropathology reports allowed us to correlate detectable changes in miRNA expression with aspects of disease severity such as Braak stage, dementia status, plaque and tangle densities, and Lewy bodies. Between PD patients and PD patients with dementia, we were able to identify miRNAs that were expressed with significantly different abundance. We found that in some cases ~70% of the deregulated miRNAs detected as significantly different in peripheral cell-free biofluids were previously identified in brain tissue from patients with neurodegenerative disease. *Summary/conclusion*: We were able to detect differentially expressed miRNAs in CSF and SER from patients with Alzheimer's and Parkinson's diseases, many of them previously identified to be misregulated in patient tissue samples. We will continue to validate many of the miRNAs identified in this paper using additional samples from patients early in their diagnosis.

P3C-064

miRNA profiling of hypoxia-induced neuronal and glial cell-derived exosomes

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Introduction: Stroke is the second leading cause of death worldwide, and the leading cause of adult-acquired disability. MicroRNAs (miRNAs) are small non-coding RNAs that function to inhibit mRNA translation. miRNAs have specific effecter roles in both the pathogenesis and repair mechanisms following cerebral ischaemia. Exosomes are small cell-derived vesicles (30–100 nm) involved in cell-to-cell signalling. Exosomes package small molecules, including miRNAs, and in a therapeutic setting they can act as biomarkers or therapeutic delivery agents – potentially useful in complex organs, such as the brain. The present study profiled exosomal miRNA release from cells of the neurovascular unit (NVU) following hypoxia/reoxygenation. *Methods*: Neuronal (B50), glial (B92) or cerebral endothelial (GPNT) cells were subjected to 9 h hypoxia (1% O_2) in serum-free media, followed by 24 h reoxygenation in complete media. Exosomes were harvested from media during both the hypoxic and the reoxygenation phase, with time-matched exosomes harvested from normoxic control cells. Exosomes were isolated using Total Exosome Isolation Buffer (Invitrogen) and total RNA extracted using the RNeasy Mini Kit (Qiagen). A miRNA OpenArray (Invitrogen) was performed assessing 754 miRNAs across the samples. Data were analysed using DataAssist v3.01 (Invitrogen) and bioinformatic analysis performed using TargetScan™. miRNA validation was performed by Taqman® qRT-PCR using specific probes. *Results*: Distinct hypoxia-induced miRNA signatures were determined within the exosomes of the different cell types. However, though miRNA signatures were on the whole distinct, 5 miRNAs were common across the B92 and B50 cells; miR-29c*,-503,-1903,-1937b & -2146; and one was common across all 3 cell types, miR-1937b. In addition, a number of miRNAs were present within hypoxic exosomes that were undetectable within the normoxic controls. Thirteen miRNAs underwent this ''off/on'' switch following hypoxia in B92, 14 in B50 and 12 in GPNT-derived exosomes. From bioinformatic analysis of all altered miRNAs

to look for relevant mRNA targets for stroke, 5 miRNAs were selected for validation from B92 (let-7d,-34b,-29b,-29c&-503) and B50 (-16*,-29c*,-342-3p,-350&-503) cell-derived exosomes. Validations from B92-derived exosomes verified miR-29b and -29c to be significantly increased following hypoxia/reoxygenation ($p < 0.05$ vs. control). Validations from B50-derived exosomes verified miR-350 to be significantly increased following hypoxia/reoxygenation ($p < 0.05$ vs. control). Further validations of miRNAs are on-going. *Summary/conclusion*: This study has profiled exosomal miRNA release in response to hypoxia/reoxygenation from NVU cells in vitro. Of these miRNAs shown to be differentially altered compared to normoxic controls several were shown to have significant targets in the setting of stroke. Further validations will allow for a full profiling of stress-induced miRNAs transported in exosomes from NVU cells in vitro.

P3C-065

Circulating extracellular vesicles can distinguish between tumor progression and pseudoprogression in glioblastoma patients

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Introduction: The current standard of care for glioblastoma (GBM) therapy includes the use of temozolomide concurrent with radiation therapy (RT), following optimal surgery. This regime increases median patient survival, but it has also increased the occurrence of pseudo-progression (PsP), making decisions regarding further treatment difficult and often delayed. None of the current imaging methods for assessing PsP have been proven sensitive and specific. *Methods*: Citrated blood (3 ml) was collected from GBM patients at the time of their RT simulation and at clinically appropriate time points during and following treatment. Blood was processed using multiple centrifugations (300 g, 2,500 g and 15,000 g) to collect MV. MV are defined herein as having a diameter >250 nm. The pellet from the final spin was analysed using flow cytometry. Antibodies to phosphotidylserine, platelets, RBC and EGFR were used to further describe the MV. *Results*: We have studied 53 blood samples on 14 GBM patients as well as 3 normal volunteers, the latter in duplicate. The mean of the control samples was $41{,}700 \pm 6{,}200$ MV/ml blood. Five patients had at least 4 MV samples analysed, with at least one sample following the completion of chemo-RT. Based on MRI and/or pathological assessment, 2 patients have recurred, 2 patients have stable disease and 1 patient is being followed for PsP vs. tumour progression (TP). MV counts in the stable patients and those being followed for PsP vs. TP show an overall downward trend over time, with later samples $<50{,}000$ MV/ml. In contrast, the MV count in patients who have progressed show an increasing trend over time, with multiple values $>50{,}000$ MV/ml. Elevated MV counts preceded MRI diagnosis of TP by 20–40 days. *Summary/conclusion*: At the current time, only imaging modalities are being investigated to assess PsP. These preliminary data suggest that the analysis of blood for MV may be useful to distinguish PsP from TP in patients with GBM.

P3C-066

Characterization of protein expression on glioblastoma multiforme exosomes and evaluation of methods for their isolation

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Introduction: Glioblastoma multiforme (GBM) is the most aggressive form of brain tumour. Their resistance to conventional treatments and high recurrence rate has been attributed to the finding of cancer cells with stem cell-like properties. The GBM stem cells are functionally defined by their ability to self-renew tumour-initiating properties and their shared genes characteristic neural stem cells. *Methods*: Exosomes were isolated from 2 commercially available GBM cell lines (U87 and U251) and an in-house generated GBM stem cell line (C16) using 3 different methods for comparison. The methods included ultracentrifugation, Total Exosome Isolation kit (Life Technologies) and ExoSpin (Cell Guidance Systems). The particle yield was evaluated by nanoparticle tracking analysis (NTA), and the shape of exosomes isolated with Total Exosome Isolation Reagent was visualized using atomic force microscopy. Characterization of the protein composition was analysed with EV array and mass spectrometry. *Results*: Particle yield was significantly lower when isolating exosomes with sequential ultracentrifugation compared to isolation with commercial kits. NTA showed differences in particle concentration from GBM stem cells compared to the commercially available cell lines, while AFM showed a rounded shape of the exosomes. Finally, protein analysis of the exosomes showed expression of several different proteins, for example, a proliferation-associated protein was found to be expressed on exosomes from all GBM cell lines evaluated. *Summary/conclusion*: The results of this study provide new insight into the characteristics of GBM exosomes, which could aid in the understanding of GBM tumourigenesis and identify new biomarkers or targets for treatment.

P3C-067

Differential effects of exosomes from stressed and non-stressed cancer cells, and from astrocytes, on brain cancer cell viability

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Introduction: Cancer transformation leads cells to selfishness behaviour, like unrestricted growth and exploitation of the intercellular signalling. Exosomes (EX) emerge as pivotal player in cells secretome. EX contain a quantum of molecules/signals that are suitable for delivering complex information. *Methods*: EX were isolated from brain tumour cell-lines and primary glioma stem cells, neurons and astrocytes by ultracentrifugation and filtration. The EX extract purification was verified by electron microscopy, NTA, sucrose gradient and western blots. Their uptake was quantified by fluorescent labelling. *Results*: Low stress ($<10\%$ cell death) induced with tunicamycin (Tu) (1 μg/ml), etoposide (Et) (5 nM) or H_2O_2 (20 μM) increase EX release from cancer cells by 1.3-fold. Adding low stress EX (lsEX) derived from Et and H_2O_2 treated cells to naïve cancer cells prior to insults like Tu (1–4 μg/ml), Et (25–70 nM) and H_2O_2 (100–400 uM) significantly decreased cell death, while EX derived from non-stress cells had no effect and lsEX derived from Tu-treated cells increased naïve cancer cell death. lsEX derived from human primary glioblastoma stem cells induce even higher protection. Thus, it seems that EX serve as a paracrine, anti-stress signal. Naturally, tumour cells are not alone in the neighbourhood. We found that astrocytes and neurons uptake cancer-derived EX in a time- and dose-dependent manner. Neuronal uptake depends on electrical activity as TTX abolishes it. Culture media of astrocytes treated with tumour-derived EX, but not neuron-derived EX, protect cancer cells from Et and H_2O_2. *Summary/conclusion*: Brain cancer cells use EX to alarm their neighbours regarding oncoming insults and to manipulate astrocytes and, possibly neurons, to generate a supportive microenvironment. All examined stresses enhanced EX release, but only lsEX derived from ER stress were toxic, which suggest a different set of EX. We postulate that cancer cells hijack exosome signalling to promote their own survival and growth. Interfering to this signalling may serve as a novel anti-tumour therapy.

Arcadis Room
Poster Session 10A - Late Breaking
Chair: *Yong Song Gho and Julia Gross* 13.00-14.00

P10A-0670

Phospholipids of tumour extracellular vesicles stratify of gefitinib-resistant non-small cell lung cancer cells from gefinitib-sensitive cells

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Introduction: Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) such as gefitinib are one of gold standard treatment options for non-small-cell lung cancer (NSCLC) patients, which eventually fail due to the acquired resistance and relapse because of the development of secondary activating mutations such as T790M in EGFR. Predicting chemo-responsiveness of cancer patients provides a major challenge in chemotherapy. The goal of the present study is to determine whether phospholipid signatures of tumour extracellular vesicles (EVs) are associated with gefitinib resistance of NSCLC. *Methods*: A sophisticated mass spectrometry-based shotgun lipidomic assays combined with bioinformatics approach were performed for in-depth analysis of the lipidomes of gefinitib-resistant PC9 and gefinitib-responsive PC9 and those of shed EVs from these cell lines. *Results*: Lipid matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) analysis showed that phospholipids in whole lysates from gefinitib-resistant NSCLC cells (PC9R) are similar to those from gefinitib-sensitive cells (PC9), while phospholipids in EVs shed from PC9R were significantly distinct from those shed from PC9. Following computational approaches identified 35 (20 positive and 15 negative ion mode) differentially regulated lipids (DRLs), which are significantly over- or underexpressed in PC9R EVs compared to PC9 EVs ($p < 0.01$, fold change > 1.5). DRLs in positive mode (e.g. PC{P-36:2} [M+H]+, PC{42:6} [M+Na]+, PC{40:7} [M+H]+, PC{32:3} [M+H]+, and PC{44:6} [M+Na]+) or in negative mode (e.g. PG {P-20:0/20:4} [M−H]−, PI {14:0/18:2} [M−H]−, and PI {18:1/14:0} [M−H]−), were significantly overexpressed in EVs shed from PC9R, compared to those from PC9. *Summary/conclusion*: Our phospholipid signatures suggest that EV associates with drug sensitivity, which is worthy of additional investigation to assess chemoresistance in patients with NSCLC treated with anti-EGFR TKIs.

P10A-0671

Impact of extracellular vesicles from leukaemia cells on coagulation and multidrug resistance transfer

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Introduction: Extracellular vesicles (EVs) play a major role during tumorigenesis. They have been associated with some cancer underlying mechanisms such as angiogenesis, inflammation, chemoresistance, metastasis and coagulation. These characteristics are partly due to their ability to impact target cells via cytoplasmic or surface constituents. About coagulation induction, it has been suggested that EVs bearing tissue factor and phosphatidylserine favour thrombosis in cancer patients. Several studies also showed a transfer of multiresistance proteins, miRNA and mRNA from multiresistant cells to sensitive ones via EVs. Chemoresistance and hypercoagulation state are major issues for the treatment of acute myeloid leukaemia (AML). To study the role of EVs in these two AML features, two strains of the promyelocytic leukaemia HL60 cell line have been analyzed: 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 was first investigated by transmission electron microscopy. To evaluate procoagulant activity of EVs from HL60/AR (EVs/AR), thrombin generation assays were performed on divers EVs fraction separated by size via filtration. The chemoresistance transfer via EVs/AR was also analyzed by treating HL60 with EVs/AR isolated by ultracentrifugation. Viability of HL60 after daunorubicin treatment was then assessed by a MTT cytotoxicity assay. *Results*: No procoagulant activity of HL60/AR and EVs/AR has been highlighted, demonstrating no procoagulant phenotype acquirement compared to HL60 cells and EVs, previously reported as non-coagulant. At the highest doses of daunorubicin investigated (0.8 and 1 μM), EVs/AR treatment confers a benefit to HL60, 72 hours after treatment. Indeed, the viability of EVs/AR-treated cells was significantly higher than viability of non-treated cells at these concentrations. *Summary/conclusion*: To our knowledge, this is the first study that demonstrates a real benefit of EVs from resistant cells on sensitive cell viability. A potential transfer of MRP1 via EVs/AR will be investigated by flow cytometry.

P10A-0672

Integrated -omics analysis uncovers a fundamental hypoxia-driven mechanism of breast cancer progression

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Introduction: Extensive studies have been conducted to understand breast cancer progression. One remaining open question is to precisely understand how hypoxia (low oxygen level), a pivotal microenvironmental factor, is involved in this process. In this work, we attempt to identify the critical genes (driver genes) and underlying mechanism involved using a systems-based approach. *Methods*: We developed a strategy based on quantitative proteomics of breast cancer cell lines presenting distinct aggressiveness properties under normoxia/hypoxia, in combination with a large-scale migration screen (siRNA screen) and protein network analysis. *Results*: We found that the less aggressive cell line temporally regulated cell migration under hypoxia and the underlying mechanism involved the PTPRG driver gene. We identified by siRNA screen a novel migration component enriched in lysosomal genes that suggested a mechanism of cell migration based on the vesicular trafficking of proteins. We dissected the underlying mechanism by phosphoproteomics and found that the regulation profile of the microtubule destabilizing protein stathmin discriminated the poorly invasive from the highly invasive cell line. Unexpectedly, stathmin knockdown dramatically enhanced cell migration of poorly invasive cells only under hypoxia. *Summary/conclusion*: These results led us to propose a model of cancer progression based on the hypoxia-dependent trafficking of vesicles containing migratory proteins

along microtubules. This study represents the first integrative-level attempt to understand the mechanism of cell migration under hypoxia and demonstrates that hypoxia might inhibit the early stages of cancer progression but promotes progression at later stages.

P10A-0673

Evaluation of the renoprotective effect of murine bone marrow mesenchymal stem cells, EVs and conditioned medium in a model of chronic nephrotoxicity

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Introduction: Mesenchymal stem cells (MSCs) treatment showed improvement of acute kidney injury, but few studies were done in chronic kidney disease models. MSC-induction of kidney repair seems to be mainly mediated by the release of soluble factors. Moreover, exchange of extracellular vesicles (EVs) between differentiated cells and stem cells could play a relevant role. *Aim*: To study the renoprotective effect of murine bone marrow (BM) MSC, EVs derived from them and their conditioned medium (CM) in a chronic nephrotoxic model induced by cyclosporine A (CsA). *Methods*: Murine BM-MSCs were isolated and their EVs and CM were obtained. Mice model was developed treating male C57BL/6 + CsA 75 mg/Kg and + BM-MSC, CM or EV treatments, in two different regimens: preventive or regenerative (4 and 2 dose, respectively). BUN levels and histopathological evaluation were determined. Fibrosis, apoptosis, regeneration, inflammation and homing were analyzed by qRT-PCR. *Results*: BM-MSCs, EVs and CM therapies showed 15% of survival improvement but not a significant decrease of renal injury except the regenerative therapy with BM-MSCs. Gene expression levels of TGF-β, caspase-3 and iNOS did not show variations between groups. PAI-1 and TIMP-1 levels were down-regulated in therapies vs. CsA alone. EGF was down-regulated and HGF up-regulated in EVs preventive and in CM regenerative and preventive treatments but not in others. IFN-Y expression was up-regulated in all groups except in EVs regenerative treatment. TLR4 levels were up-regulated in all groups, and TLR3 was up-regulated only in the EVs regenerative therapy. *Summary/conclusion*: Histological evaluation of kidney sections showed strong tissue damage because of the nephrotoxic effect of the CsA. Although we cannot reverse tissue damage, we can see an improvement in the survival and renal function of CsA-treated mice especially with BM-MSCs. It would be necessary to work with lower doses of CsA to evaluate the regenerative effect of EVs.

P10A-0674

Exosome-mediated therapeutic use of miR-7 in pancreatic disease

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Introduction: miR-7 is a highly conserved microRNA that is preferentially expressed in brain and pancreas, where it has been associated to cell growth, differentiation and hormone production. In this context, miR-7 has been proposed as a novel target for the treatment of diabetes, since its down-regulation correlates with induction of β-cell replication through the promotion of mTOR signalling in adult human islets. Exosomes (EXOs) are promising vehicles for the delivery of therapeutic microRNAs or anti-microRNA molecules. Adipose-derived mesenchymal stem cells (ADSCs) are a rich source of EXOs. In addition, the anti-inflammatory and immunoregulatory properties attributed to these cells may also be related to their EXO production. We have engineered ADSCs to have a continuous source of miR-7 and anti-miR-7-loaded EXOs and evaluated their potential for the proliferation and hormone production of pancreatic cells. *Methods*: Stable transgenic cells were obtained by lentiviral transduction of TRIPZ (Thermo Fisher) vector-based self-made constructs of miR-7, anti-miR-7 and irrelevant sequence under standard conditions. Beta-TC3 cells and ADSCs (obtained with the non-enzymatic Lipogems technology) were cultured in DMEM supplemented with 10–20% FBS under standard 37°C 5% CO_2 conditions. EXOs were isolated from FBS-free supernatants by ultracentrifugation, visualized by nanosight tecnnology and characterized by CD63 content. Total RNA (RIN > 8) was isolated with the miRVana kit (Ambion). Q-RT-PCR amplification of miRNAs was performed in a LightCycler® 480 (Roche) with the miScript SYBR Green RT-PCR system (Qiagen) and the corresponding specific primer under standard amplification conditions. *Results*: We have generated transgenic betaTC-3 pancreatic cells and ADSCs capable of highly inducible expression of miR-7 or anti-miR-7. The lentiviral system used allows for the evaluation of micro-RNA long-term effects. EXOs isolated from these cells have shown to efficiently pack these micro-RNAs as evidenced by real-time PCR quantification. Overexpression of anti-miR-7 but not of an irrelevant sequence stimulated proliferation of betaTC-3 pancreatic cells as shown by growth curve analysis, MTT assays and increased phosphorylation of histone H3. Conversely, overexpression of miR-7 inhibited cell growth. In addition, stimulation of insulin production and regulation of expression of other known targets of miR-7 were correlated with overexpression of anti-miR-7. *Summary/conclusion*: This work shows that pancreatic cells and ADSCs can be engineered to direct the loading of therapeutically active microRNAs into EXOs. This constitutes the first step towards the development of anti-miR-7-loaded EXOs-based therapeutics for the treatment of diabetes. The possibility of using autologous ADSC-derived EXOs could provide additional anti-inflammatory benefits to patients.

P10A-0675

Raftosomes: vesicles formulated from cell membranes with controlled surface composition for targeted drug delivery

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Introduction: As natural messengers between cells, extracellular vesicles provide a promising vehicle for targeted drug delivery. However, a key barrier to realizing their full potential is the risk of off-target effects due to the heterogeneous protein composition of the vesicle surface. In this study, we formulated vesicles from cell membranes, using only lipid rafts so that protein composition is restricted. We achieved targeting capability by inserting exogenous ligands into these vesicles, or 'raftosomes'. *Methods*: Extracted membrane fragments enriched in lipid rafts were assembled into vesicles. Exogenous ligands were inserted chemically by lipidation, or genetically via expression of a transmembrane construct that includes a peptide projecting into the extracellular space. Functional integrity of the ligand and targeting specificity of the engineered

raftosomes were assessed using cells expressing the corresponding receptor. Small molecules were introduced before or after raftosome formation, and release kinetics was assessed by dialysis. *Results*: Raftosomes were spherical and sized between 100 and 500 nm. Engineered raftosomes triggered target cell signalling in a concentration-dependent manner only when the raftosomes presented the corresponding ligand. Release of small molecules from the raftosomes was slow yet sustained for periods exceeding 7 days. *Summary/conclusion*: We have modulated the protein composition of the vesicle surface by simultaneously excluding non-raft proteins and inserting exogenous ligands. Unlike fusion proteins, the exogenous ligands function independent of endogenous proteins, enabling surface ligand expression to be fine-tuned. Functional integrity of the ligands further enables directed targeting of raftosomes to receptor-expressing cells with attendant sustained release of small molecules. This platform technology offers versatile control over the composition of the vesicle surface, thereby opening new opportunities for cell-derived vesicles as a targeted drug delivery system.

P10A-0676

Expansion of nucleated cells and CD34+ haematopoietic stem/progenitor cells from human cord blood induced by MSC-derived microvesicles ex vivo

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Introduction: Mesenchymal stem cell-derived microvesicles (MSC-MVs) rather than transdifferentiated MSCs have been shown to participate in tissue repair process in the animal model of acute kidney injury, which indicates a potential role of MSC-MVs with biological properties from their parental cells in cytotherapies. Since MSCs have been reported to expand nucleated cells and CD34+ haematopoietic stem/progenitor cells (HSPCs) in human cord blood, we hypothesized that MSC-MVs could also mimic the beneficial effect of MSCs on the expansion of these haematopoietic cells. For this purpose, we assessed the biologic effect of MSC-MVs on the nucleated cells and CD34+ progenitor cells in human cord blood. *Methods*: Human MSC-MVs, MSCs or MSC-MVs+MSC were co-cultured with nucleated cells or CD34+ progenitor cells from human cord blood, respectively. The effects of MSC-MVs on the proliferation of nucleated cells and CD34+ progenitor cells were evaluated, and the proportions of monocytes, granulocytes, T lymphocytes, B lymphocytes, natural killer (NK) cells and CD34+ cells in the nucleated cells were analyzed by flow cytometry. The effects of MSC-MVs on the abilities of maintenance, adhesion and chemotaxis of CD34+ progenitor cells were analyzed by colony formation assay, MTT test and transwell analysis, respectively. *Results*: MSC-MVs significantly promoted ex vivo expansion of the nucleated cells and CD34+ progenitor cells, with no effects on the abilities of adhesion to endothelium and chemotaxis of CD34+ progenitor cells. The promotive effect of MSC-MVs at 10 çg/ml was not as strong as that of the parental MSCs, however, when these microvesicles could significantly enhanced the effect of MSCs the proliferation of CD34+ progenitor cells. Besides, 7 days of co-culture with 10 μg/ml MSC-MVs alone increased the proportions of monocytes and granulocytes in the nucleated cells, which seemed to mimic the beneficial properties of MSC. Moreover, 10 μg/ml MSC-MVs significantly decreased the proportions of T lymphocytes and NK cells than their parental MSCs, suggesting that MSC-MVs also play an important role in the immunomodulatory function of MSCs. *Summary/conclusion*: Our findings indicate haematopoietic supportive and immunosuppressive properties of MSC-MVs on nucleated cells and CD34+ progenitor cells in human cord blood. These MSC-MVs can be used as a potential therapeutic approach in umbilical cord blood transplantation (UCBT).

P10A-0677

Development of exosomes-mediated oligonucleotides delivery for therapeutic silencing of Huntington's mRNA

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Introduction: Huntington's disease (HD) is a neurodegenerative disorder caused by the autosomal dominant inheritance of the mutant *Huntingtin* (HTT) allele. HD pathology includes mutant HTT protein aggregations in the cytoplasm, transcriptional disruption, impaired vesicle trafficking and mitochondrial defects in neurons. A promising therapeutic approach is RNAi against HTT mRNA. However, the most serious limitation of oligonucleotide-based therapies (ONTs) is the inefficient transit of oligonucleotides to the neurons. To improve ONT efficiency, we exploit the natural mechanism by which exosomes traffic small RNAs across cellular boundaries. *Methods*: In a screening of hydrophobically modified, small interfering RNA (hsiRNA) duplexes, we identified a hyperfunctional compound targeting both mouse and human HTT mRNA. Based on self-delivery properties of the oligonucleotide, we developed a novel approach to efficiently load exosomes derived from a glioblastoma cell line (U-87) with hsiRNA. Loaded exosomes were characterized by Nanosight and Zeta-Sizer (Malvern), which confirmed expected size distribution with a decrease in membrane zeta-potential. Electron microscopy of exosomes loaded with biotinylated hsiRNA shows the distribution of hsiRNA both inside and on the surface of exosome membranes. Incubation of hsiRNA-loaded exosomes with FVB mouse primary cortical neurons resulted in dose-dependent, efficient target silencing of HTT. *Results*: Kinetic uptake of hsiRNA-loaded exosomes, visualized by high-resolution imaging, occurs over several hours, and the hsiRNA–exosome complex is distributed to perinuclear areas. HsiRNA alone enters cells within minutes, initially localized to cell membranes, and spreads diffusely throughout the cell over several hours. Preliminary data after in vivo striatal injection of hsiRNA-exosomes complexes into wild type FVB mice brain demonstrated neuronal uptake in striatal and cortical tissue after 24 hours. *Summary/conclusion*: In conclusion, in both in vitro and in vivo experiments, hsiRNA loaded into exosomes appears to follow a distinct uptake pattern from hsiRNA alone. Exosome-based ONTs offer a promising, alternative delivery mechanism to hsiRNA alone.

P10A-0678

Techniques for detection and analysis of extracellular vesicles using flow cytometry

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Introduction: Flow cytometry (FCM) is a commonly used method for analyzing extracellular vesicles (EVs); however, accurate characterization of EVs remains challenging due to their small size and lack of discrete positive populations. Here we report the use of optimization techniques that are especially well-suited for analyzing EVs from a high volume of clinical samples. *Methods*: Platelet-poor plasma (PPP) was isolated from patient samples using a two-step centrifugation process. Samples were stained with one or more antibody-conjugated fluorochromes and analyzed using FCM. *Results*: We show that Ab filtration removes aggregates without compromising the effectiveness of the dye to label antigen-expressing cells. Furthermore, we found Ab pre-filtration to be slightly more effective at removing aggregates than a centrifugation method described in the literature: for the antibodies CD14, CD16, CD41a, CD108a, and CD235a, centrifugation removed 79–95% of aggregates on average,

whereas filtration achieved reductions of 99–100% on average ($p < 0.0001$). To reduce background fluorescence, we found that using filters to wash samples post-staining improved overall resolution of positive EV populations by enhancing detection of absolute counts by up to 40% and percentages of total EVs by >300% in some cases. Next, we demonstrate that lysed samples can be a useful alternative to isotypes for setting gates to separate negative from positive populations: in our hands, lysed controls worked as well as isotype controls for setting gates to eliminate background fluorescence 72% of the time (83/116) and better than isotype controls in 28% of cases examined (33/116). Lastly, we showed that events detected in platelet- and red blood cell-derived EV gates were directly proportional to dilution factor: plotting event count against dilution factor yielded slopes approximating -1 (R2 of 5 donors = 0.9998, 0.9952, 1.000, 0.9977, and 0.9949). Importantly, we determined that recording event count within a fixed time frame rather than collecting a set number of events with varied times of acquisition was key to yielding highly reproducible results across a wide range of concentrations in both beads and PPP. *Summary/conclusion*: (1) Centrifugal filters provide a useful alternative to current methods for removing false-positive events caused by Ab aggregates. (2) Filters provide a faster and effective alternative to ultracentrifugation and sucrose gradients for washing unbound Ab from EV samples. (3) Drawing gates around positive populations is aided by detergent lysis, which not only reveals false positive events caused by non-EVs but provides a good approximation of background fluorescence to distinguish positive from negative populations. (4) PPP samples do not need to be normalized for EV concentration prior to FCM analysis, as long as absolute numbers of positive events are counted within a fixed time frame. Taken together, the use of these optimized techniques enhances the accuracy and efficiency of EV detection using FCM.

P10A-0679

Size and concentration analysis of EVs by nanoparticle tracking analysis: a variation study

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Introduction: Cells release different types of extracellular vesicles (EVs). Exosomes are released after fusion of multivesicular bodies with the plasma membrane, whereas microvesicles bud directly from the plasma membrane. EVs, which may be used diagnostically for different disease states, are small in size and heterogeneous, thus presenting challenges for isolation and analysis. Nanoparticle tracking analysis (NTA) may be used for rapid determination of size and concentration of EVs. Here, we aimed to evaluate intra- and inter-assay variation of NTA measurements of different biological EVs and commercially available artificial vesicles/beads. *Methods*: Exosomes from PC-3 cells and Jurkat cells were isolated by sequential ultracentrifugation or precipitation, respectively. Microvesicles from human monocytes were isolated by centrifugation (17,000 g). EVs were processed in particle-depleted environments, aliquoted and stored at −80°C. Artificial vesicles (Invivofectamine® 2.0, Invitrogen), 100 nm polystyrene latex beads (Duke Scientific Corp.) and 150 nm silica microspheres (Polysciense, Inc.), all stored at +4°C, were also analyzed. Biological EVs were thawed on ice, further diluted in particle-free PBS to obtain a concentration within the recommended range ($3-10 \times 10^8$ particles/mL), and subsequently analyzed by the same operator on an NTA instrument (NS500, Malvern), using a syringe pump. Intra-assay variation of vesicle size and concentration measurements was determined analyzing samples sequentially within one day (n = 6). Inter-assay variation was assessed by analyzing sample aliquots on different days (n = 6). The results were reported as coefficient of variation (% CV) of mean vesicle size (nm), mode size (nm) and concentration (particles/mL). *Results*: (1) Size measurements: The mean and mode sizes with inter-assay CVs for biological EVs were for PC-3 cells (128 nm, 3%, 94 nm, 2%), Jurkat cells (112 nm, 2%, 79 nm, 5%) and monocytes (147 nm, 4%, 104 nm, 4%). The mean and mode sizes with inter-assay CVs were for artificial vesicles (86 nm, 2%, 76nm, 6%), 100 nm polystyrene latex beads (99 nm, 1%, 94 nm, 2%) and 150 nm silica microspheres (139 nm, 3%, 130 nm, 2%). (2) Concentration measurements: The inter-assay CVs of the mean vesicle concentrations from PC-3 cells were 18%, Jurkat cells 14% and monocytes 10%, whereas the CVs for artificial vesicles, polystyrene latex beads and silica microspheres were 15, 13 and 11%, respectively. Compared to inter-assay variation, intra-assay variation was overall better for all samples tested (data not shown). *Summary/conclusion*: The biological EVs showed, as expected, a heterogeneous size distribution using NTA. The inter-assay CVs of size measurements ranged from 1 to 6% for both biological EVs and artificial vesicles/beads. The inter-assay CVs of measured concentrations for biological EVs ranged from 10 to 18%, and for artificial vesicles/beads from 11 to 15%. In conclusion, the NTA measurements showed good repeatability.

P10A-0680

Morphological characterization of exosomes and microvesicles using transmission electron microscopy, flow cytometry and proteomic approaches

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Introduction: Exosomes (Exs) are cell-derived vesicles (originating usually as multivesicular bodies within the endosomal compartment) that are present in perhaps all biological fluids and also in conditioned media of cell cultures. Microvesicles (MVs) are larger than exosomes and originate from plasma membrane. Both types of extracellular vesicles (EVs) are composed of various proteins and lipid species reflecting the cell of origin and contain various cargos (DNA, RNA, protein). MVs and Exs are important and only recently appreciated mode of intercellular communication and thus have been receiving a growing interest also from clinical researches. However, the methods of isolation, characterization and quantification vary considerably in the literature and new standards need to be defined. Moreover, there is a lack of studies correlating the morphological appearance of these vesicles to their content and possible function. *Methods*: MVs and Exs are isolated by differential ultracentrifugation followed by a purification step in sucrose/D_2O cushion from the ascitic fluids, blood and cultures of cells. Quantification of exosomes (without coupling to beads) was done using Apogee A50/Micro flow cytometer. Next, morphological properties are assessed using negative contrasting and transmission electron microscopy (TEM). Finally, protein content is investigated using mass spectrometry (MS/MS) and western blotting (WB). *Results*: EVs isolated from human samples exhibit a great variety of morphological types and are present in body fluids in different concentrations as determined by TEM and flow cytometry of free Exs and MVs, respectively. Based on their protein content and TEM appearance, both Exs and MVs can be divided into several groups. *Summary/conclusion*: Here we present a novel approach in characterization of extracellular vesicles in which we established methods for their quantification using microflow cytometry and correlating morphological properties to protein (and lipid) content of Exs and MVs. This opens possibility for reliable stratification of Exs and MVs based on their morphology, and for extensive experimental

work on how the above mentioned characteristics reflect themselves in biological or pathological function of EVs.

P10A-0681=OP3-129

ACE2 mRNA expression in urinary exosome from patients with kidney disease

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P10A-0682=OP3-132

Urine microvesicle isolation: effect of storage and THP on RNA yield

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Scientific Program 2014 ISEV meeting
Thursday 1st May 2014
Oral Presentations

Registration 7:30-8:30

Arcadis room
Setting up posters (Poster Sessions 4A, 4C, 5A, 5C, 6C, 10B, 11) 7:30-8:30

Sponsor Exhibition Jurriaans Foyer/Mandele room 10:00-18:00

Willem Burger room
Symposium session 4A - EV and tumour development
Chair: *Aled Clayton and Peter Kurre* 8:30-10:00

O4A-140

Role of exosome-mediated tumour–stroma interaction in osteosarcoma

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Introduction: Osteosarcoma (OS) is a very aggressive malignant bone tumour that develops primarily during childhood and adolescence. It is characterized by rapid growth, a strong tendency for recurrence and an extremely high metastatic potential. In the past 3 decades, OS survival in the presence of metastasis has stagnated at a dismal 30% and no major breakthrough in the treatment of OS has been reported. To stop OS mortality, alternative therapeutical approaches need to be explored. Because OS occurs at sites of rapid bone growth during the adolescent growth spurt, microenvironmental factors and stromal cells such as mesenchymal stem cells (MSC) may have a defining role in OS development and progression but have been poorly investigated. We hypothesized a role for exosomes as microenvironmental factor in the intercellular cross-talk between OS cells and MSCs. *Methods*: The RNA content of OS exosomes was analysed by deep sequencing. The effect of purified exosomes on MSC cell cycle and cytokine expression was assessed by fluorescence-activated cell sorting and qPCR, respectively. To evaluate the effect of MSC conditioning on tumour growth and progression, we injected exosome-conditioned MSCs in a bioluminescent mouse OS xenograft model. *Results*: Our study suggests that tumour-secreted exosomes promote OS progression by reprogramming MSCs into a pro-tumourigenic phenotype. The deep sequencing analysis revealed that OS exosomes have a very complex composition dominated by tRNA, rRNA, mRNA and miRNA. Interestingly, highly abundant miRNAs have been implicated in cell cycle regulation, differentiation and oncogenesis. Consistently, upon internalization, OS exosomes stimulate cell cycle progression and induce the expression of IL6, a cytokine associated with tumour-inflammation. Preliminary results suggest that exosome-conditioned MSCs promote tumour growth and progression in a bioluminescent OS xenograft model. *Summary/conclusion*: Our studies lay a groundwork for studying tumour exosomes function in a pre-clinical mouse model of aggressive OS.

O4A-141

Chronic myeloid leukaemia–derived exosomes promote tumour growth and survival through an autocrine mechanism

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Introduction: Cancer cells can generate their own signals in order to sustain their growth and survival. Several studies have revealed the role of cancer-derived exosomes in activating signal transduction pathways involved in cancer cell proliferation and survival. Chronic myeloid leukaemia (CML) is a myeloproliferative disorder

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characterized by the expression of the chimeric BCR-ABL oncoprotein with constitutive tyrosine kinase activity. We have previously shown that CML cells released exosomes able to affect tumour microenvironment. *Methods*: Cell lines used in experiments are LAMA84, a human CML cell line. MTT assay was performed after LAMA84 serum deprivation and up to 1 week of exosomes treatment; colony formation assay was performed in methylcellulose to assess the capabilities of CML-derived exosomes to promote the formation of LAMA84-colonies. NOD-SCID mice were subcutaneously injected with LAMA84 cells and exosomes inoculation was performed in tumour site, twice a week; tumour growth was measured and tumours were removed to extract RNA. RNA was extracted from LAMA84 cells, colonies and mice biopsies. Quantitative gene expression analysis for pro- and anti-apoptotic genes was performed by TaqMan RT-PCR. *Results*: CML cells, exposed to serum deprivation and treated up to 1 week, with exosomes, result in a growth advantage. Moreover, exosomes treatment promotes the formation of LAMA84 colonies in methylcellulose. *In vivo* experiments show a greater increase in tumour size in mice treated with exosomes, compared to control. Real time PCR analyses, performed after RNA extraction from both *in vitro* and *in vivo* samples, show an increase of mRNA levels of anti-apoptotic genes, such as bcl-w, bcl-xl and survivin and a reduction of pro-apoptotic genes, bad, bax and puma. *Summary/conclusion*: CML derived-exosomes promote, through an autocrine mechanism, the proliferation and survival of tumour cells, both *in vitro* and *in vivo*, by activation of anti-apoptotic pathways.

O4A-142

Patterning the tumour microenvironment via exosomal transmission

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Introduction: Several publications have shown that tumour-derived exosomes facilitate cancer progression by altering the tumour microenvironment. We have previously shown that colorectal cancer cell–derived exosomes contain and can transfer oncogenic KRAS to recipient wild-type KRAS cells, leading to alterations in cell behaviour, including inducing significantly greater levels of transformation as assessed by cell growth in 3-dimensional matrix and cell invasion in Boyden chamber assays. *Methods*: This work uses various metabolic measurements; proteomic and RNAseq analyses; fluorescence-activated vesicle sorting (FAVS) and various microscopic techniques to delineate how exosomes might alter the tumour niche. *Results*: We have extended our previous studies to demonstrate that KRAS tumour status regulates both the metabolic and RNA patterning within the tumour niche. First, we show that the metabolic constituents of exosomes are altered with KRAS status, and Warburg effect–like changes are exhibited by wild-type KRAS-expressing recipient cells when treated with mutant KRAS exosomes. These results suggest exosomes from colorectal cancer cells can alter the tumour environment in ways that might facilitate cancer progression by altering the overall metabolism of surrounding tumour cells. Second, by RNAseq, we determined the RNA constituents present within exosomes purified from mutant and wild-type KRAS-expressing colon cells and find correlative changes in exosomal RNA content depending on KRAS status of the donor cell. *Summary/conclusion*: The implication of these RNA changes in both microRNAs and longer coding and non-coding RNAs have potential consequences for regulating cancer progression, which will be discussed. These results have important implications for how tumour-derived exosomes act to facilitate cancer progression through tumour growth, invasion and metastasis. This work has been funded by the NIH grant 1R01 CA163563-01A1 and the common fund 1U19 CA179514-01.

O4A-143

miR-134 transported in extracellular vesicles reduces triple-negative breast cancer aggression

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Introduction: Previously, we reported that extracellular vesicles (EVs) from triple-negative breast cancer (TNBC) cell line, Hs578T, and its more aggressive variant, Hs578Ts(i)$_8$, increase the aggression of recipient cells in a manner mirroring the cells of EV origin. This observation was clinically validated using EVs from TNBC sera. Here, we have globally profiled the cellular and EV miRNAs contents, aiming to identify and assess those of potential therapeutic value. *Methods*: miRNA profiling was performed by TaqMan low density array on RNA from Hs578T and Hs578Ts(i)$_8$ cells and their respective EVs. Direct miRNA transfections were performed with lipofectamine. EVs from the transfected cells were isolated and phenotypic (invasion, migration and drug response) alterations they conferred were analysed; comparing the effects of direct cellular transfection to effects of EV exposure in inducing phenotypic alterations. Publically available miRNA datasets of breast cancer patients confirmed *in vitro* observations. miRNA levels in EVs from TNBC patients' sera were analysed by qPCR. miRNA protein targets predicted *in silico* were confirmed by immunoblotting. *Results*: Most (79%) of the miRNAs detected in Hs578T cells were identified in their EVs; similarly, 75% of those in Hs578Ts(i)$_8$ cells were identified in Hs578Ts(i)$_8$ EVs. Sixty-eight miRNAs were commonly down-regulated in Hs578Ts(i)$_8$ cells and EVs compared to Hs578T cells and EVs, respectively. A number of miRNAs were validated by qPCR with miR-134 emerging as potentially important. Both direct transfection of miR-134 into Hs578Ts(i)$_8$ cells and Hs578Ts(i)$_8$ cell treatment with miR-134-enriched EVs reduced the expression levels of STAT5B and Hsp90, subsequently reducing TNBC aggression. *Summary/conclusion*: miR-134 loss plays a functional role in increasing TNBC aggression. Encapsulating miR-134 in EVs may provide a therapeutic strategy by reducing oncoprotein levels to thus reduce TNBC aggression.

O4A-144

Tumour-derived exosomes are enriched in DNp73 which promotes oncogenic potential in acceptor cells and correlates with patient survival

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Introduction: Tumour-derived exosomes are emerging as local and systemic cell-to-cell mediators of oncogenic information through the horizontal transfer of mRNAs, microRNAs and proteins during tumourigenesis. The exosomal content has been described as biologically active when taken up by the recipient cell. Identifying the specific molecular cargo of exosomes will help to determine their function in specific steps of the tumourigenic process. *Methods*: Here, we evaluate whether ΔNp73 is selectively packaged in tumour-derived exosomes, its function in the acceptor cells *in vitro* and *in vivo* and its prognosis potential in cancer. *Results*: ΔNp73 messenger is enriched in tumour-derived exosomes, suggesting its active sorting in these microvesicles. We observed the transmission of this exosome cargo to different cell types and how it confers proliferation potential and chemo-resistance to the acceptor cells *in vitro* and in animal models. *Summary/conclusion*: Finally, our data

support the potential prognostic value of exosomal ΔNp73 in colon cancer patients.

O4A-145

Calpain inhibition: a new strategy to circumvent microparticle-mediated multidrug resistance in cancer

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Introduction: Microparticles (MPs) are released from most eukaryotic cells through the outward blebbing of the plasma membrane and serve as vectors of long- and short-range signalling. MPs derived from multidrug resistant (MDR) cancer cells carry molecular components of the donor cell such as regulatory nucleic acids, membrane proteins and receptors, and can alter the activity of drug-sensitive recipient cells through the transfer of their cargo. The calpains, a family of cysteine proteinases, play a key role in MP production, and calpain inhibitors have previously been shown to significantly reduce the number of MPs released in activated platelets. Given their role in MP blebbing, we hypothesize that the inhibition or modulation of calpain activity is a promising alternate treatment target and strategy in the management of MDR in cancer. This study examines the inhibitory effect of calpain inhibitors on MP production in MDR cancer cells. *Methods*: MDR breast adenocarcinoma cells were incubated with commercially available calpain inhibitors. MPs were isolated from the supernatant through differential centrifugation and analysed with flow cytometry, using Annexin-V450 and BD TruCount™ beads for validation and quantification. *Results*: MPs were validated with phosphatidylserine expression. MDR breast adenocarcinoma cells exposed to a known calpain inhibitor showed a significant dose-dependent reduction in MP number. Cells remained viable at all concentrations tested. *Summary/conclusion*: Our findings demonstrate that MP production in MDR cancer cells can be suppressed by cellular exposure to calpain inhibitors. This work holds potential for the development of an alternate treatment strategy in the management of MP-mediated MDR in cancer. Future studies will include the synthesis and screening of novel calpain inhibitors against MP production in MDR cell lines.

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Jurriaanse room
Symposium session 4B - EV cargo selection and release mechanisms 1
Chair: *Steve Gould and Willem Stoorvogel* 8.30-10.00

Introduction (15 min) by Clotilde Thery

O4B-146

Dissecting the Wnt-trafficking routes onto extracellular vesicles

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Introduction: Spatially and temporally restricted secretion of morphogens, such as proteins of the Wnt family, governs differentiation of cell populations into heterogeneous types of tissues. Wnt signalling also contributes to a variety of pathological processes during tumour development and metastasis. We recently demonstrated how hydrophobic Wnt proteins are released in the extracellular space on exosomes, but the trafficking routes of cargo onto extracellular vesicles remain to be clarified. *Methods*: We combined surface biotinylation with inhibition of MVB sorting to follow secretion of Wnt proteins onto extracellular vesicles. These were purified by differential centrifugation, quantified and analysed by western blotting, electron microscopy and mass spectrometry. *Results*: Membrane-bound Wnts are sorted via the plasma membrane and are present on both types of extracellular vesicles, exosomes and microvesicles. Using chemical inhibitors and RNAi, we found different factors regulating their biogenesis and lipid composition. Furthermore, we investigated functional implications of Wnts packaged either on exosomes or microvesicles. *Summary/conclusion*: Using a potent signalling molecule such as Wnt as an examplary exosomal and microvesicular cargo protein allows to unravel the trafficking routes leading to extracellular communication by extracellular vesicles.

O4B-147

Live-visualization of exosome release dynamics with a pH-sensitive fluorescent reporter

Frederik Verweij[1], Juan Garcia-Vallejo[2], Serena R. Baglio[1], Hans Janssen[3], Jacques Neefjes[3], Jaap Middeldorp[1], Matthijs Verhage[4], Ruud Toonen[4] and Michiel Pegtel[1]
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Introduction: Tumour-derived exosomes are increasingly recognized as important players in cancer progression, promoting metastasis formation and stimulating invasive behaviour of cancer cells. However, lack of insight in the regulatory control and exosome release dynamics have hampered more direct demonstrations of their physiological properties. *Methods*: We designed a pH-sensitive optical reporter (CD63-pHluorin) to visualize exosome release from cancer cells following multivesicular body (MVB) fusion with the plasmamembrane (PM) on single cell level. *Results*: Live total internal fluorescence (TIRF)-microscopy demonstrates that soluble components of the tumour microenvironment (TME) stimulate MVB-PM fusion and consequent exosome release by activating GPCR signalling. MVB-PM fusion requires the SNAP23/Syntaxin-4 fusion machinery and SNAP23 phosphorylation. Notably, a defined pool of exosomes is retained within the extracellular matrix of cancer cells upon MVB-PM fusion. This pool contains high levels of MMPs and thereby has the potential to induce matrix remodelling and facilitate cancer cell migration and invasion. *Summary/conclusion*: Thus, live-visualization of MVP-PM fusion improves our understanding of the regulation and fate of tumour exosomes by revealing how TME components can promote exosome-mediated cancer cell invasion.

O4B-148

Lipid regulators prevent extracellular vesicle budding in *C. elegans* embryos

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Introduction: Cells release extracellular vesicles (ECVs) that can influence differentiation, modulate the immune response, promote coagulation and induce metastasis. Many ECVs form by budding outwards from the plasma membrane, but the molecules that regulate budding are poorly understood. In ECVs, the outer leaflet of the membrane bilayer contains phospholipids that are normally sequestered to the inner leaflet of the plasma membrane, suggesting a potential role for lipid asymmetry in ECV budding. We previously showed that the phospholipid flippase TAT-5 provides a molecular link between loss of phosphatidylethanolamine (PE) asymmetry and the dynamic budding of vesicles from the plasma membrane, providing further support for this hypothesis (Wehman et al., 2011, Curr Biol). *Methods*: In order to identify additional genes that regulate ECV budding, we used the *C. elegans* embryo as a genetic model system for ECV budding. We took advantage of transgenic reporter strains that make ECVs visible by light microscopy and used both RNA interference and deletion mutants to discover new regulators of ECV budding. *Results*: We discovered 2 additional proteins that prevent ECV budding. One protein is a conserved cofactor of TAT-5 with unknown function, further confirming the identification of TAT-5 as a potent regulator of ECV budding. The second regulates phosphatidylinositol (PI) biosynthesis, suggesting that PIs may also play a role in ECV budding. We are currently putting these genes into a pathway and discovering how they interact with each other to regulate ECV budding. *Summary/conclusion*: In conclusion, *C. elegans* genetics provides a novel approach to rapidly identify new proteins and lipids involved in ECV budding. Our research suggests that lipids and lipid regulators are important determinants of plasma membrane budding to form ECVs.

O4B-149

Senescence-associated exosome suppress tumour growth

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Introduction: Exosomes, lipid membrane vesicles secreted from cells, are known to act as a tool for cell-to-cell communication. To reveal the biogenesis mechanism and biological activity of exosomes during the cellular ageing process, we used human normal fibroblast

as an in vitro aging model and characterized the exosomes derived from replicative senescent fibroblasts. *Methods*: We isolated exosomes from human fibroblasts, TIG-3 cells by ultracentrifugation and measured the number and the size of exosomes using qNano, nanopore-based particle analyzer. siRNA for Maspin and Chmp4 are transfected using RNAiMax reagent. *Results*: We found that the size of the exosomes secreted from both young and senescent fibroblasts are very similar; however, senescent fibroblasts secreted larger number of exosomes than young cells. To elucidate the mechanisms for the increase in exosome production in senescent cells, we focused on Maspin and Chmp4C which are known as exosome regulatory gene. These genes were up-regulated in senescent fibroblasts, and, significantly, knockdown of Maspin or Chmp4C by siRNA lead to reduction of reduced exosome secretion in senescent cells. Further, forced expression of these genes enhanced exosome secretion in young fibroblasts. Taken together, these results show that Maspin and Chmp4C regulate exosomes secretion during replicative senescence. Surprisingly, we found that senescence-associated exosome inhibits tumour growth as well as tumour invasion of cancer cells. *Summary/conclusion*: Our current data suggest that senescence-associated exosome have a function of tumour suppression.

O4B-150

RAL-1 and the exocyst mediate exosome secretion in *C. elegans*

Vincent Hyenne[1], Maxime Diem[1], Ahmet Apaydin[1], Yannick Schwab[2] and Michel Labouesse[1]
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Introduction: Extracellular vesicles, including exosomes, can be secreted by various species and participate in cell–cell communication and matrix remodelling. Exosomes arise from the fusion of multi-vesicular bodies with the plasma membrane. However, the molecular pathways involved in MVB formation, their targeting to the plasma membrane, as well as their attachment and fusion remain unclear. *Methods*: To address this question, we use the nematode *C. elegans* as a model organism. *C. elegans* allows a combination of genetics, live imaging and electron microscopy in a multicellular organism. Our laboratory has previously shown that, presumably, exosomes are released at the apical surface of epidermal cells and contribute to the formation of a particular cuticular structure of the worm, the alae. *Results*: We conducted an RNAi-based screen for alae defects in *C. elegans* and identified over 60 genes that could be involved in exosome biogenesis. We focused our work on the evolutionarily conserved exocyst complex and its regulator, the GTPase *ral-1*, which are known to mediate vesicle tethering at the plasma membrane. We found that depletion of several components of the exocyst or depletion of *ral-1* results in alae defects. Similarly, overexpression of either a constitutively active (CA) or a dominant negative (DN) form of RAL-1 induced alae defects, suggesting that the GTPase activity of *ral-1* is required for exosome secretion. We show by live confocal microscopy that both RAL-1 and the exocyst component SEC-8 partially co-localize with the non-exclusive marker of the exosomal compartment in *C. elegans*, the proton pump VHA-5. Interestingly, RAL-1(DN) fully co-localizes with VHA-5, while this is not the case for RAL-1(CA). Electron microscopy analysis of *ral-1* RNAi and mutant worms suggests that *ral-1* could be involved both in the formation and in the secretion of MVBs. In addition, we show that *ral-1* is required to recruit the syntaxin SYX-5 at the plasma membrane, a protein known to be involved in membrane fusion. *Summary/conclusion*: Altogether, our results suggest a model where *ral-1* controls the exocyst mediated attachment of MVBs to the plasma membrane and the recruitment of SYX-5 at the site of attachment to fuse MVB membrane with the plasma membrane and liberate exosomes in the extracellular space.

Van Weelde/Mees room
Symposium session 4C - EV therapeutics 2
Chair: *Raymond Schiffelers and Yong Song Gho* 8.30-10.00

O4C-151

Transmigration of cell-derived membrane vesicles in tumours mediates tissue penetration of compounds

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Introduction: The major obstacle in increasing the efficacy of anti-cancer treatment lies in poor tumour penetration of therapeutic agents. In solid tumours, high interstitial fluid pressure and dysfunctional lymphatic system impede delivery of therapeutic molecules into the deep parenchyma. Despite numerous attempts to engineer synthetic nanocarriers for tumour-specific drug delivery, significant quantities of therapeutic compounds delivered systemically remain in the perivascular region, reducing the overall therapeutic effect. Naturally occurring particles play an important role in transporting biological materials within the body. In particular, membrane vesicles secreted from tumour cells migrate between them and deliver biological information for tumour progression. If we were to exploit these naturally occurring transport processes to guide the delivery and penetration of therapeutic compounds in tumours, we could overcome the abovementioned problem faced by conventional synthetic nanocarriers in drug delivery systems. *Methods*: For photodynamic therapy in vitro, tumour spheroids were treated with photosensitizer-loaded membrane fusogenic liposomes for 30 min and further incubated over 48 h. The spheroids were then irradiated with a 660-nm laser source for 5 min and the cell viability was tested using MTT assay. For tumour penetration study *in vivo*, membrane fusogenic liposomes loaded with hydrophobic dye were intravenously injected into the mouse bearing tumours. At 48 h post-injection, the tumours were excised and the tissue sections were prepared to observe tumour distribution of the dye. *Results*: Here, we show that the tumour-derived membrane vesicles can be engineered *in situ* to carry both hydrophobic and hydrophilic cargoes by treating their parent tumour cells with cargo-loaded membrane fusogenic liposomes, and mediate intercellular transmigration of the cargoes in tumour tissue. The hydrophobic photosensitizer delivered to the plasma membrane of peripheral cell layers of tumour spheroids penetrated dramatically into the centre by the membrane vesicles and induced significant phototherapeutic effect throughout the entire spheroid. Furthermore, the hydrophobic compounds delivered to tumour vasculatures by membrane fusogenic liposomes *in vivo* penetrated significantly into the tissue, presumably via tumour-derived membrane vesicles. *Summary/conclusion*: We believe that this innate biological transport system provides new insights to delivery and penetration of chemotherapeutics in solid tumours.

O4C-152

The development of exosomes as a therapy to treat metastatic melanoma

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Introduction: Metastasis causes 90% of cancer-related deaths, and novel therapies are in high demand. Exosomes and other extracellular vesicles are responsible for subverting distant microenvironments in order to support metastatic growth. We hypothesize that depending on their cargo, exosomes convey pro- or anti-metastatic effects. Pigment epithelial-derived factor (PEDF) is a type-2 tumour suppressor lost in multiple types of cancer. The expression lines links the higher PEDF expression levels with non-metastatic, poorly invasive melanoma. We have generated exosomes loaded with PEDF and assessed their therapeutic value against metastatic melanoma. *Methods*: Melanoma cells were engineered to express PEDF by lentiviral transduction. Exosomes were isolated by ultracentrifugation and characterized by dynamic light scattering (DLS), western blot and TEM. We used orthotopic and lung colonization models to demonstrate the anti-metastatic properties of exosomes *in vivo*. To identify the target organs and cell types, we labelled exosomes with a lipophilic fluorophore and analysed by immunostaining and fluorescence-activated cell sorting. The effect on specific cell types was determined using cell-based assays, Western blot and QPCR. *Results*: We achieved dramatic reduction in metastasis with PEDF exosomes treatment, in sharp contrast with control exosomes. Exosomes carrying PEDF homed to the bone marrow and lymph nodes. In bone marrow, PEDF caused a 7-fold increase in exosomes affinity to GR1+, CD11b+ myeloid cell population. *In vitro*, PEDF exosomes promoted maturation of the RAW 264.7 macrophages towards immunogenic (tumour-suppressive) M1 phenotype and inhibited inflammatory features of the tumour-associated macrophages. *Summary/conclusion*: Typically tumour-derived exosomes inhibit anti-tumour immunity. By loading melanoma exosomes with PEDF, we shift their biological activity from immunosuppressive to immunogenic and eliminate pre-metastatic niche. We are currently investigating the underlying molecular mechanisms.

O4C-153

Extracellular vesicle-mediated reversal of the malignant phenotype in prostate cancer

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Introduction: Extracellular vesicle (EV) trafficking is a fundamental cellular process that occurs in cells and is required for different aspects of pathophysiology. EV trafficking leads to changes in cellular function, including apoptosis, angiogenesis and proliferation required for increased tumour formation. In this study, we evaluated EV-mediated reversal of the malignant phenotype, drug resistance and inhibition of tumour growth in prostate cancer models. *Methods*: EVs were isolated from prostate cell lines and explant human prostate cells. EVs were co-cultured with recipient cells for 7 days and phenotypic changes including soft agar colony formation (anchorage independent growth), drug sensitivity and tumour xenograft studies performed. Transfer or induction of EV-specific proteins that may be responsible for phenotypic changes were identified via antibody and Mass spectrometry analysis. MTT assays were used to determine drug sensitivity. Mass spectrometry analysis of protein lysates using ProteoIQ was employed to identify protein candidates associated with gene ontology annotations that may be responsible for phenotypic changes. Ingenuity Pathway Analysis determined

statistically relevant canonical pathways and functions associated the protein IDs and expression values obtained using ProteoIQ. Tumour xenograft studies were performed using athymic mice to determine the ability of EVs to block tumour formation. *Results*: We report several phenotypic changes mediated by EVs isolated from non-malignant and malignant prostate cells as well as patient biopsied prostate tumour samples. EVs isolated from non-malignant PrECs (Prostate Epithelial Cells) can reverse soft agar colony formation of malignant DU145 cells, with the reciprocal effect observed. Isolation of EVs from 2 Gleason grade 8 prostate cancer patients significantly induced soft agar colony formation of non-malignant PrECs. We have identified proteins via antibody and Mass spectrometry analysis that may be responsible for the phenotypic changes. Western blot analysis confirmed the increase of 14-3-3 zeta, pRKIP and prohibitin protein levels in PrEC cells co-cultured with patient EVs. 14-3-3 proteins were also found as common proteins of 3 other Gleason grade 8 patients. Knockdown of 14-3-3 zeta lead to partial reversal of soft agar growth, indicating that it is a candidate for targeted therapy. Co-culture with EVs isolated from non-malignant RWPE-1 prostate cells inhibited DU145 tumour xenograft growth and reversed paclitaxel resistance. *Summary/conclusion*: Our study provides a rational basis for the use of EVs to reverse drug resistance, the malignant phenotype and to therapeutically inhibit prostate cancer progression.

O4C-154

Comparison of immunogenicity and anti-tumour efficacy of two DNA vaccines coding for antigens secreted in different membrane vesicle–associated forms

Clotilde Thery[1,2], Christine Sedlik[1,2], James Vigneron[3], Lea Torrieri-Dramard[3], Fabien Pitoiset[3], Jordan Denizeau[1], Caroline Chesneau[1], Philippe de La Rochere[1], Sebastian Amigorena[1,2], Olivier Lantz[1,2] and Bertrand Bellier[3]
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Introduction: The induction of an active immune response against cancer to control or eliminate tumours is still an unfulfilled challenge. We focused on plasmid DNA vaccines and developed a novative approach whereby the antigen is expressed in association with extracellular vesicles (EVs) to facilitate antigen cross-presentation. Two forms of an EV-associated antigen (OVA) have been compared: (a) OVA fused to the lipid-binding domain C1C2 of lactadherin is exposed on the surface of secreted membrane vesicles (*Zeelenberg et al., 2008, Cancer Res 68: 1228*), (b) OVA fused to retroviral Gag capsid protein is incorporated inside virus-like particles (VLP) that bud from the plasma membrane (*Bellier et al. 2006, Vaccine 24: 2643*). *Methods*: Electroporation-mediated intramuscular DNA vaccination was done in mice and OVA-specific immune responses were analysed by different immunoassays (ELISPOT, tetramers, ELISA). OVA-expressing mouse tumour cell lines were injected subcutaneously or intravenously, and tumour growth and metastasis were measured. *Results*: Both DNA vaccines induced with similar efficiency OVA-specific $CD8^+$ T cells and antibodies. Interestingly, we observed different IgG1/IgG2b ratio among OVA-specific antibodies suggesting that the 2 DNA vaccines control the polarization in different ways. Nevertheless, both OVA-C1C2 and Gag-OVA DNA vaccines were able to prevent efficiently *in vivo* outgrowth of subcutaneous tumours. More importantly, in therapeutic settings, immunization with either DNA vaccine reduced tumour progression but with variable efficacies depending on the tumour models. *Summary/conclusion*: Our 2 DNA vaccines leading to Ag secreted in association with EVs induced efficient immune responses but with some qualitative differences, and both controlled similarly tumour development. DNA vaccines should be combined with chemotherapy treatment to still improve their efficacy in therapeutic setting and be closer to pathological situations.

O4C-155

Improved therapeutic efficacy of dendritic cell vaccination by loading with laeukemia exosomes

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Introduction: Leukemia immunotherapy frequently does not meet expectation, one of the handicaps being tumour exosome (TEX)–promoted immunosuppression. We here asked, using the mouse laeukemia WEHI3B, whether dendritic cell (DC) vaccination suffices to counterregulate TEX-induced immunosuppression and whether TEX could serve as tumour antigen for DC loading. *Methods*: DCs were generated from bone marrow cells and were loaded with tumour lysate or TEX. TEX were isolated by standard protocols including sucrose density gradient purification from WEHI3B culture supernatant. WEHI3B-bearing mice were vaccinated with tumour lysate- or TEX-loaded DC or with TEX. Survival time and rate as well as immune response induction were monitored ex vivo. Lysate and TEX processing by DC were analysed *in vitro*. *Results*: DC-vaccination significantly prolonged the survival time of WEHI3B-bearing mice, TEX-loaded DC (DC-TEX) being superior to lysate-loaded DC (DC-lys), even an excess of TEX not interfering with immune response induction. The superior response to DC-TEX was accompanied by an increase in WEHI3B-specific $CD4^+$ T cells, evaluated by trogocytosis and proliferation. TEX had no negative impact on bone marrow cell-derived DC maturation in vitro and supported CD11c, MHCII and IL12 up-regulation. Importantly, TEX was more efficiently taken up by DC than tumour lysate, and uptaken TEX was recovered for a prolonged period and was, at least partly, recruited into the MHCII-loading compartment. *Summary/conclusion*: TEX did not drive DC into a suppressive phenotype and was a superior antigen due to highly efficient TEX-presentation that is supported by prolonged persistence in DC and preferential processing in the MHCII-loading compartment. As TEX can easily be recovered from patients' sera, it provides an optimized, individual-specific antigen source for DC-loading.

O4C-156

Mesenchymal stromal cells deliver exogenous miRNAs to neural cells via exosomes: mechanisms of effects and therapeutic impact

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Introduction: MicroRNAs (miRNAs) emerge as potential therapeutic targets in a variety of pathological conditions in the brain; however, their clinical application is hampered by lack of efficient delivery modes. Mesenchymal stem cells (MSCs) migrate to sites of injury and inflammation in the brain and exert therapeutic effects in a variety of pathological conditions. We recently demonstrated the ability of MSCs to functionally deliver miRNA mimics to glioma cells in vitro and in vivo. In this study we examined the ability of MSCs to deliver exogenous miRNA mimics and pre-miRNAs to human neural progenitor cells (NPCs) and astrocytes and characterized the functional and therapeutic impact of this delivery. *Methods*: MSCs were transfected with Cy3-labelled miR-124 mimic or pre-miR-124 and transferred to Green Tracker-labelled NPCs, and astrocytes was determined using fluorescence microscopy and FACS analysis. The delivery of miR-124 was further demonstrated using novel reporter plasmids which contain a sequence fully complementary to miR-124 downstream of luciferase or mCherry so that binding of miR-124 to this sequence results in decreased luciferase activity or fluorescence. These plasmids enable analysis of miRNA delivery in living cells both in vitro and in vivo. The role of gap junctions and exosomes was determined. The functional delivery of miR-124 was examined using

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a SOX9 3′-UTR reporter plasmid and by demonstrating effects on cell differentiation. In vivo impact was analyzed in Parkinson's disease and ALS animal models. *Results*: Using fluorescent-labelled miR-124 mimic and novel miR-124 reporter plasmids, we found that MSCs derived from different sources efficiently delivered this miRNA mimic to co-cultured NPCs and astrocytes after 24 hr in co-culture. The delivered miR-124 mimic significantly decreased the expression of the target gene Sox-9 and increased the neuronal differentiation of the NPCs as determined by the increased expression of beta3-tubulin and the decreased expression of nestin. In addition, the delivered miR-124 increased the expression of the glutamate transporters, EAAT1 on NPCs and EAAT2 in both NPCs and astrocytes. The increased expression of the glutamate transporters increased the glutamate uptake by the cells. Similar results were obtained with MSCs transfected with pre-miR-124. The miRNA delivery was mostly mediated by MSC-derived exosomes and was not gap junction dependent. In vivo studies demonstrated beneficial therapeutic impact of MSCs loaded with miR-124 in mouse models of ALS and Parkinson's disease. *Summary/conclusion*: These results suggest that MSCs can functionally deliver exogenous miRNAs to neural cells and provide an efficient route of therapeutic miRNA delivery to the brain in pathological conditions with clinical implications for regenerating medicine.

Networking coffee	Arcadis room	10:00-10:30
Poster Viewing Sessions 4A, 4C, 5A, 5C, 6C, 10B, 11 posters not-attended by authors		10.00-10.30 Arcadis room

Willem Burger room
Symposium session 5A - EV in infection and immune disorders
Chair: *Jeffrey Schorey and Natasha Barteneva* 10.30-12.00

O5A-157

***Legionella pneumophila* infection of macrophages alters the amount and miRNA content of released exosomes**

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Introduction: *Legionella pneumophila* (*L. pneumophila*) is a common cause of severe community- and hospital-acquired pneumonia. Upon its transmission to the human lung, *L. pneumophila* primarily enters and replicates in macrophages; however resolution of the infection requires multiple cell types and abundant cross talk between immune cells. Among other cellular interactions, secreted membranous vesicles have recently emerged as means of transport for functional proteins, messenger RNAs (mRNAs) and miRNAs between cells. *Methods*: In this study, we quantified and qualified membranous vesicles secreted by the macrophage cell line THP-1 in response to *L. pneumophila* infection. For this, we enriched the secreted microparticles and exosomes by differential centrifugation. Sizing and enumeration of particles were performed by nanoparticle-tracking analysis and electron microscopy. The protein content of the exosome fraction was further characterized by western blot. The miRNA profile of exosomes released by infected blood-derived macrophages compared to non-infected controls was analysed by miRNA arrays. In addition, the effect of the exosome fraction of infected versus non-infected THP-1 cells on THP-1 or A549 cells was analysed in comparison to the effect produced by the cytokine-containing supernatant. *Results*: Our study reveals changes in the quantity of released exosomes after *L. pneumophila* infection and in their miRNA profile. Furthermore, exosomes from infected cells are able to induce an inflammatory response in recipient cells. *Summary/conclusion*: This provides a new means of communication between the different cell types in an alveolus during infection with *L. pneumophila*.

O5A-158

Ubiquitination of soluble proteins within the phagocytic/endocytic network as a mechanism for trafficking to exosomes

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Introduction: Exosomes are extracellular vesicles formed from the fusion of multivesicular bodies with the plasma membrane and play a role in intercellular communication. Our previous studies indicate that exosomes released from *M. tuberculosis*-infected macrophages contain soluble mycobacterial proteins that can function in modulating the innate and acquired immune response. However, it was unclear how these normally secreted proteins are targeted to exosomes within infected macrophages. *Methods*: Extracellular vesicles were enriched for exosomes by differential centrifugation followed by filtration through a 0.2 micron filter. *Results*: In the present study, we determined that this protein targeting is dependent on ubiquitination and that exosome production by macrophages requires Tsg101 and HRS; known ESCRT components. Moreover, the soluble mycobacterial proteins HspX and GroES when added exogenously to macrophages were ubiquitinated and released from cells via exosomes. The trafficking of these mycobacterial proteins to exosomes was ubiquitin dependent and could be abolished by mutation of specific lysine residues. This suggested that endocytosed proteins could be released from cells through exosomes; a process not previously described. This hypothesis was supported using the tumour-associated protein He4 which when endocytosed by macrophages was found to be ubiquitinated and released from cells on exosomes. Again the trafficking of He4 was dependent on its ubiquitination. Finally, HspX but not GFP, when endocytosed by HEK293 cells, was trafficked to exosomes in an ubiquitin-dependent manner. *Summary/conclusion*: Our data suggest that ubiquitination is an important mechanism for targeting of soluble proteins within the phagocytic/endocytic network to exosomes and that proteins can be "recycled" by incorporation into exosomes.

O5A-159

Extracellular vesicles and small RNA of the cervicovaginal compartment during HIV infection

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Introduction: Components of cervicovaginal secretions may predispose to or protect against infection by HIV and other pathogens. Extracellular vesicles (EV, including exosomes and microvesicles) are found in every biofluid examined, carry cargo including proteins and RNA and may participate in intercellular signalling. Comparatively little is known about EV and their RNA cargo in the cervicovaginal compartment or how they relate to HIV infection. *Methods*: Five cervicovaginal lavage (CVL) samples from HIV-positive women were compared with samples from matched uninfected individuals in this pilot study. Whole CVL and EV fractions enriched by stepped ultracentrifugation were assessed by nanoparticle tracking analysis, and total RNA was obtained from each fraction 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*: Significantly smaller numbers of particles in the ultracentrifugation-enriched EV fraction of CVL ($p = 0.016$ by bootstrapping) were associated with HIV infection. Overall, particle counts also trended lower. miRNA content of CVL was low: <75 miRNAs detected in total CVL and <20 consistently found in EV fractions. Several miRNAs, including miR-106b and miR-223, the latter of which has putative anti-HIV-1 properties, were detected only in uninfected samples. *Summary/conclusion*: Our results are consistent with mucosal disruptions in HIV infection that may include a decline in EV trafficking. In light of these findings and reports of EV involvement in intercellular signalling and HIV-associated processes, we hypothesize that EV help maintain a healthy cervicovaginal environment. Additional studies of cervicovaginal EV and their RNA cargo are merited to characterize the potential role of EV and specific small RNAs in modulation of HIV infectivity and transmission.

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O5A-160

Mesenchymal stem cell-enriched exosomes mediate beneficial effects in a GvHD patient and in animal models for different inflammation-related diseases

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Introduction: More than 300 NIH registered clinical trials applied mesenchymal stem cells (MSCs) to treat a variety of different diseases such as myocardial infarction, stroke and graft-versus-host disease (GvHD). Initially, MSCs were thought to replace lost cells in damaged tissues. Despite contrary reports regarding the outcome of MSC treatments, MSCs seem to exert beneficial effects by the secretion of immunosuppressive factors and/or extracellular vesicles (EVs) such as exosomes. Indeed, we have successfully treated a steroid-refractory GvHD patient with EVs/exosomes enriched from MSC supernatants (*MSC-exos*) (Kordelas et al., in press). *Methods*: We are testing for the ability of *MSC-exos* to modulate symptoms of stroke in a murine model and to exert neuroprotective functions in preterm brain injuries in a neonatal rat model following LPS treatment. We also study impacts of *MSC-exos* on particle-induced osteolysis (causes aseptic loosening of joint replacements in humans) in the mice calvaria model. *Results*: Even though up to 100 times more *MSC-exos* per body weight unit were administered into the different animal models than in the patient, no side effects of repetitive *MSC-exos* treatments were detected. Remarkably, we observed beneficial effects in all systems. In the stroke model, we compared impacts of *MSC-exos* with those of the MSCs they were harvested from. Neither in histological sections nor in any of 3 behaviour tests, a significant difference between the beneficial effects of MSCs or their exosomes was observed. *Summary/conclusion*: We conclude that at least in some diseases, MSCs have been successfully applied to, exosomes and presumably other co-purified EVs exert their beneficial effects. Compared to MSCs, the application of exosomes provides a number of advantages: exosomes and other EVs are non-self-replicating and due to their small size they can be sterilized by filtration. In our on-going research we characterize the MSC-exosomes and their function in more detail.

O5A-161

Unique protein signature of extracellular vesicles in systemic lupus erythematosus

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Introduction: In the autoimmune disease systemic lupus erythematosus (SLE), impaired removal of subcellular material may contribute to disease pathogenesis. The aim of the current study was to quantify and characterize global protein signatures of EVs from SLE patients for comparison with EVs from healthy controls (HCs) and patients with other autoimmune diseases including rheumatoid arthritis (RA) and systemic sclerosis (SSc) in the search for new disease biomarkers and improved understanding of disease mechanisms. *Methods*: We profiled EV proteins from a total of 36 samples including patients diagnosed with SLE ($n = 12$), SSc ($n = 6$), RA ($n = 6$), and from HCs ($n = 12$). EVs were isolated from platelet-poor plasma (1) by 4 times wash by centrifugation at 18,890 × g, 30 min (2). Washed EVs were precipitated, resolubilized in urea and digested with endo-Lys C followed by continued digestion using trypsin. Digested samples were analysed by nano-LC-tandem mass spectrometry on an Orbitrap XL. Peptide data were processed for label-free protein quantification, and protein identification and were correlated with diagnosis and measures of disease severity. *Results*: We identify 530 unique proteins and demonstrate significant differences between HCs and SLE patients in the abundance of 268 proteins. Increased proteins in SLE-derived EVs include complement proteins, immunoglobulins, microtubule proteins, fibronectin, η isoform of 14-3-3 protein, ficolin-2 and galectin-3 binding protein. Proteins decreased in SLE-EVs include cytoskeletal, mitochondrial, and intracellular organellar proteins, Lamp1, and TGF β1. Immunoglobulin and complement associated with EVs clearly distinguish SLE-EVs from HCs and from RA and SSc cases and correlate with clinical SLE severity. Specific quantitation of galectin-3 binding protein by immunoassays confirm SLE- and disease activity-specific increase of this protein and a correlation with the expression of type I interferon-inducible gene subsets. *Summary/conclusion*: The EV protein profiles differentiate SLE patients from patients with other autoimmune diseases or HCs and show that the population of circulating EVs in SLE is atypical with fewer EVs of normal protein composition and with increased numbers of EVs that are heavily tagged for removal.

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O5A-162

Extracellular vesicles deliver pro-inflammatory viral RNAs (EBV-EBER1) into tubular epithelial cells of lupus nephritis patients

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Introduction: Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by a prolonged antiviral type I interferon response, chronic inflammation affecting multiple organs and a strong association with Epstein Barr Virus (EBV) infection. *Methods*: We investigated the role of EBV-EBER1 RNA in SLE pathogenesis. *Results*: We found that SLE patients have a distinct serology pattern consistent with deregulated EBV persistence. Moreover, we detected extracellular vesicles (EVs), in the serum of SLE patients that contain EBV-EBER1 transcripts. In vitro, non-cell autonomous EBER1 delivered through exosomes act as a potent inducer of type IFN-mediated inflammatory responses. Strikingly, SLE patients with lupus nephritis (LN) lack detectable EBER1-carrying vesicles in circulation. In LN tissues, we observed EBER1 accumulation

in the cytoplasm of tubular epithelial cells (TEC) while we failed to detect infiltrating EBV-infected cells, suggesting an exogenous source of EBER1. Apart from EBER1, we measured EBV-miRNAs in LN, but not in control tissues, suggesting EBV-modified EVs as origin and delivery device. In vitro experiments revealed that purified EBER1-containing EVs are rapidly endocytosed by primary TEC cells in a phosphatidylserine (PtdSer) Tim-receptor family dependent manner. Finally, incubation with both ivtEBER1-RNA and EBER1-containing EVs induced a type I IFN-mediated inflammatory response as evidenced by increased IL-6 production, a cytokine that plays an aggravating role in LN. *Summary/conclusion*: Thus, EBV-deregulation and production of pro-inflammatory EVs that target the TEC cells in LN tissues may aggravate local inflammation in LN kidney tissues providing new diagnostic and therapeutic opportunities for LN patients.

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Jurriaanse room
Symposium session 5B - EV as cancer biomarkers
Chair: *Dolores Di Vizio and Riccardo Alessandro* 10.00-12.00

O5B-163

Blood-based cancer diagnostic assay using digital PCR

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Introduction: Fluid biopsies are powerful because they allow for molecular characterization of tumours in a minimally invasive fashion. All tumours secrete extracellular vesicles (EVs) that are relatively stable in biofluids and can provide important information about the disease state. Tumour-derived EVs are a minor component in a pool of normal cell-derived EVs, so detecting tumour markers is a major challenge. Assay sensitivity and specificity are particularly imperative when they are used as companion diagnostic or for trial stratification. Brain tumours hold an extra level of challenge because of their enclosed location in the brain. We sought to determine the presence and levels of the mutant isocitrate dehydrogenase 1 (IDH1) in the plasma and cerebrospinal fluid (CSF) of patients with glioma. *Methods*: First, we isolated RNA from cell lines engineered to express either the mutant or wild type form of IDH1. The RNA was reverse transcribed and used as input for digital PCR, a highly sensitive and powerful technique for rare events and point mutations. To establish the limit of sensitivity for IDH1, we analysed a serial dilution of the RNA from cells and EVs. We also extracted RNA from a cohort of plasma and CSF samples of patients confirmed to have or not to have the mutation in their primary tumour. *Results*: We determined a robust sensitivity of 1 mutant copy in 10,000 wild type copies when using spiked in RNA from mutant and wild type cells. We also detected the mutant IDH1 in 63% of CSF samples. We are currently analysing plasma samples from IDH1 mutant glioma patients. We will present correlative studies between disease stage and the number of mutant IDH1 copies in circulation in an extended cohort of glioma samples. *Summary/conclusion*: This finding expands the diagnostic potential of circulating tumour EVs and demonstrates the great promise for this technology in next-generation companion diagnostics and mutational profiling of individual tumours.

O5B-164

Serum exosome miRNA signature as a multi-source biomarker of acute myeloid leukaemia

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Introduction: Current disease remission surveillance after treatment for acute myeloid leukaemia (AML) relies on cellular assays of bone marrow cells. We recently demonstrated miRNA enrichment in exosomes from AML patient cells and cell lines. In more recent AML xenograft studies, we found that miRNA levels in circulating exosomes correlated with the leukaemia burden in the bone marrow. Herein, we used a microarray survey of exosomal miRNA to improve the sensitivity and specificity of miRNA as a biomarker and expanded our investigations into AML patient serum and marrow stroma samples. *Methods*: AML engraftment in NSG mice, serum collection, tissue culture, exosome isolation, microarray, qRT-PCR and flow cytometry. *Results*: Initial investigations into AML exosomal miRNA comprised a broad survey using a microarray, revealing primarily (86%) mature miRNA. Several exosomal miRNA (miR-146a, -150, -155, -221) were chosen for serum detection. In serum exosomes from mice engrafted with AML, we found shifts in levels of these miRNA, describing a profile that correlated with disease burden. Compared to current biomarkers of disease (CBC, flow cytometry), exosomal miRNA proved to be an earlier discriminator. We next evaluated patient serum exosomes for these miRNA, and primary stromal cells from the patient samples were obtained. qRT-PCR for miRNA in exosomes produced by stromal cells revealed elevations among the candidate miRNA, supporting a previously unrecognized contribution to serum miRNA profile by AML patient stromal cells. Tests of exosome biomarker signature using xenograft models through therapy and remission cycles are currently on-going. *Summary/conclusion*: AML cells and marrow stroma contribute a group of primarily mature miRNA to the serum exosome miRNA profile. This represents, to our knowledge, the first biomarker that is representative of a transformed tissue microenvironment rather than exclusively derived from malignant cells.

O5B-165

Highly sensitive pancreatic cancer diagnosis by serum exosome stem cell and miRNA markers

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Introduction: Late diagnosis contributes to dismal prognosis of pancreatic cancer (PaCa), urging for reliable, early non-invasive detection. Serum-exosome (S-Exo) protein and/or miRNA markers might be suitable candidates. *Methods*: S-Exo was purified according to routine procedure. Pre-screening included S-Exo miRNA profile evaluation by microarray analysis, protein expression profiles were evaluated by flow cytometry. S-Exo of patients with PaCa, benign pancreatic tumours, chronic pancreatitis, non-Pa-malignancies and healthy donors (hD) were analysed for protein markers by flow-cytometry and for miRNA by qRT-PCR according to the results of pre-screening. *Results*: Pre-screenings showed high recovery of PaCIC markers and a panel of 4 miRNA in S-Exo pools of PaCa patients, though not hD. The PaCa-initiating cell (PaCIC) markers CD44v6, Tspan8, EpCAM and CD104 were expressed in 96% of PaCa patients S-Exo, but not in control groups, except non-Pa-malignancies. Recovery was tumour grading and staging independent including early stages. A panel of miR-1246, miR-4644, miR-3976 and miR-4306 was significantly up-regulated in 83% of PaCa S-Exo, but rarely in all control groups. Concomitant PaCIC and miRNA S-Exo marker evaluation significantly improved sensitivity (1.00) with a specificity of 0.80 for PaCa versus all others and of 0.92 excluding non-Pa-malignancies. *Summary/conclusion*: Combined PaCIC and miRNA S-Exo marker evaluation provides a highly sensitive diagnostic tool offering a breakthrough in PaCa diagnosis that may also account for other malignancies as suggested by the unexpectedly abundant CIC marker presence in S-Exo of cancer patients.

O5B-166

Identification of exosome-specific miRNA expression profile in bladder cancer cells and tumour-associated fibroblasts

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Introduction: Urinary bladder cancer (UBC) is histopathologically classified in non-muscle-invasive (70%) and muscle-invasive tumours (30%), which have a high risk for the development of metastases. Currently, there are no prognostic markers available which offer an early prognostic estimation of UBC. The therapeutic approaches depend on the metastatic status of the primary tumour. Interaction of tumour cells and the tumour microenvironment (TME) plays an important role in the metastasis. MicroRNAs (miRNAs), packed in exosomes, affect processes at the site of origin as well as the TME by cell–cell communication. The aim of the project is the identification of a specific miRNA expression pattern from *in vitro* obtained tumour-derived exosomes of different UBC cell lines and tumour-associated fibroblasts (TAFs). *Methods*: Exosomes were isolated from different UBC cell lines (RT-112, T-24) and TAFs by differential centrifugation. The number and size of vesicles were measured by NTA. The vesicles were examined for exosomal and contamination markers by western blotting. Total RNA was isolated from the exosomes upon treatment with or without RNase. Exosome-mediated miRNA transfer between cancer cells and TAFs was verified by (a) transfection of donor UBC cells with the *C. elegans*-specific miRNA, cel-miR-39, (b) Exosome isolation and RNAse treatment, (c) Transfer to recipient TAFs, and (d) miRNA-specific qRT-PCR analysis using total RNA from the recipient TAFs. *Results*: The isolated exosomes from UBC cells and TAFs exhibited a high amount of exosomal markers (CD63, CD81, syntenin) and less presence of contamination marker calreticulin. The external cel-miR-39 was degraded after RNase treatment. After successful transfection of RT-112 and T-24 with cel-miR-39 and transfer of RNase-treated exosomes, the presence of cel-miR-39 was observed in recipient TAFs. *Summary/conclusion*: Exosomes can be isolated from UBC cells lines and TAFs. The presence of miRNAs in different cells can be altered by transfer of exosomes from other cells. In summary, the results support the notion of exosome-mediated transfer of miRNAs in between cells constituting the TME.

O5B-167

Characterization of extracellular vesicles in chronic lymphocytic leukaemia

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Introduction: The pathogenesis of chronic lymphocytic leukaemia (CLL) is stringently associated with a tumour-supportive microenvironment. Of note, CLL cells themselves initiate tumour-promoting responses in surrounding cells, and extracellular vesicles (EVs) released by tumour cells represent a newly discovered mechanism of cell communication. As the role of EVs in the pathomechanism of CLL is unclear, we aim to characterize EVs present in blood plasma of CLL patients and released by MEC-1 cells, a CLL cell line, in order to understand their role within the microenvironment. *Methods*: EVs were isolated from blood plasma of CLL patients and healthy donors as well as from CLL cells in culture by serial centrifugation and density-based separation. Small RNA profiling was performed via small RNA deep sequencing. Proteome analysis was conducted via Coomassie-staining of SDS-PAGE gels and mass spectrometry. Uptake of PKH67-labelled EVs in myeloid cells and resulting phenotypic changes were evaluated via confocal microscopy and fluorescence-activated cell sorting analysis of myeloid differentiation markers, respectively. *Results*: We were able to isolate EVs from blood plasma of CLL patients and healthy controls as well as from MEC-1 cells. Small RNA profiling revealed a unique miRNA signature of EVs with the 5 most abundant miRNAs encompassing about 60% of all miRNAs and a selective enrichment of miR-146a in EVs. Proteome analysis indicated differences in EV and cellular composition and proteins potentially enriched in EVs are currently validated. Uptake studies showed a rapid internalization of CLL cell-derived EVs by human monocytes and macrophages and preliminary data suggest subsequent changes of macrophages towards an immune-suppressive phenotype. *Summary/conclusion*: EVs are potent vehicles in shuttling RNA and proteins to recipient cells and might be involved in the establishment of a tumour-promoting microenvironment, that is, differentiation of myeloid cells to a more suppressive phenotype.

O5B-168

Changes in subpopulations of extracellular vesicles by oncogene-targeted treatment of Melanoma cells

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Introduction: Malignant melanoma is one of the deadliest skin cancers that are developed due to UV light-induced mutations in the skin. $BRAF^{V600}$ mutations are present in more than half of the melanomas and are a key for treatment. Vemurafenib is a potent BRAFV600E kinase inhibitor that has been shown to improve progression-free survival. The aim of this study was therefore to characterize the extracellular vesicles (EVs) released by a MML-1 cell line treated with Vemurafenib to determine how this treatment affects tumour-released EVs and its effect on surrounding cells. *Methods*: Apoptotic Bodies (ABs), Microvesicles (MVs) and Exosomes (EXO) were isolated by differential centrifugation of cell supernatants. RNA was isolated using miRCURY RNA isolation kit, and quality was assessed by a Bioanalyzer. Characterization of exosomal markers and other proteins was performed by western blot and flow cytometry. *Results*: Treatment with Vemurafenib resulted in significantly increased amount of RNA and proteins in ABs, MVs and EXO as compared to the EVs from non-treated cells. Also, the RNA profiles showed a reduction in rRNA in the treated ABs and MVs compared to those untreated but no significant effect on RNA profile was observed in exosomes. Small RNA profiles indicate that greater quantities of miRNA were captured in ABs and MVs when treated, whereas no difference was seen for the miRNA levels in EXO. Western blot analysis showed that Melan-A was retained in all EVs with the highest expression in treated EXO, and TSG101 was mainly detected in EXO. Furthermore, expression of CD81 was only detected in exosomes indicating specific loading of CD81 into EXO but not ABs and MVs in MML-1 cells line. *Summary/conclusion*: This study provides the basis to determine the vesicular content during oncogene-specific therapy of melanoma cells, which can have implications for biomarker studies.

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Van Weelde/Mees room
Symposium session 5C - EV in tissue repair & remodelling
Chair: *Giovianni Camussi and Hans van Leeuwen* 10.30-12.00

O5C-169

Osteoblast-secreted extracellular vesicles stimulate the expansion of CD34$^+$ human umbilical cord blood cells

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Introduction: Umbilical cord blood (UCB) is increasingly used in haematopoietic stem cell (HSC) transplantations; however, the low cell numbers still remain a limiting factor for proper engraftment. Osteoblasts are major constituents of HSC niche and play an important role in regulating HSC self-renewal and differentiation. Recently, extracellular vesicles (EVs) have been implicated in stem cell fate regulation via horizontal transfer of proteins and nucleic acids between cells. Therefore, in this study, we focused on the characterization of human osteoblast EVs and investigated their potential in *ex vivo* expansion of human UCB-HSCs for clinical use. *Methods*: We used human pre-osteoblasts (SV-HFO cells) to isolate EVs by a series of ultracentrifugation steps. We studied the effect of osteoblast EVs on CD34$^+$ UCB cell expansion by single-platform counting and subset immunophenotyping using flow cytometry. To understand the EV function, we characterized osteoblast EVs by electron microscopy, proteomics, and miRNA sequencing, and investigated the molecular mechanism by qPCR and pathway analyses. *Results*: Treatment of CD34$^+$ UCB cells with osteoblast EVs led to 2-fold expansion of the phenotypic HSCs and 3- to 5-fold expansion of the CD34$^+$ expressing progenitors. Microscopic analyses demonstrated osteoblast EVs to be heterogenic in size and morphology. Mass spectrometry-based protein analyses identified an interesting range of novel osteoblast EV proteins primarily linked to ribosomal activity and RNA processing in addition to the well-known vesicle proteins. Moreover, EVs were enriched with small RNAs, and contained miRNAs known be involved in the regulation of early haematopoiesis. Interestingly, we discovered that EV treatment down-regulated the expression of HMG-box transcription factor 1 (HBP1), a miR-29a target in CD34$^+$ UCB cells. A quick pathway activity screen in HEK293 cells showed that Stat3 pathway was up-regulated upon EV treatment, which is of great interest for further analysis in CD34$^+$ UCB cells. *Summary/conclusion*: In this study, we demonstrated that osteoblasts secrete EVs that expand UCB-HSCs *ex vivo*, and uncovered the first clues that contributed to understand the molecular mechanism of EV function. Elucidating this molecular mechanism will provide us the means to increase the expansion efficiency and develop improved grafts for stem cell transplantations.

O5C-170

Age-dependent loss of microvesicular Galectin-3 and its consequences on bone formation *in vitro* and *in vivo*

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Introduction: Mesenchymal stem cells (MSCs) counteract the decline of physiologic functions but their regenerative power decreases with age. In particular, osteogenic differentiation capacity of MSCs has been shown to decrease with age thereby contributing to slowed down bone formation and to osteoporosis. While much is known about cellular ageing of MSCs, little is known about extrinsic factors influencing their functionality. Here, we set out to identify circulating factors of the aged systemic environment that influence osteogenesis. *Methods*: Isolation of extracellular vesicles by differential centrifugation followed by immunopurification. Determination of vesicular Galectin-3 levels by ELISA. *Results*: While searching for factors extracellular vesicles (EVs) were found. Exposition of MSCs to CD63-positive EVs isolated from plasma of human elderly donors failed to induce osteogenesis compared to EVs of young donors raising the question as to which age-dependent secreted vesicular components impact on MSCs functionality. We identified vesicular Galectin-3 as an influential component. Plasma and vesicular Galectin-3 levels were reduced in the elderly compared to young human donors, and we could demonstrate that vesicular Galectin-3 levels indeed impact on osteogenic differentiation capacity of MSCs. Overexpression of Galectin-3 in MSCs was shown to boost osteogenic differentiation capacity while reducing its protein expression by siRNA-inhibited osteogenesis *in vitro*. Moreover, intracellular Galectin-3 levels of MSCs correlated with their osteogenic differentiation potential. Nano-CT scan on Galectin-3 knockout mice revealed a reduction of femoral cortical as well as trabecular thickness compared to wild type littermates. *Summary/conclusion*: We showed that the composition of circulating EVs changes with age and that they deliver factors impacting on the osteogenic differentiation capacity of MSCs. We attributed the age-dependent impairment of osteogenesis by EVs to Galectin-3. Among other factors, osteogenesis boosting vesicular Galectin-3 was shown to be enriched within EVs isolated from young human donors. Reduction of vesicular Galectin-3 plasma levels with age might lead to a reduced uptake of Galectin-3 by MSCs, thus contributing to impaired osteogenesis with age.

O5C-171

Reversal of hematopoietic radiation damage by mesenchymal stem cell-derived extracellular vesicles

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Introduction: Recent studies have shown extracellular vesicles (EVs) from Mesenchymal stem cells (MSC) may mediate reversal of tissue damage. Here, we investigated the reversal of irradiation-induced murine marrow stem cell damage by murine MSC-EVs. *Methods*: We evaluated the effect of murine MSC-EVs on marrow from irradiated mice or on the marrow cell line, FDC-P1, irradiated *in vitro*. *Results*: B6.SJL mice were exposed to 0 or 100 cGy whole body irradiation and 7 days later lineage negative marrow cells were harvested and cultured with or without the addition of murine MSC -EVs for 48 h. The cells were then engrafted into 200 cGy exposed C 57BL/6J mice and engraftment was analysed out to 36 weeks post-transplant. EVs exposure showed 9.6% engraftment at 36 weeks, while non-EVs treated groups showed 3.7% engraftment. Murine marrow was also harvested 24 h after 100 cGy whole body irradiation, and cultured

with and without vesicles for 24 h. There was a significant increase in donor engraftment by the Lineage negative cells incubated with MSC-EVs at 3 and 6 months. The increase in engraftment with vesicle exposure persisted in serial transplantation. These studies indicate that MSC-derived vesicles can reverse radiation damage to marrow when administered either 1 or 7 days after irradiation. In in-vivo experiments, C 57BL/6 mice were exposed to 500 cGy total body irradiation. MSC-EVs were then injected intravenously 6, 24 and 72 h after irradiation. WBC recovery compared to non-irradiated mice was 17% in the non-vesicle group and 37% in the vesicle-injected group at 14 days indicating that vesicle injection enhanced leukocyte recovery following radiation damage. Marrow cells from the non-irradiated, the irradiated and the irradiated plus vesicle-treated mice were then analysed for gene expression. Twelve genes were elevated in the irradiated cells, and the elevations were reversed by vesicle exposure while 17 genes were depressed in the irradiated cells and the depressions reversed by vesicle exposure. Studies with the murine marrow cell line, FDCP-1, showed that apoptosis in irradiated cells was reversed by exposure to MSC-EVs and growth of irradiated cells was restored. *Summary/conclusion*: Our data indicate that MSC-derived vesicles have the capacity to ameliorate radiation-induced damage to marrow.

O5C-172

Impaired expression of miRNAs in mesenchymal stem cell-derived EVs reduced their regenerative potential in a model of acute kidney injury

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Introduction: Mesenchymal Stem Cells (MSC)-derived extracellular vesicles (EVs) favour recovery of acute kidney injury (AKI). It has been suggested that EV-mediated miRNA delivery contributes to this regenerative effect. To understand the role of miRNAs, we block the expression of Drosha, a critical enzyme of miRNA maturation, in MSC to alter miRNA composition within EVs. The aim of this study was to evaluate whether miRNA de-regulation affects the regenerative potential of MSC and of EVs in an *in vivo* AKI model *Methods*: MSC were transduced with an inducible RFP-lentiviral vector to knock-down Drosha (DHS-MSC). Control MSC were generated by transduction with a RFP-empty-vector. Surface proteins were characterized by fluorescence-activated cell sorting (FACS) and RNA profile by Bioanalyzer. EVs were isolated from DHS-MSC (DHS-EV) by differential ultracentrifugations and analysed by NTA and FACS to identify size and surface markers. EV-RNA was analysed by qRT-PCR for the expression of 750 mature miRNAs (Megaplex). Cells and EVs were then used in a model of glycerol-induced AKI. Glycerol was injected intra-muscle at day 0 in SCID mice, EVs or cells were then injected intravenously at day 3. Mice were sacrificed at day 5 and renal function (BUN and creatinine) and histological analysis (hyaline cast and tubular necrosis counts) were evaluated. *Results*: DHS-MSC maintained the capacity to differentiate into cells of mesodermal origin and the expression of classical surface mesenchymal markers. Characterization of DHS-EVs shows a size distribution of 150 ± 60 nm and expression of the same surface receptors of CTRL-EVs, supporting their ability to be up-taken by tubular cells. miRNA analysis showed the differential expression of 226 miRNAs between CTRL-EVs and DHS-EVs. DHS-EVs showed reduced levels of 140 miRNAs with respect to CTRL-EVs, but an unexpected up-regulation of 86 miRNAs, indicating a complex mechanism of packaging of miRNAs in EVs. Measure of damage markers and histological analysis revealed that injection of DHS-EVs or DHS-MSC, in AKI-induced mice, did not revert the renal injury even after 5 days, whereas CTRL-EVs and CTRL-MSC significantly improved function and morphology. *Summary/conclusion*: These data support the importance of miRNA-content of EVs in the regeneration process mediated by MSC.

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O5C-173

Enhancement of cell-proliferation by artificial exosomes engineered from embryonic stem cells

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Introduction: Exosomes (microvesicles) have known to deliver cellular contents as proteins and RNAs to target cells during intercellular signalling, and induce the target cells change in their characteristics. For example, some studies showed that ES cell-secreted exosomes improve proliferation of adult stem cells, whereas it is not clear that ES cell-secreted exosomes improve proliferation of stemness-deficient somatic cells. We hypothesize that higher doses of exosomes improve proliferation of stemness-deficient cells as well by delivering more RNAs and proteins to target cells. However, amount of cell-secreted exosomes is as small as 100 ng/10 million cells for a day. In this study, a large amount of artificial exosomes loaded with RNAa and proteins of ES cells was generated from ES cells, and treated to skin fibroblasts to enhance proliferation. Interestingly, the treated cells showed enhanced proliferation rate and secreted growth factors. *Methods*: Murine ES D-3 cells were used for generating the artificial exosomes. Skin fibroblasts were isolated from BL6/C57 mouse, and seeded on plate dishes. Various concentrations of the artificial exosomes were treated on the plate dishes to find the optimal condition for cell proliferation. Cell counting and CCK-8 assay were conducted to measure the number of proliferated cells. Secreted ECM protein and protein related to cell proliferation were shown by western blot, and TGF-ß was measured by ELISA to confirm changed cell activity after 2 days. *Results*: The artificial exosomes contained both cytoplasmic proteins and RNAs, especially Oct-4 and Nanog. The skin fibroblasts took up the artificial exosomes. Simultaneously, phosphorylation of MAPK, a kind of signal pathway related to cell proliferation was induced. As a result of cell counting assay, the skin fibroblasts proliferated about 1.2−1.4 times faster, compared to non-treated skin fibroblasts. TGF-ß- and collagen-secreted skin fibroblasts were expressed higher than non-treated cells. *Summary/conclusion*: The ES cell artificial exosomes induce cells to proliferate faster, secrete more ECM protein and growth factors, showing that treatment of ES artificial vesicles can play an important role in the wound healing process.

O5C-174

Dual activation of Akt and β-catenin signalling by exosomes from human umbilical cord mesenchymal stem cells enhances wound healing

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Introduction: Mesenchymal stem cells (MSCs) have been suggested as ideal cells for regenerative wound healing, but the role of MSCs-derived exosomes (MSC-Ex) in skin damage repair is largely unknown. We found that exosomes from human umbilical cord MSCs (hucMSC-Ex) significantly enhanced wound healing in comparison to normal fibroblasts. *Methods*: Exosomes from human umbilical cord MSCs were extracted and identified. We constructed rat skin deep II degree burn model and injected hucMSC-Ex, HFL1-Ex and PBS subcutaneously. Rats were sacrificed and the wound skin was removed after injection 1w or 2w. The injury was identified by HE, PNCA and CK19 staining to evaluate the proliferation and

regeneration of the wound skin. LY294002 was used to prove the function of AKT pathway in the process of hucMSC-Ex inhibiting apoptosis. We used ICG001 to prove hucMSC-Ex promoting skin cell proliferation and migration by β-catenin. We illustrated the key role of Wnt4 transferred by hucMSC-Ex by application of shRNA interference in the β-catenin pathway activation. *Results*: We demonstrated that hucMSC-Ex reduced heat stress-induced apoptosis and enhanced re-epithelialization in a skin burn model through the re-activation of Akt signalling. In addition, hucMSC-Ex promoted wound closure in a skin full-thickness excision model by accelerating skin cell proliferation and migration via β-catenin signalling. Moreover, we identified that Wnt4 in hucMSC-Ex mediated the activation of β-catenin in skin cells and knockdown of Wnt4 abolished the therapeutic effects of hucMSC-Ex in vitro and in vivo. *Summary/conclusion*: In conclusion, our findings indicate that exosomes derived from hucMSCs prompt wound healing through Akt and Wnt4/β-catenin signalling pathways.

Networking lunch	Arcadis room	12:00-13:00
Poster Viewing Sessions 4A, 4C, 5A, 5C, 6C, 10B, 11		12:30-13:00
Posters attended by authors		Arcadis room
Poster Walk, by chairperson, Session 4C, 5A, 5C, 6C, 10B, 11		13:00-14:00
Posters attended by authors		Arcadis room

Willem Burger room
Symposium session 6A - EV analysis by flow cytometry
Chair: *Frank Coumans and Marca Wauben* 14.00-15.30

Introduction (15 min) by Marca Wauben

O6A-234

Enumeration of extracellular vesicles by a new improved flow cytometric method is comparable to fluorescence mode nanoparticle tracking analysis

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Introduction: Use of extracellular vesicles (EV) as clinical biomarkers has been hampered by issues such as limited size resolution, differentiation between noise and biological events and lack of reproducible quantification in light-scatter flow cytometry. We adapted a flow cytometry (FC) method developed by Van der Vlist et al. (Nat Protoc. 7(7)) to a commercially available cytometer to address these issues and compared the results with nanoparticle tracking analysis (NTA). *Methods*: EV were isolated from human ovarian cancer cell line (HEY)-conditioned medium by sequential ultracentrifugation, stained with a membrane dye PKH67 and submitted to sucrose density gradient centrifugation. Cell-free culture medium was the negative control. For each sample, analysis of twelve 1 mL gradient fractions was performed on BD LSRFortessa flow cytometer and compared with enumeration using the Nanosight LM10 HSF instrument in both scatter and fluorescence modes. Quantification of EV by FC was performed in 96-well plates by absolute count of events in a defined volume using a high-throughput sampler with volumetric pump. Fluorescence thresholding was used to discriminate true events from background noise. Size resolution and quantification limits were tested using fluorescent beads, 100–1,000 nm. Friedman and Wilcoxon matched-pairs signed-rank tests were used to analyse differences between EV numbers in cell-conditioned medium and control. Bland–Altman analysis was used to compare NTA and FC. *Results*: Using fluorescence thresholding, FC detected fluorescent nanobeads down to 100 nm with clear differentiation from 200 nm beads. Compared to standard light-scatter triggered acquisition, thresholding on PKH67 signal eliminated 95% of events in the <500 nm range differentiating true events from noise. Quantification was linear over a wide range of concentrations. Within- and between run coefficients of variation were 2.07 and 2.17%.FC and NTA-F methodologies enumerated similar numbers of cell-derived EVs across fractions. Both methods discriminated cell-derived EV events from those in media alone. Bland–Altman analysis of bias shows that NTA-F systematically detected 1.5-fold higher EV counts than FC which may reflect greater sensitivity of NTA for <50 nm vesicles. In contrast, NTA in light scatter mode (NTA-S) detected 10–100 times more particles than either fluorescence-based method and was unable to discriminate between media alone and media with cell-derived vesicles suggesting lower specificity. *Summary/conclusion*: This adapted FC method for enumeration of fluorescent EV offers a high throughput, low sample volume, direct enumeration with high sensitivity and specificity. Enumeration of EVs was comparable with NTA-F. Due to its wide availability and higher degree of automation and standardization, fluorescence-triggered FC is a reasonable surrogate to emerging NTA-F for quantification of EV. Quantitative differences between these 2 platforms are unlikely to be clinically significant, but this will be investigated.

O6A-235

A novel flow cytometry approach for detecting and quantifying Annexin5-positive extracellular vesicles

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Introduction: Despite intense research on extracellular vesicles (EVs), our current knowledge on the mechanisms of their formation or their physiopathological roles is limited. This is mainly due to EVs' small (sub-micrometre) size and the lack of reproducible characterization and isolation procedures. Our overall aim is to describe EVs' structure at the single EV level and to quantify their main populations in various physiopathological situations. The first step of this project has recently been achieved with the elucidation of the catalogue of EVs in normal plasma (1). A major result of this study is that conventional flow cytometry (FCM) detects about 1% of EVs. Here, we present a novel FCM approach that improves tremendously the detection of Annexin5 (Anx5)-positive EVs. *Methods*: Platelet-free plasma (PFP) was prepared by two cycles of centrifugation at 2,500g for 15 min. Anx5 was labelled with one single fluorophore (Anx5-F*) (1). FCM was performed on a FC500 (Beckman Coulter) flow cytometer. *Results*: Two approaches were compared for detecting Anx5-positive EVs in PFP: (a) a conventional approach in which the trigger was set on the forward scatter (FS) and the detection was limited to objects labelled by Anx5-F* in the presence of Ca^{2+}; (b) a second approach in which the trigger was set on fluorescence (FL) (2) and Anx5-positive EVs were detected after re-calcification. The number of Anx5-positive EVs detected by FL-triggering was about 25,000 per µl PFP, about 75 $\times$ larger than the value determined by FS-triggering. Complementary experiments showed that (a) the detected events were single EVs, and (b) the detection limit corresponded to EVs bearing about 3,000 Anx5-F* molecules. *Summary/conclusion*: The approach presented here is simple, fast and of general application. The number of Anx5-positive EVs detected by this approach is close to the value determined by EM (1).

References

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2. van der Vlist et al. Nat Protoc. 2012;7:1311–26.

O6A-236

Purification of extracellular vesicles by high resolution flow cytometric sorting

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Introduction: Flow cytometry is widely used for the analysis and sorting of particles in suspension. Previously, we demonstrated the qualitative and quantitative analysis of extracellular vesicles (EVs) and other biological particles of approximately comparable size using high resolution flow cytometry. In this study, we explored opportunities and limitations of high resolution flow cytometric sorting to purify specific subsets of vesicles. *Methods*: The configuration and settings of the BD Influx flow cytometer were optimized to allow sorting of small particles. Nozzle sizes of 50 μm and 70 μm were tested and sheath pressure varied between 5 and 60 PSI. Optimization was evaluated using 100 and 200 nm polystyrene beads. EVs were derived from in vitro bone marrow-derived mast cells. Prior to vesicle isolation, cells were either left untreated and cultured overnight, or activated by crosslinking of the high-affinity IgE receptor for 1.5 h. Subpopulations of EVs were identified by PKH67 staining and additional antibody labelling of CD9 and/or CD63. *Results*: Optimal resolution for analysis of small particles on the flow cytometer (Large bore nozzle (140 μm) and low sheath pressure) implies settings least favourable for high-speed sorting. With higher sheath pressure and smaller nozzle sizes, a reduction was seen in fluorescence and scatter signals. By modifying these settings, we were able to achieve acceptable resolution, while allowing high speed sorting in a low sort volume. Using these settings, 100 and 200 nm beads could be sorted with $>90\%$ purity. Specific subsets of EVs were identified by CD9 or CD63 antibody staining and sorted based on light scattering, PKH67 staining and fluorescent antibody binding. Sorted fractions were further characterized by western blotting. *Summary/conclusion*: Specific subsets of vesicles can be detected and purified by high resolution flow cytometric sorting.

O6A-237

nanoFACS: flow cytometric analysis and sorting of single extracellular vesicles

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Introduction: Submicron extracellular vesicles are increasingly recognized as biomarkers and mediators of disease and fundamental biological processes. To facilitate subset and functional analyses of these particles, we have developed a nanoscale fluorescence-activated cell sorting (nanoFACS) method to identify, sort and study distinct subsets of single submicron particles, with distinct treatment-associated responses. Although flow cytometry of cell subsets has been used to identify and study populations of immune and cancer cells for many years, cytometric separation of submicron particles is limited by the small and overlapping sizes of these cell-derived particles. A major barrier to single submicron particle analysis and sorting has been the phenomenon of swarming. To establish reliable operating parameters for single submicron particle cytometric analysis and sorting, swarming must be detected and eliminated. *Methods*: We configured BD Influx, BD FACSCalibur, BDAria, Beckman Coulter MoFlo XDP, and Beckman Coulter AstriosEQ cytometers for maximal resolution of small particles. Non-specific background noise was reduced with 0.01–0.1 micron filters and forward scatter optical assemblies optimized for small particle detection. Using fluorescent and non-fluorescent 100–500 nm beads, with each respective instrument set at various intrinsic instrument noise rates of detection, we determined what flow rates and sample concentrations were associated with loss of single particle resolution and the onset of swarm. *Results*: Each instrument has intrinsic sample concentration and flow rate (events per second) limits for being able to discriminate single nanoparticles and analyse them without distortion. We found that the specific operational limits for each instrument depended on fluidics configurations, sample nozzle (and core stream) diameters and background instrument noise rates. Sample concentrations and sample flow rates were the major determinants of swarming. *Summary/conclusion*: Extracellular vesicles and other submicron particles are increasingly recognized as biomarkers and mediators of disease. However, cytometric separation of these particles for functional studies is limited by the small and overlapping sizes of these cell-derived particles. With nanoFACS, we have extended the range of particle sorting to an order of magnitude smaller than standard FACS sorting. Elimination of swarm is essential for data fidelity in the analysis of, and for the preparative sorting of single particles. Signal processing attributes, as well as intrinsic signal:noise levels, of each instrument were most optimal for these studies on the BD Influx, the AstriosEQ and the MoFloXDP with NanoView. Our results (a) define suitable operational protocols for nanoFACS on each of these instruments, (b) highlight which data attribute demonstrate whether a sample is swarming and (c) provide a foundation for broad use of nanoFACS in the study of extracellular vesicles and other submicron particles.

O6A-238

Differentiation of microvesicles in whole blood with respect to cellular origin

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Introduction: There is a lack of data on microvesicle (MV) release from different cell types in extracorporeal blood purification (haemapheresis). We developed a flow cytometry protocol for the detection of MVs in whole blood and their differentiation according to cellular origin. *Methods*: Flow cytometry was performed with a Gallios Flow Cytometer (Beckman Coulter) after calibration with fluorescent beads to cover the MV (0.5 and 0.9 μm) and the cell size ranges (0.9 and 3 μm). Annexin V staining was used to discriminate MVs of cellular origin from debris. The following markers were used to differentiate cell-derived MVs: monocytes ($CD45^+$, $CD14^+$, $CD11b^+$), T cells ($CD45^+$, $CD3^+$), B cells ($CD45^+$, $CD19^+$), thrombocytes ($CD45^-$, $CD41^+$), granulocytes ($CD45^+$, $CD15^+$), erythrocytes ($CD45^-$, $CD235a^+$) and NK cells ($CD45^+$, $CD56^+$). To assess MV generation during haemapheresis, freshly isolated blood (anticoagulation: acid citrate dextrose-A 1:20; 0,8IE/ml heparin; total volume 50 mL) was circulated over columns (6×1.8 cm; downscaled equivalent to clinical use) packed with acrylamide-polyacrylate-based adsorbents, which are clinically used for low-density lipoprotein (LDL) apheresis at a constant flow rate of 1.2 ml per min for 4 h. Samples were taken every hour, cells were quantified using a blood cell counter (Sysmex) and flow cytometry was performed as described above. *Results*: Passage of whole blood over the adsorbent columns resulted in increased levels of MVs derived from erythrocytes (9.0% vs. 6.9% vs. 3.1% for the untreated control after 240 min, $n = 3$) and thrombocytes (19.6% vs. 17.1% vs. 10.8%). MV generation increased with increasing surface roughness of the adsorbents. Leukocyte, thrombocyte and erythrocyte adhesion did not differ significantly for the adsorbents tested. *Summary/conclusion*: Blood contact with adsorbents induces MV release and correlates with surface roughness. MV Quantification and differentiation may provide a basis to optimize the blood compatibility of polymers.

Jurriaanse room
Symposium session 6B - Clinical implications of EV in (cardio)vascular disorders
Chair: *Joan Joseph and Peter Quesenberry* 14.00-15.30

O6B-241

Comparative analysis of extracellular vesicle-based miRNA isolated from mice with monocrotaline-induced pulmonary hypertension and humans with pulmonary arterial hypertension

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Introduction: Circulating extracellular vesicles (EVs) are increased in pulmonary hypertension (PH) but their significance is unclear. Differences in EV-based miRNA profiles between patients with PH and healthy subjects could represent a novel disease biomarker and may provide insights for PH pathogenesis. We have shown that EVs from mice with monocrotaline (MCT)-induced PH contain miRNAs that have been implicated in the pathogenesis of PH and induce pulmonary hypertensive changes when injected into healthy mice. The present study was done to determine if similar EV-based miRNA profile differences exist between normal humans and patients with PH. *Methods*: C57BL/6 mice were injected with MCT (600 mg/kg) or vehicle alone once weekly for 4 weeks and lung and plasma-derived EVs were isolated. Human subjects were enrolled at the time of right heart catheterization which was performed for known or suspected PH. Subjects found not to have PH were used as controls. EVs were isolated from blood collected from the distal port of the pulmonary artery catheter after balloon inflation with the catheter in wedge position. EV-based RNA from mice and humans was used for miRNA microarray analysis. miRNAs significantly up/down regulated or unique/absent in subjects with idiopathic pulmonary arterial hypertension (IPAH, $n=4$) compared to control subjects ($n=4$) were compared to miRNAs significantly up/down regulated or unique/absent in EVs from MCT-injured mice compared to EVs from vehicle mice. *Results*: Compared to controls, miR-145 was significantly up-regulated in both mice with MCT-induced PH (10.8-fold) and patients with IPAH (25.2-fold) and miR-340 was significantly down-regulated (5.6- and 30.3-fold, respectively). In previous studies, miR-145 has been implicated in the pathogenesis of PH in both murine and human disease (Caruso et al., 2012). The same group demonstrated high expression of miR-145 in pulmonary vascular smooth muscle cells of patients with IPAH. miR-340 appears to play an important role in preventing anaerobic glycolysis, a metabolic shift that has been demonstrated in PH. *Summary/conclusion*: We conclude that changes in circulating EVs miRNAs contribute to the pathogenesis of PH in both animal models of PH and patients with PAH.

O6B-242

Plasma exosomes as mediators of remote ischemic preconditioning: protecting the heart at a distance

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Introduction: Ischemia-reperfusion injury (IR) is a hallmark of cardiovascular disease and a major cause of death worldwide. Cardiac preconditioning is known to protect the heart from IR by activating several prosurvival pathways. The fact that cardiac protection can also be achieved when preconditioning is applied to a remote organ from the heart (Remote Ischemic Preconditioning, RIPC) means that humoral factors are released from ischemic limbs into the circulation carrying a prosurvival message. Exosomes are circulating vesicles that mediate inter-cellular communication by ferrying diverse proteins and nucleic acids. Here, we studied exosomes as possible mediators of RIPC. *Methods*: We isolated exosomes from plasma of rats or humans subjected to RIPC. We characterized control or RIPC exosomes by electron microscopy, flow cytometry, western blot and nanoparticle tracking analysis. Exosomes were used in survival experiments, and the signalling pathways leading to cardioprotection were studied. *Results*: Exosome concentration increased dramatically after RIPC in humans (from $3.5 \pm 0.3 \times 10^8$ to $1.1 \pm 0.3 \times 10^9$ exosomes/ml plasma; $p < 0.01$, $n = 6$), and administration of purified exosomes protected the heart from infarct in different settings including an *in vivo* rat model (vehicle: 48 ± 7; RIPC-Exosomes: 21 ± 4% Infarct/AAR; $p < 0.01$), *ex vivo* Langendorff (vehicle: 35 ± 3; RIPC-Exosomes: 21 ± 3% Infarct/AAR; $p < 0.01$), and *in vitro* hypoxia-reoxygenation of cardiomyocytes (43 ± 7% protection from death, $p < 0.01$). RIPC-Exosomes triggered rapid ERK and Hsp27 activation, and the inhibition of upstream PI3K or MEK abolished ERK activation and inhibited cardioprotection. *Summary/conclusion*: We demonstrate that RIPC dramatically increases the concentration of exosomes in the circulation. Exosomes acutely activate prosurvival kinases that rapidly prepare the heart against IR. Exosomes represent a novel agent with the potential to be an endogenous multi-signalling tool for cardioprotection.

O6B-243

Comparison of calcifying exosomes, calciprotein particles and blood-derived vesicles reveals distinct morphological appearance and biological properties

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Introduction: Fetuin-A is a circulating plasma glycoprotein, secreted by the liver, and plays an important role in preventing mineral precipitation in soft tissues. Notably, fetuin-A knockout mice are characterized by calcification of soft tissues, including kidney, heart and vasculature. A reduction of circulating fetuin-A levels also occurs in patients with progressive vascular calcification. Fetuin-A is thought to regulate calcium phosphate crystal precipitation by forming soluble calciprotein particles (CPPs) in the circulation. It also accumulates in exosomes, which are secreted by vascular smooth muscle cells (VSMCs) and act as calcification niduses in the vessel wall. However the relationship between fetuin-A-containing calciprotein complexes and calcifying exosomes remains unknown. In the present study, we compared exosomes isolated from calcifying VSMCs, extracellular vesicles (EVs) from the blood and CPPs. *Methods*: Exosomes and EVs were isolated from cell media or platelet-free plasma by differential centrifugation. CPPs were synthesized in vitro. The size, morphology and composition of vesicles were compared using Nanosight LM-10, immunogold labelling and transmission electron microscopy and western blotting. *Results*: VSMC-derived exosomes and blood-derived EV are enriched with fetuin-A and CD63, and immunogold labelling confirmed the presence of CD63

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and size similarity. Nanosight analysis revealed that calcium phosphate crystals have the smallest size (mode 107 ± 7 nm) whereas fetuin-A-containing CPPs are the largest (mode 182 ± 9.3 nm). The average size of exosomes isolated from calcifying VSMCs and blood-derived EVs was 136 ± 3.6 nm and 146 ± 14 nm, respectively. Moreover, calcifying VSMCs-derived exosomes were morphologically distinct from CPPs. Interestingly, only exosomes secreted by calcifying VSMCs contained amorphous calcium phosphate crystals as detected by EDX analysis. In addition, we compared the relationship between circulating free fetuin-A and fetuin-A bound to EVs and found that there is a strong correlation between these suggesting that a significant part of circulating fetuin-A is associated with EVs. *Summary/conclusion*: Though the biochemical composition of the proposed initiators of calcification bare some similarities and fetuin-A has been found in calcifying exosomes and circulating CPPs, our data strongly indicate that these calcifying entities have different morphological and biochemical composition. Exosomes may be the most likely possible triggers of soft-tissue calcification inducing further formation of CPPs and large calcium phosphate crystals.

O6B-244

Extracellular vesicle-derived CD14 is independently associated with the extent of cardiovascular disease burden in patients with manifest vascular disease

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Introduction: In patients with established cardiovascular disease, high levels of extracellular vesicle (EV)-derived proteins cystatin C, CD14 and α2-antiplasmin predict recurrent cardiovascular events. We examined whether these proteins are associated with the extent of vascular disease. *Methods*: In 1,062 patients from the SMART (Secondary Manifestations in ARTerial disease) study, EVs were isolated from plasma at baseline. Cystatin C, CD14 and α2-antiplasmin were measured in these vesicles using a multiplex assay. The extent of vascular disease burden was determined by a sum score that incorporates history and current presence of clinically manifest coronary, cerebrovascular, peripheral arterial and abdominal aneurysm disease, and parameters of atherosclerosis that were assessed during the SMART screening protocol (ankle brachial index, common carotid intima-media thickness, carotid stenosis and aorta diameter). The relation between EV protein levels and extent of vascular disease was evaluated using ordinal multivariable regression models. *Results*: EV-derived CD14 was significantly associated with the number of affected vascular territories (OR 2.4, 95% CI: 1.4–4.1) as represented by the sum score, independent of cardiovascular risk factors. Cystatin C and α2-antiplasmin EV levels did not show an independent association with vascular disease extent. When investigating parameters of the sum score separately, we did not observe a strong association between any of the EV-derived proteins and the markers of atherosclerosis. EV-derived cystatin C, CD14 and α2-antiplasmin showed a moderate correlation with their plasma equivalents in a subset of 522 patients from the cohort (Spearman's R 0.47, 0.55, and 0.65, respectively (all with p-value <0.001)). Only plasma CD14, however, was found to be associated with vascular disease extent (OR 1.2, 95% CI: 1.01–1.5)) but to a much lower extend compared to EV CD14 (OR 2.4. For cystatin C and α2-antiplasmin these ORs 1.1, 95% CI: 0.92–1.4 and 1.1, 95% CI: 0.93–1.3. *Summary/conclusion*: EV-derived CD14 levels are strongly correlated to the extent of vascular disease but not specifically to markers that reflect atherosclerosis burden, in patients with manifest cardiovascular disease.

O6B-245

Effects of antiplatelet therapy on platelet extracellular vesicles in health and disease

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Introduction: Dual antiplatelet therapy with aspirin and clopidogrel is commonly used to prevent recurrent ischaemic events. It is unclear, however, whether antiplatelet therapy inhibits platelet extracellular vesicle (EV) formation. The aim of this study was to investigate the effects of antiplatelet therapy on platelet EV formation. *Methods*: Blood samples from 10 normal individuals not receiving antiplatelet therapy were incubated *in vitro* with aspirin or a P2Y12 inhibitor (MeSAMP). In addition, 48 patients receiving long-term dual antiplatelet therapy and undergoing coronary angiography were also studied. Multiplate™ impedance aggregometry was used to assess platelet inhibition. Platelet EV formation and procoagulant activity in response to adenosine diphosphate (ADP), arachidonic acid (AA) and thrombin-receptor-activating peptide (TRAP) stimulation were assessed by flow cytometry and Procoag-PL assays, respectively. *Results*: In normals, *in vitro* P2Y12 inhibition resulted in a significant inhibition of platelet aggregation and EV formation with ADP and AA, whereas aspirin only significantly inhibited AA-induced platelet aggregation and EV formation. In patients receiving dual antiplatelet therapy, there was a significant decrease in platelet aggregation in response to ADP, ADP-HS, AA and TRAP; however, platelet EV-associated procoagulant activity was inhibited in response to AA only and not to ADP or TRAP. *Summary/conclusion*: *In vitro* P2Y12 inhibition is more effective at preventing platelet EV release compared to aspirin; however, in patients, dual antiplatelet therapy fails to completely inhibit EV release, particularly in response to ADP and TRAP. This could explain why some patients administered dual antiplatelet therapy continue to experience ischaemic events.

O6B-246

Exosomes derived from human umbilical cord mesenchymal stem cells prevent cisplatin-induced nephrotoxicity by activating autophagy

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Introduction: Mesenchymal stem cells (MSCs)-derived exosomes have been implicated as a novel therapeutic approach for tissue injury, but whether hucMSC-Ex administration before injury can prevent kidney damage in the early stage, and the underlying mechanism are not well understood. In this study, we demonstrated that hucMSC-Ex could prevent cisplatin-induced renal toxicity by activating autophagy. *Methods*: The hucMSC-Ex was extracted, purified and identified. In vitro, normal rat kidney epithelial (NRK-52E) cells were pre-incubated with exosomes from hucMSC or PBS, HFL1-Ex (human foetal lung fibroblast-1-Ex), 3MA, Rapa, followed by treatment with cisplatin for 8 h, and then collected cells and culture supernatant. The moleculars related to autophagy, apoptosis and inflammation were detected by qRT-PCR, TUNEL, mitochondrial membrane potential analysis, immunofluorescence, immunohistochemistry, western blot, and enzyme-linked immunosorbent assay. In vivo, rats were injected hucMSC-Ex or PBS, HFL1-Ex, 3MA, Rapa under bilateral renal capsules followed by injecting cisplatin intraperitoneally. Serum was collected before the injection of cisplatin (0d) and after injection of cisplatin 1d, 2d and 3d. Rats were sacrificed and kidneys were removed after injection 3d. The injury was identified by HE staining and levels of serum creatinine (Cr) and blood urea nitrogen (BUN). The moleculars related to autophagy, apoptosis and inflammation were detected by western blot, immunohistochemistry

and Luminex assays. *Results*: *In vitro*, hucMSC-Ex inhibited cisplatin-induced mitochondrial apoptosis and secretion of inflammatory cytokines in renal tubular cells. In vivo, hucMSC-Ex alleviated cisplatin-induced damage of renal function and structure. HucMSC-Ex increased the expression of autophagic marker protein LC3B and autophagy-related genes ATG5 and ATG7 by inhibiting the phosphorylation of mammalian target of rapamycin (mTOR). Autophagy inducer mimicked the effects of hucMSC-Ex in protecting against cisplatin-induced renal injury, while the effects of hucMSC-Ex were abrogated by autophagy inhibitor, 3-methyladenine (3MA). *Summary/conclusion*: Taken together, our findings indicate that hucMSC-Ex prevents cisplatin-induced nephrotoxicity through the activation of autophagy in renal tubular cells, suggesting that exosomes may contribute to the protective role of hucMSC in renal protection.

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Van Weelde/Mees room
Symposium session 6C - EV as biomarkers
Chair: *Hidetoshi Tahara and Shona Pedersen* 14.00-15.30

O6C-249

Advanced molecular tools for proteomic analyses of microvesicles
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Introduction: The possibility to detect and analyse biomolecules in their biological environments with increased specificity and sensitivity will provide opportunities to use very rare molecules as reliable biomarkers of diseases. We have recently developed a proximity ligation assay, in which high specificity and sensitivity for detection of target molecule result from the requirement of multiple recognition events, combined with extremely high efficiency of signal detection due to amplification of DNA molecules that are used as probes in combination with affinity binders. A multiplex form of the assay allows parallel analyses of panels of proteins in minute amounts of samples, while another form of the assay facilitates detection of high-order biological complexes such as microvesicles *Methods*: In proximity ligation assays, affinity probes, such as antibodies, are attached to DNA oligonucleotides to form proximity probes. Once a target molecule is recognized by a set of proximity probes, the DNA strands are connected to each other via enzymatic ligation to form a DNA template that can be amplified and quantified as the measure of the target molecule's concentration. In the *in situ* PLA the PCR, the amplification step is replaced by rolling-circle-amplification. *Results*: Here, we illustrate the application of different formats of PLA for detection and characterization of microvesicles including the surface protein profiling and analyses of the total protein content using different protein panels. *Summary/conclusion*: The PLA technology provides tools with extreme sensitivity and specificity for screening and characterization of microvesicles, and for validation of microvesicles as biomarkers in cancers and other diseases.

O6C-250

Characterization of exosome-like vesicles in uterine aspirates
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Introduction: Extracellular vesicles (EVs), including microvesicles and exosomes-like vesicles, are small membrane vesicles that may carry bioactive shipments that include proteins, lipids as well as mRNA and non-coding RNA, released from the cell of origin, to the microenvironment. EVs can be found in a variety of body fluids, including, blood, urine, saliva, breast milk, ascites as well as uterine aspirates. Recent studies have revealed the role of EVs in cell–cell communication, pathogenesis, drug, vaccine and gene-vector delivery, and as possible reservoirs of biomarkers. A major bottleneck in this field is the lack of standardization for already challenging techniques to isolate EVs. *Methods*: This study obtained approval from the institutional review board. Written informed consent was obtained from all patients. EVs from uterine aspirates were obtained by ultracentrifugation and characterized at nature level (by electron microscopy and nanosight tracking particles) at protein level (by protein quantification and immunoblotting) and at RNA and miRNA level (by bioanlayzer and RTqPCR). *Results*: Here, we standardized the protocol for EVs isolation from uterine aspirates by ultracentrifugation and further characterized their number, shape and their protein, RNA and miRNA cargo. *Summary/conclusion*: Our data demonstrate that isolation of EVs from uterine fluids is feasible and promising for the identification of critical molecules involved in proximal organs, such as genital organs, which can be used as diagnostic markers.

O6C-251

Proteomic analysis of urinary extracellular vesicles in ADPKD patients
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Introduction: Autosomal dominant polycystic kidney disease (ADPKD) is the most common inherited kidney disease that is characterized by progressive cyst growth often leading to renal failure. At present, there is no good marker to predict disease progression. Urinary extracellular vesicles (uEVs) are nanometer-sized particles that contain many disease-related proteins from all renal epithelial cells and form a potential source of biomarkers. Therefore, we conducted a proteomic study of uEVs and compared the findings with the conventional acetone precipitation method in ADPKD. *Methods*: We collected spot urines of 6 ADPDK patients and 6 age- and sex-matched healthy volunteers. These samples were normalized for urinary creatinine, pooled and precipitated using acetone or subjected to ultracentrifugation to isolate uEVs. Samples were trypsinized and subsequently analysed using liquid chromatography coupled to mass spectrometry (LC-MS/MS). uEVs were labelled with isotopomeric dimethyl labels, allowing quantitative analysis using bioinformatics. *Results*: A total of 1,048 proteins in acetone precipitated urine and 1,245 proteins in uEVs were identified, of which 718 were unique for uEVs. Quantitative analysis of the uEVs revealed a 2-fold up-regulation of 227 proteins and a 2-fold down-regulation of 116 proteins. Many proteins previously implicated in the pathogenesis of ADPKD were identified, including junctional proteins, cilium-related proteins and proteins involved in cell-polarity. A selection of the most promising candidate biomarkers is currently made and these will be validated in a separate set of patients using immunoblotting. Interestingly, most of the up- or down-regulated disease-related proteins have not been found in acetone precipitated urine. This supports the notion that the isolation of uEVs enriches the urinary proteome and therefore appears a promising strategy for biomarker research. *Summary/conclusion*: uEVs enrich the urine proteome and are potential biomarkers for ADPKD. Several candidate biomarkers are currently selected for prospective analysis and confirmation in an independent cohort.

O6C-252

Impact of age on urinary extracellular vesicles from healthy humans
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Introduction: The incidence of kidney and urological diseases increases with age. However, the underlying mechanisms and cellular processes associated with these diseases are not fully known. Activated cells release biologically active vesicles which contribute to and reflect pathophysiology. This study was designed to characterize specific cell-derived extracellular vesicles (EV) in urine from different regions of the nephron/urinary tract in healthy humans. *Methods*: Random urine samples (n = 95; 49 men and 46 women) were obtained from age- and sex-matched living healthy kidney donors (20–70 years of age) prior to kidney donation from the Mayo Clinic. EV (>0.2 micron) of cell-free urine were analysed by digital flow cytometry using fluorophore conjugated cell-specific antibodies with or without annexin-V. EV counts were calculated as EV/μL urine and normalized to EV/mg creatinine and correlated with age. *Results*: The levels of EV (EV/μL urine and EV/mg creatinine) positive for surface markers of exosomes, podocytes, cuboidal epithelium of proximal tubule, simple cuboidal epithelium of thick loop of Henle, principal and intercalated cells of collecting duct negatively correlated ($p < 0.05$) with age. In contrast, there was no statistically significant correlation between age and urinary EV positive for parietal epithelium of Bowman's capsule, squamous epithelium of thin loop of Henle, cuboidal epithelium of digital tubule, transitional epithelium of renal pelvis and urothelium of bladder in these healthy kidney donors. *Summary/conclusion*: These results demonstrate that there is a decline in nephron segment-specific EV release into the urine with normal aging whereas the urinary tract showed no significant change in EV release with normal aging. These data also provide age and sex specific reference ranges for urine-borne larger EV, which could be useful for future studies regarding their clinical diagnostic and prognostic value in more diseased patient populations.

O6C-253

Exosome-based serum analysis reveals reliable cancer tissue-related miRNA signatures

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Introduction: The current interest and dispute in biomarker discovery provoke using of more careful strategies for detection of disease-related miRNAs. Many reports claimed to use whole blood as a suitable, robust and non-invasive tool, while others focussed on concentrated and stable miRNA signals in exosomes. Meanwhile, different studies warn against using blood miRNAs in (cancer) biomarker discovery down to the suggested contribution of miRNAs of blood-cell origin and implications of cell type composition, count and haemolysis. This conflict may result from the heterogeneity of the studies with respect to differences in sample type and size, age of participants, phenotype, examined tissue and technical procedure. To address this challenge, we present here a combined approach to identify reliable miRNA markers. *Methods*: Using a qRT-PCR panel of 742 miRNAs, we compared the expression profiles in matched pairs of total and exosome-enriched serum samples of 10 colorectal cancer (CRC) patients at stage IV. We assessed the efficiency of the applied exosome isolation method by examining the miRNA content in the exosome-depleted serum fraction. Two different data normalization methods were then independently applied and only the overlapping miRNAs were considered for follow-up. *Results*: More than half of the overlapping miRNAs are known to be dysregulated in and related to CRC progression. Next, we tested 45 potential miRNA candidates in independent 38 matched total and exosome-enriched serum samples (9 CRC patients at early stages and 10 controls) and respective tissue samples (n = 22). The results revealed: (1) a better performance of tissue samples preserved in frozen liquid N2 than those in RNA-later; (2) all serum markers (n = 13) can also be detected in exosomes; (3) the exosome-based approach however provided additional "hidden" miRNA biomarkers (n = 10) that would have been missed by the conventional method; (4) exosome patterns reflect the dysregulation of miRNA expression in corresponding primary tissue (n = 15). Interestingly, about half of these markers are reported to interact with NF-kB/IL6/STAT3 pathway and their expression seems to be STAT3-dependent. *Summary/conclusion*: Exosome blood-based miRNA diagnostics thus seems to evolve as a promising strategy for identifying complex malignancy-related biomarker patterns.

O6C-254

Proteomics and metabolomics analyses to unravel novel markers and physiological role of hepatocyte-derived EV

Juan M Falcon-Perez
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Introduction: Hepatic function is essential for homeostasis of the organism and outcome of different endogenous and external estresses greatly depends on the integrated response ejected by this organ. Liver injury ranging from mild infection to life-threatening liver failure is a serious worldwide health issue and a major goal in liver pathology is the identification of molecular markers for its early detection, that is, before clinical manifestations are produced. Our group is studying the physiological role of extracellular vesicles in the hepatic function in normal and pathological conditions to identify novel low-invasive markers for liver injury. Our group demonstrated that hepatocytes are able to secrete exosome-like vesicles enriched in metabolic enzymes. *Methods*: We are currently achieving a thorough analysis by transcriptomics, proteomics and metabolomics of hepatocyte-derived extracellular vesicles in normal conditions or challenged to different model toxins as well as the effect that these vesicles have on homeostasis of different body fluids. *Results*: We have detected a significant number of RNAs and proteins in these vesicles that are altered by the liver toxins. Our work provides a repertoire of low invasive candidate markers for liver damage. In addition, we have detected a number of metabolites that are enriched in hepatocytes-released vesicles that support a physiological role of these vesicles in several cellular pathways. *Summary/conclusion*: Our integrated multi-omics study of hepatocyte-derived EVs reveal a number of low-invasive markers for liver damage, and also support a role of these hepatic vesicles in the clearance of endogenous compounds and drugs.

Networking coffee	Arcadis room	15:30-16:30
Poster Viewing Sessions 4A, 4C, 5A, 5C, 6C, 10B, 11 Posters not-attended by authors	Arcadis room	15:30-16:30
Plenary Session Chair: *Clotilde Thery and Jan Lötvall*	Willem Burger room	16:30-17:30

Philip D. Stahl, Washington University School of Medicine, St Louis, USA

Exosomes: Looking back 3 decades and into the future

ISEV general assembly	Willem Burger room	17:30-18:30

General business

Finances

ISEV Scientific Journal

Election of The Executive ISEV Board

Any other business

ISEV Networking dinner & Social Event	19:00-22:00

The Networking Dinner will take place aboard the largest paddle steamer of Europe ''De Majesteit.''

Poster Presentations

Arcadis room
Poster Session 4A - EV in cancer 1
Chair: *Irina Nazarenko and Aris Panaretakis* 13.00-14.00

P4A-175

Extraction and characterization of vesicles from patient samples in Helsinki urological biobank

Maija Puhka[1], Taija af Hällström[1], Tuomas Mirtti[2], Kimmo Pitkänen[1], Antti Rannikko[3] and Olli Kallioniemi[1]
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Introduction: Helsinki urological biobank (HUB) is a joint project of Hospital district of Helsinki and Uusimaa and Institute for molecular medicine Finland (FIMM) collecting tissue, blood and urine samples together with comprehensive clinical information from urological patients. We are setting up isolation protocols of extracellular vesicles (EVs) from the biobank samples starting from urine in order to serve the needs of biobank users and our own research projects on prostate cancer. *Methods*: EVs from patient urine samples were isolated using differential ultracentrifugation, ultrafiltration or commercial kits. Isolated EVs were characterized by whole mount EM, immuno-EM, western blotting, NTA and RNA analysis. The size range of the EVs was analysed by EM and nanoparticle tracking analysis and compared to the size range of inner vesicles within multivesicular bodies of NRK-52E and Huh-7 cell lines derived from kidney and liver, respectively. *Results*: EVs from patient urine samples contained several exosome-enriched markers and a lot of small RNA species but contained no or minor amounts of organelle markers and ribosomal RNA. Some of the isolation protocols gave improved EV yields upon dilution of the samples. The size range of the isolated EVs was roughly similar to that of vesicles in the multivesicular bodies. *Summary/conclusion*: Patient urine samples stored in the biobank are well preserved and amenable for EV isolations using several approaches. In future, HUB will start processing plasma samples according to the most recent recommendations.

P4A-177=OP1-106

Exosomes: future diagnostic tool in prostate cancer

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P4A-178

Prostate cancer cell-derived extracellular vesicle subpopulations contain different gDNA fragments

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Introduction: Extracellular vesicles (EVs) have an important role in the intercellular transfer of genetic information. EVs have been shown to contain nucleic acids such as mRNA, microRNAs, ncRNAs and even DNA. However, less is known of the genomic DNA (gDNA) packed into EVs. It is also unknown, whether the gDNA cargo is randomly sorted to the different EV subpopulations, or if it is preferably packed into specific vesicle types. The aim of this study was to analyse whether different prostate cancer (PCa) cell-derived EV subpopulations (apoptotic bodies, microvesicles and exosomes) carry different gDNA fragments. *Methods*: EV subpopulations from 3 PCa cell lines (LNCaP, PC-3 and RC92a/h) were separated by differential ultracentrifugation (1,200 $\times g$, 20,000 $\times g$ and 110,000 $\times g$). The different EV subpopulations were verified through transmission electron microscopy and characterized by total protein content and nanoparticle tracking analysis (NTA). gDNA fragments of different genes were detected by qPCR and confirmed by DNA sequencing. *Results*: We report that the PCa EV subpopulations were different in terms of total protein and DNA content. Although the particle concentration of microvesicles and exosomes by NTA were similar, the total protein content was significantly different. Particle concentration and total protein content correlated with each other for some, but not all PCa cell-derived microvesicles and exosomes. Analysis of the gDNA content of *TP53*, *PTEN* and *MLH1* fragments in the EV populations from the different PCa cell lines showed, that different EV subpopulations carry different gDNA content, which could indicate a selective mechanism of nucleic acid packing depending on the cell and the EV subtype. *Summary/conclusion*: PCa EV subpopulations carry different gDNA sequences, which could potentially be used as diagnostic and prognostic biomarkers.

P4A-179

Potential of the mRNA carried by urinary extracellular vesicles for the diagnosis of prostate and bladder cancers

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Introduction: Bladder and prostate cancers are the most common malignancies of genitourinary tracts in males. Since prognosis ameliorates with early detection, it will be beneficial to have a repertoire of diagnostic markers that could complement the current diagnosis protocols. Cell-secreted extracellular vesicles have received great interest as a source of low invasive disease biomarkers because they are found in many body fluids, including urine. *Methods*: We collected samples of patients which have been diagnosed with bladder and prostate cancer, as well as individuals without cancer. We obtained a signed informed consent from the patients involved in the study, which was approved by Basque Ethical Committee for Clinical Research. The samples were stored at -80°C. Subsequent purification of urinary Extracellular Vesicles

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(uEVs) was performed by ultracentrifugation and RNA extracted, and an array-based catalogue of the mRNA associated to uEVs was generated. *Results*: More than 4,000 different transcripts were detected in uEVs. We observe high variability among samples, with low correlation both intra and inter-groups. In order to validate the array results by PCR, we collected a new set of samples and designed primers for transcripts which were differentially present between groups. *Summary/conclusion*: We have generated a catalogue of transcripts present in uEVs, observed a high inter-individual variability and we have found genes differentially present in samples from cancer patients when compared with samples from non-cancer patients.

P4A-180

Procoagulant activity of microparticle from patient with acute Leukaemia has a predictive value for thrombotic events

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Introduction: Thrombosis is a common complication of patients with malignancies. Among patients with acute myelogenous leukaemia (AML), the incidence of venous thromboembolism (VTE) is 5.2%, and among patients with acute lymphoblastic leukaemia (ALL), the incidence of VTE is 4.5% in the first 2 years of disease. Despite their influence on mortality and morbidity, the mechanisms inducing a hypercoagulable state are not fully understood to date. Recent studies demonstrate that cancer cells shed microvesicles (MVs) harbouring tissue factor (TF-MVs). Tissue factor, the most potent initiator of the coagulation cascade, plays a critical role in haemostasis. The TF-MVs play a major role in thromboembolism also by expressing procoagulant phospholipids (PL) such as phosphatidylserine in addition to TF. Several recent studies suggest that TF-MVs and their activity may have prognostic value in identifying cancer patients with increased risk of developing VTE or disseminated intravascular coagulation (DIC). The primary object of this study is to assess the association between procoagulant state in patient with acute leukaemia and MVs' procoagulant activity (PCA). A secondary objective is to assess the potential of MVs-PCA as biomarker to predict thrombotic events. *Methods*: Blood samples from 17 patients with acute leukaemia newly diagnosed are obtained at Day-0 (without treatment), D-3 and D-7 (3 and 7 days after treatment). The platelet-poor plasma was obtained from the supernatant fraction of the blood tubes after a double centrifugation for 15 min at 2,500 g. MVs are isolated by ultracentrifugation for 90 min at 100,000 g and then concentrated 6,6-fold. PCA of these MVs is measured by thrombin generation with an increased sensitivity to TF-MVs. *Results*: Among 17 AL patients, 3 patients have an increased MV-PCA at D-0 in comparison to healthy subjects. At D-0, the remaining 14 patients without thrombotic events do not show an increased PCA. Among these 3 patients, 2 developed a thrombotic event (DIC and Clot in pyelon). In these 2 patients, a significant PCA was attributed to TF. The patient with an increased PCA without thrombotic event showed a moderate TF activity in comparison to patients with thrombotic events. Moreover, the study of MVs-PCA showed in 1 patient at D-3 an increased activity. This patient developed a DIC at D-5 for which the causal associated with the chemotherapy was possible. *Summary/conclusion*: The preliminary results from this study confirm the hypothesis of an association between thrombotic events and MVs-PCA, and suggests the role of MVs derived from leukemic blast and other cells in procoagulant state in AL. Moreover, MVs PCA could have a predictive value for VTE and DIC in patients with acute leukaemia.

P4A-181

Exosomal biomarkers for primary and recurrent lung cancer

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Introduction: Unlike significant progress made in the prognosis of certain cancers, the prognosis for lung cancer remains grim with 5-year survival around 10%. Even after resection, many patients die of relapse. Poor outcomes and relapses indicate need for new screening and early biomarkers for detection of recurrent lung tumours. Exosomes are endocytic vesicles that mediate cell-to-cell communication. Tumour-derived exosomes (TDE) are suggested to contribute to the pathogenesis and recurrence of cancer. *Methods*: Athymic nude mice ($n = 25$) were inoculated with H1299 cells (1.5×10^6 cells). When tumours reached 300–400 mm^3, tumours were excised from 18/25 mice; the primary tumours (PT) in 7 mice were allowed to grow. At euthanasia, blood and tumours were collected. Exosomes were isolated from serum using ExoQuick reagent. TDEs from serum were enriched with anti-EpCAM magnetic beads. Western blot was performed for EpCAM and recurrent disease markers. Exosomal miRNA profiles were determined by Human MicroRNA A Card containing 377 TaqMan Assays. *Results*: The incidence rate of recurrent tumours (RT) was about 60% with average tumour volume of 431 ± 236 mm^3. Serum exosomes had particle size of 34–97 nm. Significantly higher levels of tumour marker, EpCAM was observed in exosomes from tumour-bearing versus control mice. Increased expression of the recurrent disease markers, Notch1, MMP2, CCND1, VEGF and HSPs was observed in RT versus PT. Serum exosomes from RT mice also had higher expression of these proteins compared to exosomes from PT mice. A similarity of several miRNAs between serum exosomes and PT was observed, indicating their origin. Higher levels of recurrence-associated miRNAs were observed in RT-bearing compared to PT mice. *Summary/conclusion*: TDE can be a true representation of tumour-profile and a more stringent validation of miRNAs as circulatory biomarkers. Enrichment of serum exosomes for tumour markers would further increase the prognostic significance. (Supported from Duggan Endowment and Helmsley Funds).

P4A-182=OP1-105

Characterization of circulating microparticle origin in patients with myeloproliferative neoplasms (MPN) by flow cytometry

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P4A-183

Proteomic analysis of exosomes from highly metastatic and non-metastatic murine breast cancer cell lines

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Introduction: Exosomes play a major role as mediators of carcinogenesis and metastasis. Previous studies have shown that exosomes from both the tumour microenvironment and the tumour itself are important in tumour progression and dissemination. In this study we focus on the exosomes derived from breast cancer cells with different metastatic potential. *Methods*: Exosomes from metastatic and non-metastatic breast cancer cell lines were isolated by ultracentrifugation and characterized by expression of exosomal markers Tsg101, Alix, Rab5, CD63 and CD81 on sucrose density fractions where exosomes are known to float. Quantitative targeted LC/MS proteomic analysis was performed on the isolated exosomes and their parental cells. *Results*: Bioinformatic analysis revealed that about half of the proteins identified were found in both the metastatic and non-metastatic derived exosomes. Over 30% of the proteins

identified in metastatic exosomes were part of pathways involved in extracellular matrix organization. The most enriched proteins found in exosomes isolated from metastatic cells compared to non-metastatic exosomes have been linked to breast cancer metastasis and thus indicate that these exosomes may play a role in this process. The functional importance of the metastatic exosomes was investigated by educating non-metastatic cells with these exosomes and examining the activation of key oncogenic signalling cascades. *Summary/conclusion*: The molecular profiling of exosomes secreted from metastatic breast cancer cells has revealed a distinct set of proteins that may play a role in breast cancer dissemination and distant colonization.

P4A-184

Plasma extracellular vesicles are not suitable to detect tumour-specific mutations

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Introduction: Extracellular vesicles (EVs) originate from many different cell types. They contain proteins as well as genetic information. It has been reported that tumour cell-derived EVs contain mRNA encoding tumour-specific mutations. In our study we were aiming to detect BRAFV600E-mutation in EVs derived from melanoma patients. *Methods*: In preliminary experiments we confirmed the presence of the BRAFV600E mutation in EVs isolated from primary melanoma cell cultures using differential centrifugation. In contrast to cell culture, the EV population in blood plasma is expected to be from many different cell types. In order to detect tumour-specific mutations in plasma, we reasoned that the selective analysis of melanoma cell-derived EVs would improve the oncogene detection frequency. Therefore we developed antibodies specifically binding melanoma EVs. Monoclonal antibodies were generated by immunizing mice against EVs originating from melanoma cell cultures. Antibodies were selected based on their binding affinity to melanoma EVs. Subsequently antibodies were coupled to microbeads in order to isolate EVs from plasma. For comparison, EVs were purified using differential centrifugation. *Results*: Using real-time PCR analysis, we detected BRAFV600E in samples from consenting melanoma patients surprisingly in healthy individuals (15 and 10 individuals, respectively) as well. Conversely no NRAS mutation was found in either study population. Interestingly using immunoisolation, a subpopulation of EVs was identified that preferentially contained the V600E mutation in melanoma patients and healthy individuals. Of note, this subpopulation was CD81-negative. *Summary/conclusion.* Our findings suggest that EVs are not suitable to detect tumour-specific mutations. Our data raise the question from where the non-UV light-induced BRAF mutation originates, especially in healthy individuals.

P4A-185

Different exosome cargo from plasma/bronchoalveolar lavage in non-small-cell lung cancer

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Introduction: Tumour-derived exosomes are emerging mediators of tumourigenesis, facilitating tumour growth, metastasis, development of drug resistance and immunosuppression; however, little is known about the presence and characteristics of exosomes isolated from BAL in patients with lung neoplasm. *Methods*: Exosomes isolated in plasma and bronchoalveolar lavage from 30 and 75 patients with tumour and non-tumour pathology were quantified by acetylcholinesterase activity and characterized by western blot, electron microscopy and nanoparticle tracking analysis. Differences in exosome cargo were analysed by miRNA quantitative PCR in pooled samples and validated in a second series of patients. Exosomes were detected in greater amounts in plasma than in BAL in both pathologies ($p < 0.001$). *Results*: The most miRNAs evaluated by PCR array were detected in tumour plasma, tumour BAL and non-tumour BAL pools, but only 56% were detected in non-tumour plasma pool. Comparing the top 10 miRNAs with the highest levels detected in each pool, we only found elevated homology between the BAL samples of the two pathologies. In tumour plasma we found a higher percentage of miRNAs with increased levels than in tumour BAL and non-tumour plasma. *Summary/conclusion*: Data show the differences between BAL and plasma exosome amount and miRNA content and support the view that tumours may preferentially use blood to spread the release of specific genetic material, in relation with the local airway.

P4A-1860

Phenotyping of exosomes from various cancer cell lines using Extracellular Vesicle Array and nanoparticle tracking analysis

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Introduction: Exosomes and other microvesicles are one of several communicational tools cells use to communicate with each other. It is therefore expected that cell culture supernatants will contain microvesicles with specific phenotypes reflecting the cells that produced them. The amount of exosomes have been observed to be upregulated in patients with cancers compared to healthy individuals and are therefore believed to play a part in cancer in general and in the spread of cancer. Consequently, we investigated the phenotype of exosomes produced by different concentrations of eight cancer cell lines subjected to either normoxic or hypoxic conditions. In this study, the Extracellular Vesicle (EV) Array (Jørgensen et al. 2013, JEV) is used for capturing, detecting and profiling exosomes directly from unpurified cell culture supernatants from several different cell lines. *Methods*: Phenotypes of the exosomes were analysed by the EV Array with more than 30 different exosome biomarkers and subsequently detected with biotin labelled anti-tetraspanin antibodies (CD9, CD63 and CD81). The cancer cell lines LS180, SKOV-3, PC3, A549, OAW42, COV504, HeLa and SW948 were grown in different concentrations and subjected to normoxic or hypoxic conditions (O_2, 3%). The cell culture supernatants were harvested after 24, 48 or 72 h and analysed on EV Array and on nanoparticle tracking analysis (NTA). *Results*: Using EV Array it was possible to detect and phenotype exosomes for more than 30 markers simultaneously using only 100 μl of cell culture supernatant. The amount of microvesicles and the size distribution were also measured by NTA. The distribution of the markers varied among the cell lines. The phenotype of the exosomes derived from cells from the same cancer type was more similar than those from different cancer types. The results indicate that the production of exosomes is unrelated to the O_2 condition but more influenced by the culture period and cell concentration. *Summary/conclusion*: The phenotype of the exosomes produced by the eight different cancer cell lines varied, but the exosomal profile appeared to be unaffected by hypoxic culture conditions. The results stress the importance of characterizing the cell line of interest and the corresponding exosome phenotypes.

P4A-1861

Expression of surface antigens in subpopulations of exosomes released into the urine from prostate cancer patients

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Introduction: Exosomes are small (30–100 nm) vesicles secreted by cells found in all body fluids including urine. For possible early prostate cancer detection, critical for the prognosis and also for monitoring treatment response, we analyse exosomes from prostate cancer patients in terms of surface markers and RNA content to find a suitable surface marker on prostate cancer exosomes. *Methods*: Urine exosomes from prostate cancer patients were pre-enriched using Total Exosome Isolation kit followed by magnetic isolation using Dynabeads coated with anti-human CD9, CD63 and CD81 antibodies. The isolated exosome subpopulations were subjected to extensive flow analysis, qRT-PCR, ELISA and western blotting. *Results*: Initial data suggested variation between patients in expression of typical exosomal markers. In-depth analysis was performed by expanding the number of surface markers examined in the search for a prostate cancer-specific maker. Exosome surface marker expression detected by flow analysis was confirmed by ELISA and western blotting. In addition, qRT-PCR identifying known exosomal related nucleic acids. *Summary/conclusion*: A method for pre-enrichment and specific isolation of exosomes found in urine from prostate cancer patients was developed. The magnetic bead-based exosome isolation method was proven to be compatible with several downstream analysis methods, including flow cytometry, western blotting, ELISA and qRT-PCR. Variation in exosome surface marker expression was shown for the prostate cancer patients examined indicating there may exist exosome subpopulations with potential to serve as prognostic biomarkers.

Arcadis room
Poster Session 4C - EV therapeutics 2
Chair: *Ramesh Gupta and Soon Sim Tan* 13.00-14.00

P4C-187

Biodistribution of milk-derived exosomes and exosomal curcumin

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Introduction: Milk has been identified as a scalable source for producing large quantities of exosomes with applications in drug delivery (Munagala et al., *ISEV*, 2014). Here, we investigated the effect of route of administration of milk exosomes on tissue distribution, as well as the effect of exosomal drug delivery on tissue accumulation using curcumin as a model compound. *Methods*: Exosomes were isolated from the mature milk and labelled with fluorescent dye PKH67 to study in vitro uptake. Near-IR fluorescent dye DiR was used to label exosomes for in vivo distribution studies in nude mouse model. Various tissues were collected at euthanasia and imaged by PhotonIMAGER. To determine the tissue drug distribution, S/D rats were treated daily by gavage with curcumin-loaded milk exosomes or equivalent dose of free curcumin. Tissue curcumin levels were determined by HPLC. *Results*: Milk exosomes labelled with the PKH67 were readily taken up by macrophages and human cancer cells in vitro. In vivo the tissue distribution varied with route of administration: intravenous administration resulted in hepatic accumulation ($>$70%), while nasal delivery resulted in predominant ($>$50%) accumulation in the lung. The other tissues (kidney, spleen, pancreas, ovary, heart, brain and colon) showed largely similar distribution. Oral and intraperitoneal routes, however, resulted in a more uniform biodistribution. Tissue analysis of rats treated with exosomal curcumin showed significantly higher (3–6-fold) levels of curcumin in various tissues, including the brain compared to free curcumin. These data show that exosomal delivery significantly increased the bioavailability and could cross blood–brain barrier. *Summary/conclusion*. Our data indicate that bovine milk-derived exosomes serves as an excellent carrier to enhance the drug's bioavailability, provide target tissue delivery in some cases and cross the blood–brain barrier. Supported from USPHS grants CA-118114 and CA-125152, KLCRP grant, Duggan Endowment, and Helmsley Funds.

P4C-188

Biodistribution of exosomes: evaluation of different cell sources, doses and time points

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Introduction: In the light of the recent findings of extracellular vesicles (EVs) as important players in the intercellular communication, the biodistribution and clearance of EVs are of utmost importance, not only in order to understand their function, but also for other studies, for example, when regarding EVs as potential diagnostic markers or therapeutic carriers. *Methods*: We investigate the biodistribution of systemically delivered, fluorescently labelled EVs and the impact of using different doses, different cell sources (ES, DCs, HEK293T, C2C12), the impact of targeted EVs (RVG insertion) as well as the effect of tumour burden on the biodistribution. *Results*: *In vitro*, EVs were found to be stable in serum at 37°C. In vivo, the fluorescent signal in serum decreased after the 1st h, following iv injection. During the first 24 h post injection, tissue fluorescence increased in all organs, with a similar distribution pattern among the analysed organs. However, a change in the amount of injected EVs affected the distribution, particularly altering the pattern at the highest dose. The distribution of EVs from different cell sources displayed a common trend with liver, spleen and lungs being the top accumulation sites. Yet, distinct differences were also detected, with higher liver accumulation of cell line vs. primary cell derived EVs, and an increased accumulation of DC-EVs in spleen. EVs expressing RVG, which has been shown efficient for CNS targeting, resulted in an increased signal from organs expressing the AChR: brain, heart and muscle. Furthermore, in a mouse melanoma model, EVs accumulated in the tumour tissue. *Summary/conclusion.* EVs could be detected in all analysed organs, as well as tumour tissue, but mainly distributed to the organs of the mononuclear phagocyte system (liver, spleen and lungs). The distribution can be manipulated, for example, by varying the dose or expressing a ligand such as RVG for targeting. EVs also appear to possess an innate homing capacity as, for example, DC-EVs displayed the highest accumulation in the spleen.

P4C-189

Extracellular vesicles for delivery of small RNAs

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Introduction: Extracellular vesicles (EVs) are important mediators of intercellular communication. Because transfer of their RNA content can lead to functional changes in recipient cells, EVs are increasingly being considered for therapeutic RNA delivery. However, realization of their potential awaits development of efficient methods for loading of exogenous RNA. *Methods*: EVs were isolated from various cell types and characterized using western blot and NTA analysis. Small RNA was overexpressed in donor cells to prepare small RNA-loaded EVs. Transfection of donor cells with shRNA-overexpressing plasmids or siRNA duplexes was compared. Uptake and knockdown efficiency in recipient cells was evaluated in vitro. *Results*: Small RNA overexpression led to increased release via EVs. In HEK293T cells, release was ceramide-dependent. Transfection of siRNA duplexes was more efficient than transfection of shRNA-overexpressing plasmids to prepare small RNA-loaded EVs. EVs were able to deliver small RNAs to recipient cells in vitro. *Summary/conclusion*: EVs can be loaded with small RNAs of interest, and EVs can deliver these to recipient cells. These results underline their potential as therapeutic RNA delivery systems.

P4C-190

Extracellular vesicle-mediated delivery of components of a new prodrug therapeutic regimen

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Introduction: Specific gene delivery can be useful for both treatment and diagnosis. We have developed a prodrug system consisting of a non-toxic prodrug, called CNOB, and a highly active nitroreductase, ChrR. We successfully used this strategy to treat malignant xenografts with approximately 40% complete remission in mice. The activated drug, MCHB, can be non-invasively monitored by fluorescence, thus offering another substantial advantage over other therapeutics. We describe delivery of both components of our prodrug system by extracellular vesicles (EVs) such as exosomes and microvesicles. We have designed EVs that specifically target the human epidermal growth factor 2 (HER2), which is overexpressed in an aggressive form of breast cancer. This system will enhance prodrug solubility and cancer specificity, resulting in effective and safe therapy. *Methods*: RAW264.7 murine macrophage cells were transfected with a plasmid containing the HER2-targeting ligand. These cells served as the source of EVs. MCF7 human breast cancer cells overexpressing HER2 (MCF7/HER2) were used as target cells. The specificity of EV binding to HER2 was measured by flow cytometry using functionalized beads. The binding specificity in vitro was also measured by flow cytometry and fluorescent microscopy after specific labelling of the murine CD9 using an APC-conjugated monoclonal antibody. Incubation of EVs with CNOB resulted in CNOB-loaded EVs. Flow cytometry and fluorescent imaging were used to determine the efficiency of CNOB uptake by EVs and delivery to HER2/ChrR overexpressing MCF7 cells. *Results*: CD63/CD81-positive EVs (80–170 nm in diameter) were isolated by differential ultracentrifugation. The targeted exosomes specifically adhered to HER2-functionalized beads after incubation at 4°C overnight. The targeted exosomes bound to and were taken up by MCF7 cells in vitro at 37°C as early as 6 h incubation. CNOB-loaded exosomes also bound specifically to MCF7/HER2 cells, and the prodrug was activated in the presence of the ChrR enzyme. Exosomes containing the ChrR mRNA also specifically targeted MCF7/HER2 cells in vitro. *Summary/conclusion*: We now have preliminary evidence of the delivery of both our prodrug and its activating enzyme specifically to a breast cancer cell line using EVs. This will serve as a basis for improved delivery using EVs.

P4C-191

The evaluation of a hybrid extracellular vesicle–polymer nanocomposite for RNAi therapy

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Introduction: Following the discovery that extracellular vesicles (EVs) are nature's own nucleic acid delivery system, great interest has gone towards exploiting these cell-derived vesicles as a platform for siRNA delivery. However, a major impediment in using EVs as siRNA carriers is the lack of an efficient siRNA-loading procedure. Here we report the limitations of using electroporation (EP) for loading isolated EVs with exogenous siRNA. As an alternative, we demonstrate the construction of a hybrid nanocomposite composed of a siRNA-loaded polymer matrix core enveloped by purified EVs. *Methods*: EVs were isolated from conditioned cell culture medium of various cell lines using differential centrifugation. SiRNA encapsulation was quantified by fluorescence fluctuation spectroscopy or PAGE. Nanoparticle tracking analysis and confocal microscopy were applied to analyse aggregate formation following EP. The association of nanogels (NGs) and EVs was characterized using subresolution fluorescence based single particle tracking. Size and zeta potential were measured using DLS. Cell uptake and RNAi-induced gene silencing were evaluated using confocal microscopy and/or flow cytometry. *Results*: In contrast to previous reports, we observed that using EP for siRNA loading in EVs results in the formation of aggregates that co-precipitate EVs and siRNA, thus biasing the measured siRNA encapsulation. To overcome this drawback, we aimed to design a hybrid lipid–polymer nanocomposite for siRNA delivery based on EVs. We successfully created a nanoparticle composed of a NG core enveloped by purified EVs based on electrostatic interactions. Moreover, high amounts of siRNA could be stably encapsulated in the NG core (20 nmol/mg NG). Despite the overall negative charge, efficient intracellular siRNA delivery was still observed. As a proof of principle, we showed silencing of a model protein (CD45) in the JAWSII cell line. *Summary/conclusion*: EP showed strong limitations towards efficient siRNA loading into EVs. An alternative, nanohybrid siRNA delivery system is presented here, combining the beneficial characteristics of a polymeric matrix and an EV lipid shell. We anticipate that the described nanocomposites can be exploited as combinatorial EV-based nanomedicines, merging efficient siRNA delivery with the inherent biological characteristics of EVs.

P4C-192

Functional polymer gel–exosomes hybrids for drug delivery system and tissue engineering

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Introduction: Exosomes were utilized as delivery systems of biological molecules. However, it is difficult to control the delivery of exosomes to various cells and tissues. We propose new strategy for application of exosomes to DDS and tissue engineering by modifying the surface of exosomes with functional polymers and by trapping them in functional hydrogels as a scaffold. We developed nanogel engineering by self-assembly of functional associating polymers for nanomedicine. The nanogels were useful for protein delivery and applied them to cancer or nasal vaccine. Nanogel-integrated hydrogels act as new scaffold for tissue engineering. We report here functional polysaccharide nanogel–exosome hybrids. *Methods*: Exosomes were prepared from the supernatant of macrophage-like cells (RAW264.7) by differential ultracentrifugation. The exosome solution was added to nanogel solution of ethylenediamine-modified cholesteryl group-bearing pullulan (CHP-NH_2) at various concentrations. The complexation of exosome with CHP-NH_2 nanogel was evaluated by dynamic light scattering and TEM observation. Cellular uptake and localization of the CHP-NH_2 nanogel/fluorescent-labelled exosome hybrids were determined by flow cytometer (FACS) and confocal laser scanning fluorescence microscope (CLSFM). *Results*: The average sizes of exosomes were approximately 100–150 nm, and zeta potential of the exosomes was -12 mV. After mixing the nanogel and exosome, the sizes increased by about -20 nm in comparison of exosome alone. HeLa cell did not uptake well the exosomes alone. Cationic nanogel–exosome hybrids, however, were effectively internalized into HeLa cell. CLSFM measurements showed that the exosomes were taken by endocytosis and partly escaped to cytosol. *Summary/conclusion*: Cationic nanogels improved intracellular uptake of exosomes by coating the surface. We developed cell-specific nanogels and reactive nanogels as building blocks to prepare new gel materials for tissue engineering. Functional nanogel hybrids offer a new option of wide utilization of exosomes in DDS and tissue engineering.

Arcadis room
Poster Session 5A - EV in infection and immune disorders
Chair: *Tom Groot Kormelink and Francesc Borrás* 13.00-14.00

P5A-193=OP2-120

The effect of rosuvastatin on circulating microparticles in treatment-naïve HIV-patients

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P5A-194

Differences in complement factors on BAL fluid exosomes between sarcoidosis patients and healthy controls

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Introduction: Sarcoidosis is a systemic granulomatous disease and predominantly affects to the lung. Although the aetiology is still unclear, there are some evidence that mycobacterial infection and autoimmune components are involved in the physiopathology of sarcoidosis. Sweden has the highest incidence of this disease in the world, with 30–50 new cases per 100,000 individuals per year. In two-thirds of acute patients occur spontaneous remission of the disease, but some develop chronic disease that may result in fibrosis and respiratory failure. Analysis on bronchoalveolar lavage fluid (BALF) has shown exosomes and their concentration is higher in sarcoidosis patients than in healthy controls. *Methods*: iTRAQ (isobaric tag for relative and absolute quantification) semi-quantitative proteomic technology was used to compare BALF exosomes between 5 healthy controls and 15 sarcoidosis patients. Verification of these results was made by FACS phenotyping on CD63-beads. *Results*: Using the iTRAQ approach, we observed that the levels of complement components such as C3, C4b-binding protein alpha-chain, C5, C6, C7, C8 and C9 were at least twofold higher in BALF exosomes from sarcoidosis patients compared to healthy individuals. In contrast, inhibition factors of the complement system, like CD55 and CD59 proteins, were reduced in patients compared to healthy controls. FACS verification showed that CD55 levels were higher on the surface of sarcoidosis exosomes than healthy exosomes ($p = 0.042$). When adding sarcoidosis and healthy BAL fluid exosomes to peripheral blood mononuclear cells, we observed an increased exosomes uptake by T cells. While C3 levels on the T cell surface did not changed, CD55 expression changed at different time points being maximum after 1 h. *Summary/conclusion*: In summary, our data suggest that BALF exosomes have a role in complement activation or regulation in sarcoidosis. Further studies are necessary to elucidate whether BALF exosomes transfer CD55 molecules or increase its expression on T cells.

P5A-195

Exosomes from Epstein–Barr virus infected gastric cells contain ebv miR-BART15-3p to target anti-apoptotic TAX1BP1 gene

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Introduction: MicroRNAs (miRNAs) are a class of non-coding RNA molecules approximately 19–25 in length that downregulate the expression of target genes by binding to the 3′-untranslated region (3′-UTR) at the post-transcriptional level. Epstein–Barr virus (EBV) generates at least 44 miRNAs, but the functions of most miRNAs have not been clarified yet. *Methods*: We have searched for target genes of miR-BART15-3p using bioinformatics analyses. Luciferase activity was measured using the Dual-Glo Luciferase Reporter Assay System. Cell apoptosis was measured by PE Annexin V apoptosis detection Kit or propidum iodide staining. TAX1BP1 mRNA and the protein were assessed using quantitative reverse transcription polymerase chain reaction and western blots. Exosomes were isolated from the culture media of the gastric cell lines by the ultracentrifugation method. *Results*: We found possible seed match site in the 3′-UTR of Tax1-binding protein 1 (TAX1BP1). The luciferase activity of the reporter vector including the 3′-UTR of TAX1BP1 was decreased by miR-BART15-3p. miR-BART15-3p downregulated the expression of TAX1BP1 mRNA and protein in AGS cells, while the inhibitor against miR-BART15-3p upregulated the expression of TAX1BP1 mRNA and protein in AGS-EBV cells. miR-BART15-3p was secreted from EBV-infected gastric carcinoma cells, and the level of miR-BART15-3p was 2- to 16-fold higher in exosomes than in the corresponding cells. *Summary/conclusion*: Our results suggest that miR-BART15-3p targets anti-apoptotic TAX1BP1 gene and can be transmitted to neighbouring cells via exosomes from EBV-infected gastric cell lines.

P5A-196

Elevated levels of TNFα contained extracellular vesicles in HAART treated HIV patients

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Introduction: Although highly active antiretroviral therapy (HAART) has dramatically decreased mortality from HIV infection, many patients develop drug resistance and/or remain on a low CD4 count. Recent studies suggest that extracellular vesicles (EVs) play a role in HIV pathological processes as EVs derived from HIV-infected cells contain the viral protein Nef. In our previous work, we reported that HIV-1 Nef induced the release of ADAM-containing EVs, inducing potentially detrimental effects on non-infected bystander cells. Previously, we demonstrated that Nef signalling complex interacted with the integrin-associated adaptor protein Paxillin, leading to the packaging of ADAM proteases into secreted EVs. Here we extend these findings and demonstrate their presence in vivo. *Methods*: EVs were isolated from healthy donors and HIV patients plasma through the sequential ultracentrifugations and sucrose gradient. The purified EVs were subsequently analysed by immunoblot and microRNA array. *Results*: By analysing plasma samples from HIV-infected individuals, we demonstrate that there is an approximately 20-fold increase of microRNA levels and concentration of EVs in HAART-treated HIV patient blood as comparted to healthy donor. These EVs harboured Nef, but no viral capsid protein p24, excluding the presence of viral particles. In addition to ADAM17, we found the pro-inflammatory

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cytokine TNFα in Nef-containing vesicles. Conversely, in the absence of Nef only the immature TNFα precursor was present. *Summary/conclusion*: The here presented data are consistent with our pervious in vitro findings. We conclude that Nef-containing EVs contribute to immune activation in HIV infection by stimulating TNFα maturation and release through EVs.

P5A-197

HIV-infected and Nef-transfected microglia and astrocytes secrete exosomes carrying Nef

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Introduction: Despite the highly effective HAART administration, the neurological manifestations of AIDS remain a huge problem in diagnosis and treatment of HIV-infected patients. Although HIV protein Nef is implicated in NeuroAIDS, the mechanism remains unclear. We previously showed that Nef, expressed from transfected or HIV-infected primary T-cells, stimulates its own export via exosomes, which causes activation-induced cell death of resting lymphocytes. To examine, if Nef is capable of inducing its own release from CNS cells by promoting secretion of exosomes, we examined the effect of ectopic Nef expression or HIV infection on microglia and astrocytes. *Methods*: VSV-G pseudotyped HIV viruses NL4-3, YU2 and NL4-3 ΔNef were produced in 293T cells and tested for infectivity on TZM-bl cells by luciferase assay. Later we used these viruses to infect human microglia and astrocytes. Alternatively, CNS cells were transfected with Nef-GFP or GFP as a control. After incubation, exosomes/viruses were isolated by ultracentrifugation and subsequent separation on Optiprep gradient. Exosomes, viruses and cell lysates were then analysed by western blot for specific protein markers. *Results*: We successfully prepared pseudotyped viruses as shown by the composition analysis by western blot and measuring infectivity with luciferase assay. When microglia and astrocytes were infected with NL4-3 or YU2 pseudotyped viruses, the production of exosomes was increased compared to NL4-3 ΔNef-infected or -uninfected cells. Western blot analysis showed that the released exosomes contained Nef protein, which was especially obvious from YU2-infected cells derived exosomes. Astrocytes or microglia expressing Nef-GFP showed four- to ninefold increase in secreted exosomes relative to control, respectively. Exosomes secreted from microglia cells contained Nef protein. *Summary/conclusion*: We conclude that Nef stimulates its own export via the release of exosomes from microglia and astrocytes, when expressed from plasmid or in the context of HIV infection.

P5A-198

Exosomes from human immunodeficiency virus type-1-infected cells license quiescent CD4$^+$ T lymphocytes to replicate HIV-1 through a Nef- and ADAM17-dependent mechanism

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Introduction: CD4$^+$ T lymphocytes are the preferential target of human immunodeficiency virus (HIV) infection and replicate HIV when activated, but resist HIV infection when they are in a quiescent/resting state. HIV-1 infected cells release nanovesicles in the forms of viral particles and exosomes. We were interested in establishing whether exosomes released by HIV-1 infected cells may impact the activation state of quiescent lymphocytes and their susceptibility to HIV-1 infection. *Methods*: Exosomes from HIV-1-infected cells can be isolated through iodixanol gradients of vesicles concentrated from the supernatants. To simplify the procedure, we considered the use of exosomes from a human CD4$^+$ T lymphocyte cell line (F12/Hut-78), infected by a full-length, non-producer HIV-1 variant. *Results*: We provide evidence that exosomes from HIV-1-infected cells can activate resting human primary CD4$^+$ T lymphocytes, which thereby become permissive for HIV-1 replication. The expression of HIV-1 Nef in exosome-producing cells is both necessary and sufficient for cell activation as well as HIV-1 replication in target CD4$^+$ T lymphocytes. These results were obtained using exosomes from HIV-1-expressing cells, and confirmed with transwell co-cultures of HIV-1-infected cells with quiescent CD4$^+$ T lymphocytes in the presence of inhibitors of exosome release. By inspecting the underlying mechanism, we found that ADAM17, that is, a disintegrin and metalloprotease converting pro-TNFα in its mature form, associates with exosomes from HIV-1-infected cells, and plays a key role in the HIV-1 replication in quiescent CD4+ T lymphocytes. Treatment with an inhibitor of ADAM17 abolished both activation and HIV-1 replication in resting CD4+ T lymphocytes. TNFα appeared to be the downstream effector of ADAM17 since the treatment of resting lymphocytes with anti-TNFα antibodies blocked the HIV-1 replication. *Summary/conclusion*: The data presented here are consistent with a model where Nef induces intercellular communication through exosomes to activate bystander quiescent CD4+ T lymphocytes, and thus stimulating viral spread. Moreover, Nef, ADAM17 and TNFα could represent targets of new therapies aimed at containing virus spread, hampering primary infection, and limiting the emerging of drug-resistant HIV-1 quasispecies.

P5A-199

Extracellular vesicles from *Haemophilus influenzae*-infected macrophages induce pro-inflammatory responses

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Introduction: As major effectors of respiratory tract immunity, macrophages provide protection against infection. In order to resolve such infections effectively, intercellular communication is of major importance. Extracellular vesicles (EVs) provide the cell with sophisticated means for intercellular communication. These nanosized (40–100 nm) vesicles facilitate the transfer of mRNAs, miRNAs and proteins. Most cells constitutively release EVs, but upon exposure to stress (e.g. infections) the release of EVs may be significantly enhanced. Therefore the aims of this study were to determine (a) whether macrophages infected with non-typeable *Haemophilus influenzae* (NTHI), a pathogen able to cause infections of the lower respiratory tract, release EV and (b) if these EVs are able to modulate the innate immune response. *Methods*: THP-1 macrophages were either infected with live NTHI or exposed to heat-inactivated NTHI for 2 and 16 h. Next, EVs were isolated from the medium by differential ultracentrifugation and characterized by western blotting and electron microscopy. In addition, the functional activity of the EVs was assessed by determining the inflammatory responses of naive macrophages following EV exposure for 16 h. *Results*: EVs derived from either condition (control, NTHI-infection or stimulation with heat-inactivated NTHI) were between 30 and 100 nm in size and expressed the exosome-specific markers CD63 and CD81. Moreover, when naive macrophages were exposed for 16 h to these EVs, EVs derived from infected macrophages or macrophages exposed to control EVs, irrespective of the duration of the infection/stimulation (*2 h:* control: 113 ± 91 vs. NTHI: 2,931 ± 1,014 vs. HI-NTHI: 1,941 ± 1,040; *16 h:* control: 45 ± 21 vs. NTHI: 873 ± 450 vs. HI-: 695 ± 476, all in pg/ml). *Summary/conclusion*: In conclusion, immunostimulatory EVs were released both by NTHI-infected macrophages and by macrophages stimulated with heat-inactivated NTHI. Future experiments will be performed to further assess the transcriptomic and proteomic characteristics of these EVs as well as their immunological properties.

P5A-200

The role of exosomes in the inflammatory response to high-intensity exercise

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Introduction: Several studies have reported that high-intensity exercise is associated with depression of immune function and induces an acute phase response of increased levels of plasma cytokines such as TNF-a, IL-6 and IL-10. Alongside the secretion of cytokines, studies have shown that cells of the immune system also secrete small vesicles which are called exosomes, as part of the immune response to inflammation. This study was designed to explore exosome levels and the acute inflammatory response of healthy individuals in response to single bouts of high-intensity exercise. *Methods*: Eleven healthy male subjects were recruited to participate in this study. All participants attended two sessions. In the first session, blood samples were collected at regular intervals for 10 h. In the second session, participants were asked to engage in high intensity ''Delayed-onset muscle soreness'' exercise for approximately 15 min, which involved stepping up and down with weights of 3.5% of the individual's total body weight to induce muscle damage. A post-exercise blood sample was collected immediately after the exercise and at various time points equivalent to the resting control. Cytokine and exosome levels were measured in all samples with ELISA and Nanosight, respectively *Results*: As expected high-intensity exercise induced IL-6 and IL-10 and reduced TNF-alpha plasma cytokine levels. Interestingly, the levels of exosomes in plasma were reduced shortly after the muscle-damaging exercise, and levels remained lower than the control samples for 4–6 h. *Summary/conclusion*: These data suggest that following exercise the levels of plasma exosomes drop whilst inflammatory cytokine markers increase. The mechanisms and interactions between exosome and cytokine secretion and their role in the effect of exercise on immune function warrant further investigation.

P5A-201

Cigarette smoke extract induces airway epithelial cells to secrete a compositionally and functionally distinct population of extracellular vesicles

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Introduction: Cigarette smoke-induced changes in epithelial signalling towards the pulmonary immune system are thought to contribute to impaired innate immunity in chronic obstructive pulmonary disease (COPD). As extracellular vesicles (EVs) play a prominent role in intercellular communication, we hypothesized (a) that airway epithelial cells secrete EVs with immune-regulatory properties and (b) that composition and functionality of these EVs change when airway epithelial cells are exposed to cigarette smoke extract (CSE). *Methods*: Alveolar epithelial A549 cells were exposed to CSE or PBS as a negative control. After 24 h, cells were collected and EVs isolated from conditioned medium by sequential ultracentrifugation. EV samples were analysed by BCA microplate assay for their protein concentration and by western blotting for several marker proteins. Moreover, the EVs were visualized with electron microscopy. THP1 macrophages were exposed to different exosome doses. TNFα secretion by THP1 macrophages was determined using ELISA. *Results*: The size distribution of the isolated vesicles (40–100 nm) and their enrichment in exosome marker proteins (CD9, CD63, CD81, TSG101) indicated that our isolation protocol yielded an exosome-enriched vesicle population. Although there was no difference in protein yield between CSE EVs and control EVs, the tetraspanins CD9 and CD81 were significantly upregulated in CSE EVs. Moreover, CSE EVs, but not control EVs, could induce TNFα release by THP1 macrophages. *Summary/conclusion*: CSE EVs differed from control EVs in their tetraspanin composition and in their ability to induce TNFα release from THP1 macrophages, suggesting that EVs may indeed be involved in aberrant immune regulation upon smoke exposure and in COPD. Further compositional and functional properties remain to be investigated. The physiological relevance of our model has to be verified by studying EVs from body fluids of COPD patients and control subjects.

P5A-202=OP2-116

Characterization of neutrophilic granulocytes derived apoptotic bodies

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P5A-203

The role of post-translational modifications of microvesicles in systemic immune responses of mice

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Introduction: Post-translational protein modifications are considered to play an important role in orchestrating immune responses. However, there is very limited information on how post-translational modifications of extracellular vesicles impact the immunogenicity of these structures. The goal of our study was to assess immune responses of mice immunized with either native or post-translationally modified microvesicles (MVs). *Methods*: Murine Th1 T cell hybridoma (5/4E8) cells were used as a source of MVs. The secreted MVs were isolated by differential centrifugation and gravity-driven filtration. Groups of mice ($n = 5$/each group) were immunized subcutaneously with a stable emulsion of complete Freund's adjuvant and MVs [native, citrullinated (citMVs), deglycosylated (dgMVs) or oxidized MVs (oxMVs)]. Total blood plasma IgM and IgG levels were measured by ELISA. Isolated MVs with/without post-synthetic modifications were used as recall antigens to stimulate draining lymph node cell cultures of mice immunized with the respective antigens. *Results*: Total IgM and IgG antibodies of mice immunized with either native MVs or dgMVs did not differ from those in the controls. In contrast, we found significantly elevated total IgM and IgG levels in sera of mice immunized with oxMVs ($p < 0.05$ and $p < 0.01$, respectively). Furthermore, total IgG but not IgM levels of mice immunized with citMVs was significantly elevated ($p < 0.05$) as compared with adjuvant controls. In lymph node cells cultures, in vitro restimulation with dgMVs induced a more than twofold elevation in IL-2 expression, while restimulation with autologous MVs, ox MVs and citMVs did not have an effect. *Summary/conclusion*: The elevated Ig levels induced by the immunization with autologous oxMVs may reflect the reported immune anti-oxidant function of natural autoantibodies. Moreover, our data suggest that post-synthetic modifications of MVs (such as deglycosylation) may result in breaking T cell tolerance.

P5A-204

Level of procoagulant microvesicles in-steady state-sickle cell anaemia patients

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Introduction: Sickle cell disease (SCD) is an inherited, inflammatory disease, characterized by many complications including thrombotic state. Increased level of circulatory microvesicles (MVs) has been reported in SCD. Plasma MVs are membrane-bound vesicles shed from cells upon activation or during apoptosis. MVs have been associated with the risk of thrombosis due to their thrombotic feature, which comes from the negatively charged phospholipids. This thrombotic feature increased in the presence of tissue factor (TF) on their surface. *Aim*: To compare the level of circulating MVs and TF-bearing MVs in steady-state SCD patients with matched controls. *Methods*: Citrated whole blood was drawn from 54 SCD patients and 34 healthy controls (aged from 2 to 12 years old). Patients were recruited from sickle cell clinic at Assir and Mahil Assir Hospitals. The control group included healthy children recruited from home rotation in Abha city. The study was approved by the ethical committee of King Khalid University and all participants signed an informed consent. Platelet free plasma was obtained by separation of whole blood at 1,800x g for 30 min at 22°C followed by a sharp spin of the plasma at 13,000x g for 10 min and then immediately stored at −80°C. The level of MVs and TF-bearing MVs in the plasma was measured using the Zymuphen MP-activity and Zymuphen MP-TF respectively. *Results*: The anaemic condition was confirmed in steady state SCD patients by looking at the complete blood count results (haemoglobin in patients vs. control; 8.2 ± 1.0 vs. 12.7 ± 0.7; $p < 0.001$) and the presence of sickle cell in the blood film. SCD patients showed a significant increase in the level of MVs in their plasma compared to the healthy matched control (patients vs. control, 31.0 ± 3.6 nm vs. 16.4 ± 4.1 nm; $p = 0.0095$). Although, the level of TF activity on MVs was comparable in the two groups and had low levels, it reached statistical significance (patients vs. control, 0.83 nm ± 0.39 vs. 0.48 ± 0.21; $p < 0.05$). *Summary/conclusion*: These results showed increased level of MVs and TF-MVs in the circulation of steady state SCD patients. However, the procoagulant activity and size of plasma MVs need to be investigated.

Arcadis room
Poster Session 5C - EV in tissue repair & remodelling
Chair: *Pamela Russell and Jess Morhayim* 13.00-14.00

P5C-206

Mesenchymal stem cell-derived exosomes containing microRNAs accelerate skeletal muscle regeneration

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Introduction: Mesenchymal stem cells (MSCs) transplantation has been applied for treatment of several diseases. Although the paracrine effects from MSCs have been proposed as the major mechanism that contributes to tissue regeneration, the details of paracrine effects are still not well understood. We hypothesized that exosomes from MSCs function in a novel paracrine factor for tissue repair such as skeletal muscle, in addition to cytokine or growth factor. To examine function of exosomes in conditioned media from cultured MSCs, we evaluated inducibility of myogenesis, angiogenesis and skeletal muscle repair in vitro and in vivo. *Methods*: Human BMMSCs were cultured with serum-free DMEM for 48 h. Exosomes from conditioned media were collected by ultracentrifuged. Supernatants also were collected as exosome-depleted conditioned media. We verified isolated exosomes by western blots, using an antibody against the commonly found exosome marker protein such as flotillin-1. Furthermore, we measured nanoparticles such as exosomes by qNano. The mouse myoblast cell line, C2C12, was seeded, and the media were changed to DMEM (control), MSC-conditioned medium (MSC-CM), MSC-exosome (MSC-exosome) suspension DMEM or MSC-exosome-depleted conditioned media [MSC-CM (exo-)] containing 2% horse serum to induce myogenic differentiation. To evaluate the angiogenic functions of MSC-CM, MSC-exosome and MSC-CM (exo-), we examined whether migration and tube formation in human umbilical vein endothelial cells (HUVECs). To evaluate the effects of MSC-exosome on muscle regeneration in vivo, C57BL/6 mice were used as a model of injured muscle induced by CTX. MSC-exosomes were injected into injured TA muscle after muscle injury. Muscle-related cytokines and miRNAs in MSC-exosomes were analysed by ELISA or BioPlex, and nCounter. *Results*: MSC-conditioned media (MSC-CM) and MSC-exosomes significantly promoted myogensis in C2C12, and migration and tube formation activity in HUVECs compared with control media. MSC-exosomes local injection into mouse model of cardiotoxin-induced muscle injury accelerated histological muscular regeneration with enhanced angiogenesis and reduced fibrosis after skeletal muscle injury. Although MSC-CM was high concentrations of muscle-repair-related cytokines, VEGF and IL-6, MSC-exosome was significant lower VEGF and IL-6 level than MSC-CM. A number of miRNAs were presented in MSCs-exosomes. We found several miRNAs in MSCs-exosomes. Among them, miR-21, anti-apoptotic miRNA, was highest amount. Another two miRNAs induce myogensis and migration activity in vitro. *Summary/conclusion*: The present study demonstrated that exosomes derived from MSC promote the regeneration of skeletal muscle via myogenesis and angiogenesis, and miRNAs in its exosome participate as a new factor of paracrine. We conclude that MSC-exosome is involved in myogenesis and angiogenesis via miRNAs and contributes to regeneration of skeletal muscle in mouse injury model.

P5C-207

MSC-derived exosomes enhance myelination in cerebral hemisphere brain slices from neonatal rats

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Introduction: Administration of bone marrow-derived mesenchymal stem cells (MSCs) is well known to support functional recovery of animals after stroke and brain injury. Among the beneficial effects of MSC treatment is enhancement of white matter regeneration, including axonal outgrowth and remyelination. In previous studies, we have found that exosomes released by MSCs contribute to MSC-induced axonal sprouting and functional recovery. Here, we examined the role of MSC-derived exosomes on oligodendrocyte progenitor cell (OPC) maturation and myelination. *Methods*: In this study, exosomes were obtained from primary cultured MSCs of no older than passage 2. Exosomes were isolated via ExoQuick reagent, resuspended in culture medium, and their concentration and size distribution were determined using an Izon qNano. Serial dilutions of exosomes were added to culture medium of either primary cultured OPCs, or to organotypic brain slice cultures from the cerebral hemispheres of postnatal rat pups. In OPC cultures, proliferation and differentiation were assayed by immunochemistry and western blot after 4 days of culture in exosome containing medium. In brain slice cultures, myelination was assessed after 7 days by immunostaining and confocal microscopy. *Results*: The presence of exosomes inhibited differentiation of OPCs in culture, as measured by the number of myelin basic protein (MBP) positive cells. However, their proliferation rate was significantly increased. Surprisingly, and in contrast to OPC data, exosomes significantly increased MBP positive processes that were co-localized to axons in brain slice cultures, indicating an increase in myelination. *Summary/conclusion*: These contrasting results suggest that the direct effect that exosomes exert on OPCs in culture is not representative of what may happen in vivo, where exosomes may interact with many brain cell types. The increased myelination observed in cerebral hemisphere brain slices may be due to some unknown paracrine effect mediated by other neural cells or endothelial cells. This work was supported by American Heart Association grant 13SDG16330024.

P5C-208

The potential role of iPSCs-derived microvesicles in delivery of bioactive content to target cells: implications for tissue regeneration

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Introduction: Microvesicles (MVs) are membrane-enclosed cell fragments released into the extracellular environment by direct budding from the cell plasma membrane or derived from exocytosis of multivesicular bodies. MVs may act as mediators of cell-to-cell communication by transferring their vast bioactive content from parent cells to cells of other origins. In this study, we compared MVs derived from mouse induced pluripotent stem cells (miPSCs) obtained in our laboratory to their parental cell line in terms of phenotype and transcript level for selected mRNAs. We also investigated transfer of mRNAs by miPSC-MVs to human recipient cells. *Methods*: MVs were isolated by ultracentrifugation (100,000 g for 1 h/twice). The levels of transcripts related to pluripotency (Oct4, Nanog and Rex1), angio- and cardiomyogenesis (Gata4, Gata2, Tie2, Flk1 and EphB2) in miPSC-MVs and miPSCs were compared by real-time RT-PCR method.

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Furthermore, the expression of selected surface antigens including markers related to stemness was evaluated by cytofluorometric analysis on miPSC-MVs and miPSCs. We also investigated the transfer of bioactive content of MVs by incubation of miPSC-MVs with human cell line and analysis of the selected murine transcripts in target cells. *Results*: We detected the presence of all tested transcripts in miPSC-MVs and established that MVs are enriched in several mRNAs compared to their parental cell line. Cytofluorometric analysis of miPSC-MVs showed the presence of several surface antigens expressed on the surface of miPSCs: SSEA-1, Sca-1 and other molecules such as CD29, CD105 and CD49e. The transfer assay demonstrated that MVs can mediate the transfer of mRNAs from pluripotent donor cells to target cells. *Summary/conclusion*: These findings suggest that iPSC-derived MVs may be important mediators of signalling within cell niches influencing phenotype and function of target cells, as well as potential therapeutic tools for tissue regeneration via activation of proliferation and differentiation of target cells.

P5C-209

Exosomes derived from human platelet lysate affect MSC functions in vitro

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Introduction: Despite the popularity of platelet lysate (PL) treatments in orthopaedics, the mechanism of action and the effectiveness of this therapeutic tool are still controversial. So far, the activity of PL has been associated with different growth factors (GFs) released upon platelet degranulation. However, PL activity might also be due to the efficient cell to cell transport system of GF and other bioactive molecules by their encapsulation into exosomes. In this study, we characterized exosomes from human PL and investigated their effect on MSC proliferation, migration and osteogenic differentiation. *Methods*: Exosomes were isolated from human PL by differential ultracentrifugation. Their purity was assessed by electron microscopy and evaluating CD63 expression by western blot analysis. To test the effect of exosomes on MSC functions, bone marrow-derived MSCs were cultured in presence of two different exosome concentrations or with PL. At specific time points, cell proliferation, migration and osteogenic differentiation were evaluated by Alamar blue assay, Boyden chamber assay and Alizarin red staining, respectively. *Results*: Electron microscopy revealed the presence of vesicles within the expected size range of exosomes (30–100 nm) which expressed the specific exosomal marker CD63. MSC treated with PL-derived exosomes showed a significant and dose-dependent increase of cell proliferation and migration. Furthermore, osteogenic differentiation assay demonstrated that exosome concentration differently affected the ability of MSC to deposit mineralized matrix. *Summary/conclusion*: In this study, we demonstrated that exosomes can be successfully isolated from human PL. PL-derived exosomes increase cell proliferation and migration at a higher extent than PL. In addition, exosome concentration affects osteogenic differentiation of MSC. Our results provide evidence of exosomes as putative effectors of PL, and highlight the importance of these vesicles as a potential nanodelivery system for cell-free regeneration therapies.

P5C-210=OP1-109

Extracellular vesicles isolated from equine synovial fluid bind to and are internalized by chondrocytes and synoviocytes in vitro

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P5C-211

Abundance of active lysyl oxidase like 2 on the surface of endothelial cell-derived exosomes is increased upon stimulation with hypoxia

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Introduction: Endothelial cells secrete exosomes that can stimulate migration and angiogenesis in an autocrine manner. A quantitative proteomics analysis of these exosomes previously identified 1,354 proteins. Exposure of endothelial cells to hypoxia resulted in altered abundances of several proteins which are mainly involved in cytoskeletal and extracellular matrix remodelling. One of the upregulated proteins, lysyl oxidase like 2 (LOXL2), was of specific interest given its multiple roles in extracellular matrix (ECM) remodelling, angiogenesis and cell differentiation. *Methods*: Exosomes were collected from the cell culture supernatant of HMEC-1 cells by differential ultracentrifugation and analysed by sucrose density gradient analysis. In vitro LOXL2 cross-linking activity of intact exosomes was determined using an Amplex red-based assay, with cadaverine as a substrate. To further investigate the role of exosomal LOXL2, knockdown and overexpression endothelial cell lines were established using lentiviral vectors. Exosomes from these cell lines were tested for LOXL2 abundance and activity. *Results*: LOXL2 association with exosomes was determined using sucrose density gradient analysis, showing peak fractions of both LOXL2 and flotillin-1 at a density of 1.10–1.11 g/ml. This association was confirmed by immune electron microscopy. Using a proteinase K protection assay, we found that LOXL2 is present on the surface of exosomes, placing it in the same compartment as the ECM. Immunoblot analysis showed a twofold increase of LOXL2 in exosomes grown in 2% oxygen. LOXL2 abundance on exosomes was also successfully modulated by lentiviral knockdown and overexpression, as confirmed by immunoblotting. Furthermore, a lysyl oxidase activity assay showed that LOXL2 is enzymatically active in intact exosomes and that exosomal lysyl oxidase activity increases 1.5-fold when endothelial cells are cultured in hypoxic conditions. *Summary/conclusion*: We show that functional LOXL2 is associated with endothelial cell-derived exosomes and that its abundance on these exosomes is increased by culturing of endothelial cells under hypoxia. We also show that LOXL2 abundance in exosomes can be substantially altered by lentiviral knockdown/overexpression. These data suggest a role for endothelial cell-derived exosomes in ECM remodelling through LOXL2 delivery, under the regulation of hypoxia.

P5C-213=OP3-130

Uptake of macrophage exosomes by the human placenta

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P5C-214

Extracellular vesicles from renal tubular cells induce an epithelial commitment of human bone marrow mesenchymal stromal cells

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Introduction: Mesenchymal–epithelial interactions play an important role in renal tubular morphogenesis and in maintaining the structure

of the kidney. In different experimental models of acute kidney injuries, the injection of mesenchymal stromal cells (MSCs) facilitates renal repair. We have hypothesized that extracellular vesicles produced by renal proximal tubular epithelial cells (RPTEC-EVs) may induce MSC differentiation. This hypothesis has been tested, characterizing the phenotype and RNA content of RPTEC-EVs and evaluating the in vitro effects of RPTEC-EVs on MSCs. Furthermore, the possible contribution of epithelial–mesenchymal transition (EMT)-related microRNA-200 family (miR-200a/b/c, miR-141 and miR-429) on mesenchymal–epithelial transition (MET) of MSCs has been evaluated. *Methods*: EVs were obtained from RPTECs by differential ultracentrifugation and characterized for their surface protein expression and RNA content. Bone marrow-derived MSCs were incubated with RPTEC-EVs, or co-cultured in a transwell system with RPTECs. Epithelial commitment of MSCs was assessed by analysis of cellular expression of specific mesenchymal and epithelial markers. Since RPTEC-EVs were found to contain miR-200 family, we studied its capacity to induce MET in MSCs by transient transfection of MSCs with these miRNA mimics. *Results*: RPTEC-EVs contain specific epithelial mRNAs (aminopeptidase A and cytokeratin 18). Moreover, these EVs contain miRNAs, such as miR-200 family, that are reported in literature to be involved in EMT. After 1 week of incubation with RPTEC-EVs, levels of mesenchymal markers (TWIST and vimentin) were significantly reduced in MSCs. However, the expression of specific epithelial markers by MSCs was observed only in co-culture experiments. After 1 week of transfection with miR-200 family mimics, MSCs showed an epithelial-like morphology, with an increase in epithelial marker expression. *Summary/conclusion*: RPTEC-EVs contribute to the MET of MSCs by decreasing the mesenchymal markers. However, the full epithelial differentiation requires co-culture with RPTECs but not direct contact between the two cellular types. This suggests the need of soluble factors. Experiments with miR-200 family mimics indicate the possible involvement of these miRNAs.

P5C-215

Prostate cancer exosomes can differentiate mesenchymal stem cells into myofibroblast-like cells

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Introduction: The reactive stroma in prostate cancer (PCa) often contains α-smooth muscle actin (αSMA) positive myofibroblasts, which are thought to promote disease progression. Their origin remains uncertain but infiltrating bone marrow mesenchymal stem cells (BM-MSCs) may contribute towards this. Transforming growth factor-β (TGFβ) is a well-studied soluble factor responsible for controlling myofibroblastic differentiation from assorted precursor cell types. Here we hypothesize that cancer cell exosomes, expressing TGFβ, can modulate BM-MSC differentiation into myofibroblast-like cells. *Methods*: PCa exosomes isolated using a sucrose cushion was characterized by western blot, nanoparticle tracking analysis and TGFβ ELISA. BM-MSCs were defined by flow cytometry of surface phenotype and adipogenic differentiation capacity. PCa exosomes, or cancer cell conditioned media rendered exosome deficient (by ultracentrifugation or RAB27A knockdown), were used as a stimulus for MSC differentiation. Myofibroblastic differentiation of MSCs in response to exosomes was assessed by immunohistochemistry for αSMA expression, as well as VEGF-A and HGF ELISA. Exosomally differentiated BM-MSCs were examined for functional changes, in modulating endothelial migration as well as proliferation of both endothelial and tumour cells. *Results*: PCa exosomes can override the adipogenic differentiation programme of BM-MSCs and instead trigger differentiation of MSC into αSMA myofibroblastic cells. Although myofibroblastic differentiation of MSCs are largely dependent upon exosomal-TGFβ, the myofibroblastic phenotype generated cannot be reproduced by adding sTGFb. Exosomally differentiated BM-MSCs produce heightened angiogenic factors (VEGF and HGF) and accelerate endothelial cell proliferation and migration as well as directly promote tumour cell proliferation. Such effects are lost from cancer cell conditioned media upon exosome depletion by either ultracentrifugation or using a RAB27A knockdown approach, pointing to exosomes as the principal factor involved. *Summary/conclusion*: BM-MSC differentiation is profoundly controlled by the presence of PC exosomes, resulting in a unique pro-angiogenic phenotype resembling stromal cells extracted from cancerous prostates. Future studies will examine the functional importance of exosomally differentiated BM-MSC in tumour progression using 3D co-cultures and *in vivo* model systems.

P5C-216

Exosomes derived from mesenchymal stem cells during osteogenic differentiation induce osteogenic differentiation of mesenchymal stem cells

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Introduction: Mesenchymal stem cells (MSCs) are promising candidates for bone regeneration because of their potential for osteogenic differentiation. Nevertheless, it is unclear whether and how MSCs secrete any paracrine factors to modulate MSCs differentiation. It is known that cells can communicate either by direct cell–cell contact or by paracrine factors such as exosomes. The aim of this study was to investigate whether exosomes from different time points during differentiation promote the differentiation of MSCs towards the osteogenic lineage. *Methods*: Human MSCs were cultured with osteogenic additives in exosome-free media for 21 days. Media were collected every 3 days (D3-D21). Exosomes were isolated from conditioned media by ultracentrifugation and added to undifferentiated MSCs without any osteogenic additives. The osteogenic differentiation of exosome-treated MSCs was evaluated by ALP assay after 14D, calcium/phosphate assay and Alizarin red staining after 21D. Internalization of D3 and D21 exosomes, labelled with PKH67, in MSCs was evaluated after 24 h by flow cytometry and confocal microscopy. *Results*: An increased ALP level was demonstrated in all groups of exosome-treated MSCs compared with untreated group. However, only D18- and D21-exosomes treated MSCs had increased calcium and phosphate content and positive staining for Alizarin red, indicating mineralization. Flow cytometry showed that ~2.5% and ~23.0% of MSCs were positive for the PKH67-labelled D3 and D21 exosomes, respectively. Confocal microscopy revealed that both PKH67-labelled D3 and D21exosomes were localized in recipient MSCs. *Summary/conclusion*: The present observations indicate that MSCs internalize exosomes from other MSCs undergoing differentiation towards osteogenic lineage. Further, these exosomes promote a variable degree of osteogenic differentiation and mineralization of MSCs.

P5C-217

Characterization of extracellular vesicles from the secretome of human induced pluripotent stem cells during the differentiation process into dendritic cells

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Introduction: The secretome of a cell comprises of both secreted extracellular vesicles (EVs) and non-vesicular components. EVs are produced constitutively from most cell lines, serving as an efficient form of communication between cells through the transfer of function RNAs and proteins. Previous studies have shown that the secretome of cells greatly differs between different cell types. However, little is

known about how the stem cell secretome, in particular the EVs, changes with cellular differentiation. *Methods*: Conditioned media were collected from undifferentiated human pluripotent stem cells (iPSCs) and at various stages of directed differentiation of these iPSCs into dendritic cells (DCs). The media were cleared of cell debris and concentrated using a 10 kDa cut-off filter. The retentate was then applied on a sephacryl S-400 size-exclusion column, and fractions were pooled to generate three subgroups: EVs, an intermediate pool after first EV peak, and a third group consisting of the second peak based on UV 280 nm chromatograph. EVs were then characterized with nanoparticle tracking analysis (NTA), western blotting, electron microscopy (EM), RNA bioanalyser and proteomics. *Results*: Few EVs were detected in the secretome of undifferentiated human iPSCs, but there was an increase in EV particle concentration and expression of exosomal markers including CD9 and Alix in the EV subgroup as the DC differentiation progressed. EM pictures on EVs from the DCs were measured to be around 100 nm in size, and this corresponds nicely with the NTA data. Interestingly, small RNA profiles showed that EVs have a smaller percentage of miRNAs out of the total small RNAs as compared to the non-vesicular components of the secretome. *Summary/conclusion*: The secretome of iPSCs changes greatly as they differentiate toward a DC fate, where we see a significant increase production of EVs in the later stages of differentiation. Importantly, this technique allows us to separate EVs from the non-vesicular pool such that we can characterize how and which proteins and RNA are secreted from cells across the differentiation process. Differences in the cellular, EVs and non-vesicular protein and RNA content will give insight into how the secretome changes with differentiation and the possible role of EVs in this process.

P5C-218

Extracellular vesicles secreted during human myoblast differentiation stimulate skeletal myogenic differentiation of human adipose-derived stem cells

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Introduction: The skeletal muscle is a metabolically active tissue that secretes various signalling molecules. Although numerous signalling molecules, so called exosome, have been identified via recent profiling studies, there are still poorly understood about how it regulates biological functions. In this study, we investigated whether exosomes secreted during human skeletal myoblast (hSkM) differentiation can trigger a differentiation of human adipose-derived stem cells (hASCs) towards a myoblast phenotype. *Methods*: Exosomes from conditioned media obtained during hSkM differentiation were isolated by concentration and ultracentrifugation and analysed by transmission electron microscopy, dynamic light scattering liquid chromatography tandem mass spectrometry (LC-MS/MS), and growth factor array. Myogenesis of hASCs was monitored in the presence exosome-containing medium (Ex-medium) for 21 days. *Results*: We showed that hSkM release exosomes (approximately 30 nm in diameter) including various proteins and soluble factors during myotube formation. The exosome-treated hASCs fused with neighbouring cells and exhibited the myotube-like phenotype with increased expression of myogenin, myosin heavy chain and desmin. *Summary/conclusion*: Our results suggest that hSkM-derived exosomes could act as ''endocrine signals'' for control of hASC fate toward myogenic lineage.

P5C-219

Characterization of the extracellular vesicles released by C2C12 cells during the early phase of myogenic differentiation process

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Introduction: Skeletal muscle is a highly plastic tissue able of adapting to different stresses, in part due to its remarkable regenerative capacity. In addition to soluble proteins, it has been found that C2C12 myoblasts and myotubes release exosome-like vesicles in the extracellular environment during myogenic differentiation. Cells can release different types of extracellular vesicles (EVs): exosomes, shedding vesicles and apoptotic bodies. The aim of this study was to investigate the type of EVs released by C2C12 cells in the early phases of myogenic differentiation process. *Methods*: C2C12 cells were cultured in DMEM containing 10% FBS and then shifted to low-serum medium to induce myotube formation. Conditioned medium was collected every 48 h. EVs were purified by serial centrifugations and finally pelleted by ultracentrifugation at 110,000 *g*. The EVs collected during myogenic differentiation process were characterized using TEM, western blot, density gradient and real-time PCR analyses. *Results*: The EVs from myoblasts showed a mean size of approximately 53 ± 8 nm in diameter with the outer dense wall and the inner less dense region. At the early stage of myogenic differentiation, although some EVs were similar to those of myoblasts, the most abundant EVs had a larger diameter if compared to those of myoblasts and contained an electron-dense material. At late differentiation stage, size distribution analysis of EVs revealed a mean diameter size of 205 ± 86 nm with an abundant quantity of vesicles containing electron transparent material and a few of them presented an electron-dense material. Moreover, at the first days of differentiation, using density gradient separation it was possible to identify two subpopulation of EVs, one rich in Tsg101 and the other characterized by abundant quantities of DNA and miR-206. *Summary/conclusion*: The results herein reported suggest that differentiating C2C12 cells may release different types of EVs which could have different effects on neighbouring cells.

P5C-220

Heterogeneity of skeletal muscle-derived extracellular nanovesicles and role of protein lipidation

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Introduction: Several cell types have the capacity to secrete small vesicles that contain cell-specific collections of proteins, lipids and genetic material. Research on secreted vesicles has been focusing primarily on the immune system and tumour cells. Recently, we have reported that skeletal muscle (SkM) cell can release nanovesicles. The subcellular architecture of SkM is very different from that of mononucleated cells, and it is now apparent that preserving muscle structure and function, proper myogenesis and regeneration requires muscle-specific elaborations of known membrane trafficking pathways. For this reason, and on the basis of our findings, we believe that vesicle biogenesis in muscle may differ with that of other mononucleated cells, being sustained by a direct budding of nanovesicle from the plasma membrane. Furthermore, dramatic changes occur during myogenesis, and the C2C12 cell system mimics these events in vitro: migrating myoblasts proliferate (D0), after inducing differentiation they recognize each other (DI), align and adhere (DII) and fuse to form terminally differentiated multinucleated myotubes (DIII), which eventually start to contract at later stages (DVI). During these events profound modifications occur at the cellular (membrane and cytoskeleton) and molecular levels. In this context, two recent papers, from us and another group, showed high heterogeneity of the C2C12-derived nanovesicles, depending

upon the differentiation stage of muscle cells. This heterogeneity may reflect differences in the membrane modifications that occur in myocytes (DII) vs. myotubes (DIII-DVI) in a phase that is characterized by a fusion event occurring at the plasma membrane. Here, we investigated how muscle cells generate these vesicles and what are their regulators. *Methods*: The skeletal muscle cell line C2C12 was used as cell system; cells were treated or not with specific inhibitors of protein lipidation, and then the SkM-derived nanovesicles were isolated from cells at different stage of SkM differentiation, using differential ultracentrifugation. To characterize nanovesicles, we applied well-established biophysical techniques, for example, dynamic light scattering, coupled to immunoblot analyses of protein content. *Results*: To date, exosomes have mainly been studied with a biological approach; here, SkM-derived nanovesicles were analysed by biophysical techniques, at different stage of SkM differentiation and after inhibition of protein lipidation. We coupled this information with analyses of nanovesicle-specific biomarkers/regulators, after the inhibition of protein lipidation. *Summary/conclusion*: Our analyses qualify these techniques as valuable methods to analyse and compare the size and integrity of nanovesicles in dispersion, in different experimental settings. The research leading to these results has been funded by the Italian Ministry for Education, University, and Research in the framework of the Flagship Project NanoMAX.

P5C-221

Human plasma microparticles are specific source of pro- and antiangiogenic miRNAs

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Introduction: Membrane-derived microvesicles – microparticles (MPs) – are important conveyors of secreted molecular mediators. Plasma MPs originate mainly from platelets, but also from leukocytes, erythrocytes and endothelial cells. MicroRNAs (miRNAs) can regulate angiogenesis by controlling the expression of pro- and antiangiogenic factors. *Objective*. As MPs have angiogenic potential, we tested the hypothesis if plasma derived MPs can carry pro- and antiangiogenic miRNAs. *Methods*: Platelet poor plasma from eight middle-aged men was harvested. All subjects gave their informed consent with accordance with the requirements of the institutional local Ethics Committee. MPs were separated by centrifugation method (16,000 g, 90 min at 4°C). MiRNA was extracted following the miRNeasy (Qiagen) protocol. Reverse transcription was performed with the polyadenylation and cDNA synthesis kit (Exiqon). The expression of 15 potentially proangiogenic (miR-10b, 126, 132, 21, 210, 23a, 23b, 27a, 27b) and antiangiogenic (miR-15a, 16, 17, 24 320a, 320b) miRNAs was analysed with serum/plasma miRCURY LNA Universal panel (Exiqon) by the 7900HT Applied Biosystem system. Expression levels were globally normalized using DDCt methods. *Results*: Both proangiogenic (miR-126, 21, 23a) and antiangiogenic (miR-15a, 16, 24) miRNAs were overexpressed in human platelet poor plasma MPs. The down-expression of some specific proangiogenic (miR-10b, 132, 210) miRNAs was also observed. *Summary/conclusion*: Centrifugation is a method of choice for MP separation. Circulating MPs transfer diverse miRNAs which act in various angiogenic settings.

P5C-222

Effects of different extracellular vesicles population on reversal of FDCP -1 cells radiation damage

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Introduction: We have previously shown mesenchymal stem cell (MSC)-derived vesicles separated by differential centrifugation (300 g, 10 kg and 100 kg) can reverse radiation whole bone marrow (WBM) damage. This is a classic method for preparations of exosomes. However, this separation isolates the smaller vesicles, discarding larger vesicles. Biologic healing effects of larger vesicles with or without smaller vesicles have not been investigated. Here, we investigated the healing effects of three different preparations of vesicles: 10 k pellet (large vesicles), 100–10 k pellet (small vesicles), and 100 k pellet (no 10 k spin). *Methods*. Murine/human MSC or murine WBM derived vesicles were isolated by differential centrifugation. Three different vesicles were collected: 10 k pellet, 100–10 k pellet and100 k pellet. The effects of three different preparations of vesicles on reversal of growth of irradiated murine cell line FDCP-1 cells were analysed by CyQuant NF cell proliferation assay. This assay measures cellular DNA content as an index of cell proliferation. Absorbance reading was converted to percentages of non-vesicles treated control. *Results*: FDCP1 cells were exposed at 500 cGy irradiation and cultured with or without the addition of three different preparations of murine WBM vesicles for 7 days. A significant increase in cell proliferation was observed after three different vesicles treatment. Cells treated with 100 g pellet showed the best effect on reversal of radiation damage by $745 \pm 252\%$ of non-vesicles treated control compared to 10 k pellet treated group ($429 \pm 111\%$) and 100–10 k pellet ($198 \pm 80\%$) ($p < 0.05$). In a similar experiment, FDCP1 cells were exposed at 500 cGy irradiation and cultured with or without the addition of three different preparations of human MSC cell vesicles for 7 days. Cells treated with100 k pellet had the highest effect on reversal of radiation damage. *Summary/conclusion*: There are different biological effects in different extracellular vesicles populations. For reversal of radiation toxicity, the most effective vesicle population would include both smaller (exosome) and larger vesicles (microvesicles).

Arcadis room
Poster Session 6C - EV as biomarkers
Chair: *Rebecca Dragovic and Marei Sammar* 13.00-14.00

P6C-223

Establishing the core proteome of human platelet exosomes using differential isolation techniques

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Introduction: Platelets are released daily from the bone marrow into the blood stream, with an approximate life-cycle of 10 days. Traditionally considered the principle effectors of haemostasis and thrombosis, increasing evidence now suggests that platelets fulfil a much broader role in health and disease. Activated platelets release 2 distinct membrane vesicle populations: exosomes and microparticles; circulating levels of which can be markedly increased in disease. To date platelet exosomes have been characterized only by transmission electron microscopy (TEM), therefore, our objective was to establish the core exosomal proteome of human platelets. *Methods*: We adopted a comparative evaluation of exosomes isolated from activated platelet releasates from 3 healthy volunteers by (a) differential ultracentrifugation (DC) and (b) ExoQuick precipitation (ExoQ). Assessment of exosome isolation was performed for both techniques by nanoparticle tracking analysis (NTA), TEM and western blot analysis. All exosomal populations were then subjected to proteomics analysis in triplicate using a high performance Q-Exactive Quadrupole Orbitrap mass spectrometer coupled to quantitative computational analysis using MaxQuant software. *Results*: Western blotting revealed that both techniques isolated a fraction enriched for exosomal markers, with NTA and TEM further characterizing differentially isolated vesicles by size and structure. Using strict criteria, a total of 704 proteins were identified from all 18 MS runs, 119 of which were present in all 18 runs across both isolation strategies and represent the core human platelet exosome proteome. *Summary/conclusion*: We have established the human platelet exosomal proteome highlighting differences in reproducibility, vesicle size and yield for each isolation strategy. Such insights will aid in understanding the broader role of platelets and platelet-derived vesicles in health and disease. Research funded by Science Foundation Ireland.

P6C-224

Characterization and quantification of circulating microparticles in patients with Diamond Blackfan anaemia

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Introduction: Diamond Blackfan anaemia (DBA) is an inherited anaemia characterized by a deficiency of red cell progenitors in bone marrow (BM). The inability to study erythroid progenitors in the BM is the major factor limiting DBA research. Study of microparticles (MPs) shed from erythroid progenitors can be useful for an investigation of the pathophysiology of the disease. *Methods*: MPs have been isolated from plasma of eight DBA patients and 22 controls by differential centrifugations and analysed by flow cytometry. The number of events in the MPs and Trucount™ gates has been utilized to calculate the absolute MPs number. To identify MPs from erythroid progenitors we tested three markers: CD34, CD71, CD235a. *Results*: Looking at CD71 and CD34 markers, two distinct clusters have been identified, that is, $CD71^+/CD34^-$ and $CD34^+/CD71_{low}$. The mean of MPs $CD71^+/CD34^-$ was not statistically different when comparing controls and patients. On the other hand, the mean of MPs $CD34^+/CD71_{low}$ was significantly lower in patients ($p < 0.05$ using Student t-test). Moreover, a well-represented cluster $CD235a^+$ has been observed in all samples. MPs $CD235a^+$ are released by circulating reticulocyte or by mature red cell. However, in some individuals, the $CD235a^+$ population was also variably positive to CD71. This population could be released by erythroid precursors from peripheral blood. *Summary/conclusion*: We identified MPs derived from red cell progenitors in vivo. MPs population $CD34^+/CD71_{low}$ distinguishes DBA patients from controls. The behaviour of MPs positive to CD34 and CD71 (early markers of erythroid differentiation) is in accordance with the low number of erythroid progenitors in DBA.

P6C-226

Identification of circulating exosomes as biomarkers for drug-induced liver injury

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Introduction: Drug-induced liver injury (DILI) is a frequent side effect of many drugs, and serum aminotransferases have been the clinical standard for evaluating liver injury for the 50–60 years. However, these tissue enzymes showed low specificity, that is, non-specifically increased by muscle injury. Emerging evidence suggested that exosomes may provide biomarkers to reflect organ status. Thus, it is important to identify specific biomarkers to detect the early signs of DILI using circulating exosomes. *Methods*: To evaluate the potential of circulating exosomes as biomarkers of liver injury, mice were treated with hepatotoxic doses of acetaminophen (APAP). In our search for biomarkers of APAP-induced liver injury, we isolated circulating exosomes from plasma of control and APAP-treated mice and they were characterized using electron microscopy and western blot analysis. To further characterize exosomes induced by APAP-induced liver injury, we performed a proteomic analysis for circulating exosomes. *Results*: Serum aminotransferases were significantly increased in plasma with APAP. Characterization of the circulating exosomes was confirmed by CD63 expression as an exosomal marker using western blot and FACS analysis. We show for the first time that APAP treatment increased the amounts of circulating exosomal proteins in plasma and liver-specific exosomal proteins were elevated in the liver damage by APAP. *Summary/conclusion*: These findings suggest that specific circulating exosomal proteins could be potential biomarkers at the early stage of APAP-induced liver injury.

Arcadis room
Poster Session 10B - Late Breaking
Chair: *María Yáñez-Mó and Jeffrey Schorey* 13.00-14.00

P10B-2260

Human thymic epithelial cells release TRAs carrying exosomes

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Introduction: Exosomes have been shown to be abundant in thymic tissue and are potentially important for presentation of antigens to developing thymocytes. The cellular source of thymic exosomes is however still an enigma, and it is not known if thymic epithelial cells (TECs) are able to produce exosomes. In this work, we have cultured human thymic stroma, characterized the resulting cultured cells and isolated exosomes from the cultures. *Methods*: Thymii were collected during cardiac surgery of children, parents gave informed consent and the study was approved by the local ethics committee. Pieces of tissue were cultured and removed after 6 days of culture, leaving only cultured adherent cells in fresh media. After 3 and 6 days of further culturing, media were collected and used for EV isolation (stepwise centrifugation and ultracentrifugation). Isolated EVs were analysed using nanoparticle trafficking analysis, flow cytometry, quantitative PCR and proteomics. Cells were trypsinized and analyzed using confocal microscopy, flow cytometry, quantitative PCR and proteomics. *Results*: The cultured thymic cells have a morphologic appearance typical of epithelial cells and resemble cultured TECs. In addition, Hassall's corpuscle-like structures spontaneously appear after approximately 1 week in culture. Both confocal microscopy and flow cytometry confirm cultured cells as EpCAM+. In addition, the mTEC marker K5 is expressed on the cultured cells and visualized using confocal microscopy. EVs released by human cultured TECs have the size distribution expected of exosomes, peaking at 100 nm. Flow cytometry of the exosomes shows that they are positive for TSG101, CD9 and CD81. PCR and proteomic analyses show that cultured TECs and TEC-derived exosomes carry tissue-restricted antigens (TRAs), indicating a role in thymic selection processes. *Summary/conclusion*: Isolated exosomes from TEC cultures hold open the possibility for these vesicles to be involved in transfer of antigens from the thymic epithelia to developing thymocytes. This mechanism could dramatically increase the TRA coverage of the thymus.

P10B-2261

Extracellular vesicles and exosomes from adult stem cells in the regeneration of organ injury

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Introduction: EVs play a relevant role as mediators of stem cell-induced regeneration by reprogramming injured cells. The EVSteMInjury project was designed to promote EVs as new potential therapeutic strategies in the field of regenerative medicine. *Methods*: We will seek to define the most promising ''active biological ingredients'' in the EVs, by characterizing their macromolecular content and their mechanisms of action in kidney tissue injury. To take advantage of the opportunities offered by two different design strategies, we will produce both biological and synthetic EVs and will assess their efficiency. Finally, we have planned to explore the renoprotective effect of EVs developed within the project by using a large number of experimental in vitro as well as in vivo models of acute and chronic kidney injury. *Results*: Four partners have joined in a consortium and are contributing to the project with different expertise and technical resources from the private sector (i.e. Fresenius Medical Care Italia S.p.A.). Dr. Ciro TETTA is the project coordinator, and the Translational Centre for Regenerative Medicine under the scientific coordination of Prof. Giovanni Camussi is performing most of the molecular and phenotypic characterization of EVs from different stem cell sources; from London Metropolitan University, Dr. Jameel INAL and the Cellular & Molecular Immunology Research Laboratory are responsible for performing 2D-DIGE analysis of MVs and exosomes and for defining biological roles of MVs in infection and cancer therapy; Dr. Raymond M. Schiffelers and the Laboratory of Clinical Chemistry & Hematology at the University Medical Centre, Utrecht, are taking charge of the manufacture or the biologic engineering of EV mimics; the Nephrology and Urology Department directed by Dr. Josep M. CAMPISTOL at Consorci Institut D'Investigacions Biomèdiques August Pi I Sunyer (IDIBAPS) in Barcelona is setting up in vivo and in vitro models of CKI in combination with immunosuppressant drugs. *Summary/conclusion*: The studies performed will provide the bases for the pre-clinical development for the use of EVs in defined clinical conditions not only in renal diseases but also in other organ injuries, such as the liver and the heart.

P10B-2262

Extracellular vesicle-mediated immune regulation during pregnancy

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Introduction: Successful pregnancy is based on the perpetual immunological communication between the foetus and the mother. Foetal cells (by direct cell-cell interactions), extracellular vesicles (EVs) and soluble mediators induce local and systemic immune tolerance during pregnancy. Although the regulatory role of trophoblasts has been well established, many open questions remain unanswered in the field of trophoblast-derived EVs. By the using of an in vitro cell culture system (BeWo choriocarcinoma cell line), we have shown that trophoblast-derived EVs play a role in the induction and maintenance of local cytokine milieu at the foeto-placental interface. We demonstrated for the first time that trophoblast-derived EVs modify the sensitivity of CD4+ lymphocytes for IL-6 by down-regulation of their IL-6Ra expression which favours Treg cell differentiation. *Methods*: We characterized the molecular basis of the interactions between trophoblast-derived EVs and lymphocytes. The effects of trophoblast-derived EVs were followed up by carrying out cell cycle analysis and by the assessment of Th1/Th2/Th17 cytokines using cytometric bead arrays and LPS, concanavalin A or zymosan stimuli. *Results*: We found that trophoblast-derived EVs bind to T lymphocytes. This binding was partially inhibited by antibody blocking of PSR and CD95 on the surface of lymphocytes or by blocking phosphatidylserine, CD95L or HL-G on the

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surface of trophoblast-derived EVs. The presence of EVs did not affect the cell cycle of lymphocytes, but it inhibited the ConA-induced proliferation. BeWo-derived MVs enhanced the production of all investigated cytokines in the ConA-stimulated cells and induced higher production of IL-4 and IL-6 in the LPS pre-treated lymphocytes. In contrast, BeWo-derived EVs did not modify the cytokine secretion pattern after zymosan pre-treatment. *Summary/conclusion*: Our findings indicate that circulating trophoblast-derived EVs play an important role in the regulation of the immune responses during pregnancy.

P10B-2263

Latent EBV-infected B cells trigger antiviral immunity in dendritic cells through exosome-mediated EBV-EBER1 transmission

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Introduction: The human herpesvirus Epstein Barr (EBV) establishes an asymptomatic persistent infection in 90% of the world population by modulating host-immune responses. EBV encodes for two highly abundant polymerase III transcripts with suspected pro-inflammatory properties, of which EBER1, but not EBER2 is sorted and released with exosomes. *Methods*: We investigated the possible effects of EBER1-containing exosomes on antiviral immune responses upon internalization by sensory dendritic cells (DCs) in vitro and in vivo. *Results*: EBV-EBER1 small RNA transcripts evade antiviral immune responses in latent infected cells since forced expression of the cytosolic RNA sensor RIG-I activates antiviral immunity in wild-type, but not in EBER-deficient EBV-infected B cells. IP-studies further support that 5′-triphosphate-EBER1 evades recognition by viral sensors in latent infected B cells during nucleo-cytoplasmic shuttling and sorting. In contrast to observations in infected B cells, latent EBV is sensed by conventional and plasmacytoid DCs through internalization of EBER1-containing exosomes. We confirmed that the IFN-inducing capacity of these exosomes is dependent on the exosomal (small) RNA content and show that the effect is lost upon enzymatic removal of 5′-triphosphate residues. Exosomes exposure with phosphatidylserine, specific targeting and induced maturation of pDCs present in heterogeneous populations of mononuclear tonsil cells suggest relevance of our findings in vivo. Indeed EBER1 was detected in skin lesions with suspected pDC infiltrations, while EBV-infected cells were absent as shown by two independent techniques. *Summary/conclusion*: Thus latent EBV activates antiviral immunity and maturation of pDCs that in susceptible individuals could promote chronic tissue inflammation.

P10B-2264

Characterization of mouse lung tissue-derived extracellular vesicles

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Introduction: Extracellular vesicles (EVs) isolated from cell lines, primary cell cultures and body fluids have been extensively described previously, but studies on tissue-derived vesicles are fewer. The aim of this study was therefore to isolate and characterize the EVs from the lung of an allergic inflammatory mouse model. *Methods*: C57Bl/6 mice (5-6 weeks old) were sensitized intraperitoneally twice, 5 days apart, to chicken OVA bound to aluminium hydroxide. Eight days after the second sensitization, the mice were exposed intranasally to OVA on five consecutive days to induce allergic airway inflammation (OVA/OVA). The control group was exposed to PBS only (OVA/PBS). Mice were sacrificed 24 h after the last exposure, and the lung tissues were harvested, cut in to small pieces and incubated in medium for 30 minutes to allow for vesicles in tissue to enter the medium. EVs were isolated by differential centrifugation and a 0.2 μm filter before final 120,000æg centrifugation. RNA was isolated using miRCURY total RNA isolation kit, and quality was assessed by a Bioanalyzer. EVs were analyzed by transmission electron microscopy. *Results*: The bioanalyzer analysis showed that both the vesicles isolated from OVA/OVA and OVA/PBS lung tissue contained RNA profiles similar to previously described as exosomal RNA profiles, containing primarily short RNA and no peaks for the ribosomal S18 and S28 subunits. The size distribution of EVs isolated from the OVA/OVA mice were not significantly different from the OVA/PBS mice when analyzed by transmission electron microscopy, as both of them had a mean size of approximately 100 nm. *Summary/conclusion*: These data suggest that EVs can be isolated from lung tissue and that the overall morphology and diameter of lung tissue-derived EVs do not change overtly by allergen exposure.

P10B-2265

Migratory activity in human bone marrow-derived mesenchymal stem cells by mast cell-derived exosomes

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Introduction: The most common response of any inflammatory signal is the activation of various kinds of immune cells at the bone marrow progenitor's population. Stem cells population of these niches is critical for physiological homeostasis of immune system. In allergy and inflammation of lung, mast cell has been shown to be one of the primary components that get activated in response and have various paracrine- and endocrine-like signals. Understanding the crosstalk of mast cell-derived extracellular vesicles and their site of origin in bone marrow is currently not known. This preliminary study aims to understand the crosstalk between activated human mast cell-derived exosomes and bone marrow-derived stem cells. *Methods*: To understand the cross talk, first exosomes were isolated using differential centrifugation protocol which includes long (3 hours) centrifugation in last step. Human bone marrow-derived stem cells (BMSCs) were isolated incubated with different dosage of human mast cell (HMC1) in serum-free condition. Migratory and morphological assessment was performed on BMSC in its early passage at different time point. *Results*: In the reverse migration assay, BMSCs showed migratory response towards exosomes derived from mast cells (HMC1) in dose-dependent manner. Our preliminary result also showed altered morphology (elongated) of BMSC cells by HMC1 exosomes indicating altered cell behaviour. *Summary/conclusion*: Mast cell-derived exosomes induce migratory phenotype in bone marrow stem cells and potentially communicate with their site of origin in the bone marrow.

P10B-2266

How many beer extracellular vesicles are taken by human beings?

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Introduction: From simple organisms (archaea, Gram-negative and Gram-positive bacteria, or yeast) to complex multicellular organism, almost all living organisms in earth shed extracellular vesicles (EVs) into extracellular milieu. Fermentation is a natural conversion process of sugars into products such as beer, alcohol and lactic acid by yeasts and bacteria. In many societies, human beings take huge amount of fermented foods. For example, beer is a common alcoholic beverage and the world consumes over 100 billion litres of beer per year. Beers are produced by *Saccharomyces cerevisiae*-dominated alcoholic fermentation of grain malt. Since yeast cells actively secrete EVs, we wonder how many EVs are present in commercial beer and how many beer EVs are taken by human beings. *Methods*: EVs were purified from the bottom-fermenting beer by the combination of filtration, ultracentrifugation and gel filtration chromatography. The characteristics of purified beer EVs were determined by transmission electron microscopy. The presence of yeast rRNA/rDNA and proteins in purified beer EVs was determined by RT-PCR-based DNA sequencing and by quantitative enzyme-linked immunosorbent assay, respectively. Moreover, the total numbers of EVs in beer and in purified beer EVs were determined by nanoparticle tracking analysis. Cy7-labelled beer EVs were introduced to mice, and distribution of injected EVs and EV-mediated systemic inflammatory responses were examined. Finally, beer EV-induced inflammatory responses were studied in macrophages and endothelial cells *in vitro*. *Results*: Nanoparticle tracking analysis showed that 0.22-μm filtered beer contained $5.32 \pm 1.06 \times 10^{13}$ EVs/litre. By the combination of ultracentrifugation and gel filtration chromatography, we isolated $3.72 \pm 0.53 \times 10^{13}$ EVs (1.3 mg of EVs measured on the basis of total protein) from 1 litre of beer: purification yield is around 70% based on particle number. Examination of the purified beer EVs using transmission emission microscopy revealed that they were closed nanosized bilayered vesicles. These purified EVs harboured significant amount of yeast rRNA/rDNA and proteins, suggesting most of beer EVs are derived from *S. cerevisiae*. After single intraperitoneal injection of Cy7-labelled beer EVs, we observed strong accumulation of Cy7-labelled beer EVs in lung, liver, spleen and kidney without any systemic inflammation. Furthermore, purified beer EVs did not cause any effect on the expression of macrophage cytokines (TNF alpha, IL-6 and IL-10) and endothelial ICAM-1, while EVs derived from *Escherichia coli* significantly induced their expression in macrophages and endothelial cells. *Summary/conclusion*: Beer is a rich source of yeast EVs, and these yeast EVs have no inflammatory activity both in vitro and in vivo. Our findings suggest that all human beings in the world annually take more than 10^{24} EVs (10^5 kg of EVs on the basis of total protein) without any significant pro-inflammatory effect.

P10B-2267

Biogenesis and biological activity of exosomes in replicative senescent cells

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Introduction: Exosomes, lipid membrane vesicles secreted from cells, are known to act as a tool for cell-to-cell communication. To reveal a biogenesis mechanisms and biological activity of exosomes during the cellular aging process, we used human normal fibroblast as an in vitro aging model, and characterized the exosomes derived from replicative senescent fibroblasts. *Methods*: First, we isolated exosomes from human fibroblasts, TIG-3 cells, and measured the number and the size of exosomes using qNano, nanopore-based particle analyzer. We found that the size of exosomes secreted from both young and senescent fibroblasts is very similar; however, senescent fibroblasts secreted larger number of exosomes than young cells. Next, to elucidate the mechanisms for the increase in exosome production in senescent cells, we focused on Maspin and Chmp4C, which are known as exosome regulatory gene. These genes were up-regulated in senescent fibroblasts, and significantly, knockdown of Maspin or Chmp4C by siRNA leads to reduction of exosome secretion in senescent cells, respectively. Further, forced expression of these genes enhanced exosome secretion in young fibroblasts. *Results*: Taken together, these results show that Maspin and Chmp4C regulate exosomes secretion during replicative senescence. In addition, we would like to discuss biological activity of exosomes purified from fibroblasts, such as cell growth, survival rate and cell invasion in cancer cell lines. Moreover, we could detect exosomes by using the spectral cell analyzer SP6800, which is novel technology based on Blu-ray disk technology. *Summary/conclusion*: In this meeting, we would like to discuss the biological significance of exosomes derived from senescent cells and the new technology of exosomes detection.

P10B-2268=OP1-110

Extracellular proteasomes are generated by blebbing of microvesicles

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P10B-2269=OP1-111

The role of extracellular vesicle-mediated transfer of donor genomic DNA to recipient cells in genetic influence between cells

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Arcadis room
Poster Session 11 - EV in reproduction & pregnancy
Chair: *Rebecca Dragovic and Marei Sammar* 13.00-14.00

P11-227

Galectins as major components in amniotic fluid-derived exosomes

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Introduction: Exosomes are small membrane vesicles with a size of 40–100 nm that are secreted by different cell types from a late endosomal cellular compartment and are present in various body fluids including plasma, malignant ascites, urine, amniotic fluid and saliva. Exosomes cargo functional proteins, miRNAs and mRNA and can be taken up by other cells. Galectins, an evolutionarily conserved family of endogenous lectins, share unique features, including their highly conserved structure, exquisite carbohydrate specificity by their affinity for β-galactosides, and ability to differentially regulate a myriad of biological responses. We analysed the presence of S-type lectins (galectins) in exosomes and tested the secretion mode of exosomes from cell lines. *Methods*: Exosomes were purified from various body fluids such as amniotic fluid, liver cirrhosis ascites and malignant ascites of ovarian cancer patients and from the condition medium of OVM, and SKOV3 cell lines by differential centrifugation and sucrose gradient fractionation. Exosomes were characterized by specific markers using western blot and flow cytometry analyses after adsorption of exosomes to latex beads. The secretion mode of exosomes was tested by treating OVM and SKOV3 cells with calcium ionophore and MCD-methyl-b-cyclodextrin in vitro. *Results*: Exosomes purified from amniotic fluids and ascites were characterized by western blot analysis and revealed to be positive for known exosomal proteins such as annexin I, CD9, HSP70, TSG101, LAMP-1 and CD24. These exosomes displayed the correct buoyant density and orientation of antigens. Amniotic fluid-derived exosomes contain Galectin-3, -9, -13, whereas Galectin-1 and -9 were detected in ascites-derived exosomes. Galectin-8 was not detected in exosomes derived from amniotic and ascites fluid or liver cirrhosis ascites. PLAP was detected in ovarian ascites but not in amniotic fluid-derived exosomes. Secretion of exosomes from OVM and SKOV3 cells was intensively induced by calcium ionophore, whereas treating of cells with MCD in vitro leads to increase in secretion of exosomes from OVM and to less extend from SKOV3 cells. *Summary/conclusion*: Our results demonstrate that members of galectin family have been differentially detected within exosomes from body fluids. Secretion of exosomes is not only dependent in calcium efflux but could also be modulated by the membrane composition as resulted from cholesterol depletion. Galectins may act as a bait to recruit glycoproteins and glycoconjugates to the exosomes. Alternatively, galectins are routed to the exosomes in cross-linked mode bound to their counter receptors and ligand glycans.

P11-228

Glucocerebrosidase and extracellular vesicle shedding from a placental cell line

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Introduction: Inhibition of neutral sphingomyelinase results in decreased intracellular ceramide and extracellular vesicle (ECV) release in a mouse oligodendroglial cell line (Trajkovic K et al., 2008). In placenta of pre-eclamptic pregnancies, we observed increased levels of the lysosomal membrane protein glucocerebrosidase (GBA) that hydrolyses glucosylceramide to ceramide. Here we investigate if increased expression of GBA in a human choriocarcinoma cell line (JAR) results in increased levels of ECV release. *Methods*: JAR cells were transduced with an expression construct containing the full length GBA ORF under control of a CMV promoter 72 h, and post-transduction cells were extensively washed and placed in ECV-free medium for a further 72 h. Cells were harvested for RNA analysis, and GBA expression was quantified using qPCR. ECVs were harvested from the culture medium through differential centrifugation as described by Théry C et al. (2006). Nanoparticle tracking analysis (NTA) was used to determine the amount and size distribution of shedded ECVs. Flowcytometry using CD81, CD63 and CD9 antibodies (Jorgensen M et al., 2013) was used to distinguish ECV subpopulations. *Results*: Transduction resulted in a 45-fold increase in GBA mRNA levels compared to mock transduction. NTA analysis showed a 2.5-fold decrease in ECV shedding of GBA overexpression cells, largely due to a substantial decrease in the number of vesicles sized between 100 and 300 nm. Flowcytometry analysis showed a threefold decrease of CD 81 positive ECVs from JAR cells overexpressing GBA. *Summary/conclusion*: Our preliminary data show that increased expression of the lysosomal enzyme GBA in a placental cell line decreases the total amount of released ECVs compared to mock transduced cells with a preference for vesicles ranging from 100 to 300 nm expressing CD81 on the surface. The role of ECV shed after increased GBA expression in placental signalling and pre-eclamptic pregnancy is currently under investigation.

P11-229

Glycan profile of amniotic fluid extracellular vesicles

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Introduction: Amniotic fluid is valuable diagnostic material in monitoring of normal/pathological pregnancy. Extracellular vesicle (EV) analysis represents novel aspect of amniotic fluid diagnostic potential. However, there have only been a few published analysis of amniotic fluid EVs (AEVs), related to their protein/marker and RNA content. None, so far, was related to glycosylation of AEVs. We aimed to establish the glycan profile of AEVs from normal pregnancy using lectin-based solid phase assay. *Methods*: AEVs were isolated from two pools of eight amniotic fluids from normal mid-trimester pregnancies, according to local ethical standards. AEVs were isolated using differential centrifugation, and further purified by sucrose gradient cushion and ultrafiltration. Presence of CD63 marker in EVs isolate was determined by western blot. AEVs were immobilized on poly-L-lysine coated microtitre plates and probed with anti-CD63 MoAb and a panel of biotinylated plant lectins, specific for N- and O-glycans. *Results*: Western blot revealed AEVs to be positive for CD63 EVs marker. Lectins WGA, RCA and AAA exhibited highest reactivity with immobilized AEVs; binding of ConA, SNA, PHA-L and PHA-E was

moderate while MAA and UEA did not react. This indicated the presence of N-glycans of high mannose or complex bi- or multi-antennary chains, with core fucose (Fucα1,6GlcNAc) or bisected GlcNAc. Sialylation is found to be of Siaα2,6Gal type. O-glycans-specific lectins PNA, SBA and JCA showed considerable binding indicating presence of T or Tn antigens. All lectin-binding reactions were dose-dependent and could be inhibited with appropriate inhibitory sugars. *Summary/conclusion*: Obtained glycan-profile of AEVs can be used as a reference in comparative analysis of AEVs from pathological pregnancies and so contribute to full exploitation of amniotic fluid diagnostic potential.

P11-230

Deep sequencing analysis of prostasomal RNA

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Introduction: Prostasomes are extracellular vesicles that are released by the epithelial cells of the prostate gland and secreted into the seminal fluid. Their proposed roles include regulation of sperm cell function and immune suppression to prevent destruction of sperm cells in the female reproductive tract. Prostasomes that are released by hyperplastic, malignant and metastatic prostate tumour cells display characteristics similar to those of normal cells but may also contain tumour-specific molecular content. Prostate tumour cells may release prostasomes into the interstitial compartment or even into the blood circulation. Therefore, the presence of prostasome-specific markers in the blood might be indicative for prostate cancer. To identify potential prostasome-specific markers, we analysed the RNA content of prostasomes derived from healthy vasectomized men. *Methods*: Prostasomes were isolated by differential centrifugation, column chromatography and floatation in linear sucrose gradients from two independent pools of seminal fluid obtained from 4–5 vasectomized men. Total RNA was extracted, and a cDNA library of small RNAs was constructed and sequenced with a SOLid sequencer. Fragments were sequenced both starting from the 5′- and 3′- end, producing 73 nt and 35 nt long reads respectively. Sequences were aligned to the human reference genome using the Lifescope alignment software and alignments were visualized with the Integral Genome Viewer. *Results*: Only one out of two sub-populations of isolated prostasomes contained significant amounts of RNA with the majority within the 100–150 nt range. These included ncRNAs, which were categorized. *Summary/conclusion*: Our data indicate a prostasome-specific RNA profile.

P11-231

Effect of extracellular vesicles secreted by bovine oviductal epithelial cells in in vitro bovine embryo production

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Introduction: Bovine oviductal epithelial cells (BOECs) have been used extensively in in vitro culture systems and as a model to study embryo-maternal interactions. Extracellular vesicles (EVs) are being recently unveiled as an unexpected mode of intracellular communication. *Methods*: We evaluated the developmental capacity of bovine zygotes and the quality of the produced embryos when cultured in vitro with EV purified from confluent BOEC co-cultures (DMEM or TCM199). EVs were quantified by NTA and their integrity and size assessed by electron microscopy. First, fresh or frozen EV of different concentrations were used for embryo culture: SOF + 5%FCS (C +); C+ with 3×10^5; 1.5×10^5; 7.5×10^4 EV/mL. Thereafter, frozen EVs in the absence of FCS were assessed: SOF (C-), and C- with 3×10^5 EV/mL from DMEM or M199. Blastocysts on days 7/8 were used for quality evaluation through cell count, survival after vitrification/warming and gene expression by qRT-PCR. *Results*: No differences were found between groups for blastocyst yield on Day 7 (40.7 ± 1.9). Total cell number was significantly higher in embryos derived from fresh or frozen EVs (175.3 ± 8.0) when compared to C+ (160.8 ± 7.3). Significantly more embryos survived at 24 h for EV groups (67.2 ± 3.4), compared to C+ (44.1 ± 4.4). PAG1 and CX43 were similar in C- and DMEM, but significantly higher to TCM199. IFN-T was higher in C- compared with both EV groups. PLAC8 was significantly higher in C- compared with DMEM. GAPDH was significantly higher in both EV groups compared to C-, and G6PD were higher in C- and TCM199, compared to DMEM. *Summary/conclusion*: In conclusion, the use of EV secreted from BOEC in vitro culture has a positive effect on the quality of in vitro produced bovine embryos. Moreover, these data reveal that EVs have a role in communication between the oviduct and the embryo in the early stages of development.

P11-232

Improvement of cloned embryos development via co-culturing with parthenotes: a possible role of exosomes/microvesicles for embryos paracrine communication

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Introduction: It is well known that embryos cultured in group can create a microenvironment through secretion of autocrine and paracrine factors that can support and improve the embryos development when compared to the embryos cultured individually. *Methods*: Parthenogenetic (PA) embryos were co-cultured with cloned (NT) embryos using 0.4 μm transwell polyester membrane inserts. Blastocyst formation and relative quantification of pluripotency mRNAs via real-time PCR were compared. Embryos conditioned medium (CM) was subjected to differential centrifugation (final step at 200,000 × *g* for 1 h). The resulted pellet contents were identified through transmission electron microscope (TEM), fluorescence-activated cell sorting (FACS) and immunofluorescence (IF) against exosomal/membrane marker CD9. *Results*: Co-culture of PA embryos significantly improved blastocyst development of NT embryos when compared to the control NT embryos (threefold, $p < 0.05$). The relative quantification of pluripotency mRNAs (Pou5f, Klf4 and Nanog) showed significant increase in the NT embryos co-cultured with PA embryos when compared to the control group. Exosomes/microvesicles (EXs/MVs) were visualized in the PA embryos CM pellets through TEM and were CD9+ via FACS and IF. Small particles tend to be in the early stages embryos CM when compared to the blastocyst stage (35.4 ± 6.9 nm vs. 101.66 ± 18.4 nm, respectively). Furthermore, these EXs/MVs were found to contain mRNAs Pou5f, Sox2, Klf4, c-Myc and Nanog. Relative quantification of Pou5f, Sox2, Klf4, c-Myc and Nanog in the PA-CM was significantly higher than those in NT-CM either on day 2 or day 4 of embryo culture. *Summary/conclusion*: In vitro produced embryos can secrete EXs/MVs as communication tools within their microenvironment during in vitro culture. We showed the usefulness of co-culturing PA and cloned embryos to improve porcine cloning efficiency and provided a new paradigm for embryo-to-embryo communication *in vitro*. Supported by IPET (#311011-05-3-SB010), MOTIE (#10033839-2013) and BK21 PLUS.

Scientific Program 2014 ISEV meeting
Friday May 2nd, 2014
Oral Presentations

Registration		8:00-9:00
Arcadis Room Setting up posters (Poster Sessions 7A, 7B, 8A, 8B, 8C, 9A, 10C)		8:00-9:00
Sponsor Exhibition	Jurriaans Foyer/Mandele room	10:00-18:00
Plenary Session Chair: *Marca Wauben and Philip Stahl*	Willem Burger room	9:00-10:00

Hans Clevers, University medical Center Utrecht, Holland
The self-renewal force of specialized stem cells in the gut epithelium: Wnt signalling, lgr5 stem cells and disease

Networking coffee	Arcadis room	10:00-10:30
Poster Viewing Session 7A, 7B, 8A, 8B, 8C, 9A, 10C posters not-attended by authors	Arcadis room	10:00-10:30

Willem Burger room
Symposium Session 7A - EV cargo selection and release mechanisms 2
Chair: *Clotilde Thery and Maria Yáñez-Mó* 10:30-12:00

O7A-257

Mesenchymal stem cells use extracellular vesicles to outsource mitophagy

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Introduction: Mitochondrial quality control is mediated by mitophagy, and retention of dysfunctional mitochondria leads to cell death. Previously, we reported that mesenchymal stem cells (MSCs) transfer their mitochondria to macrophages, but the biological meaning of this observation is poorly understood. *Methods*: We studied the mitochondrial potential and ROS production of human MSCs, and labelled with baculovirus, the mitochondria (GFP) and phagosomes (RFP) of hMSCs, to determine the fate of these mitochondria. Subsequently, we co-culture these hMSCs in the presence of macrophages to follow the transfer of the GFP-labelled mitochondria. MSCs secrete micro vesicles (MV) that can be isolated from culture media by centrifugation. Larger MVs are isolated from MSC-cultured media by low-speed (10,000 g) centrifugation while typical exosomes require differential centrifugation (100,000 g/18 h) followed by sucrose gradient separation. We conducted proteomic and RNA analysis of MVs to understand the mechanism by which MSC extrude mitochondria and enhance their survival. *Results*: During culture, hMSCs undergo mitochondrial oxidative stress that compromise their differentiation and proliferation potential. hMSC mitochondria are partially depolarized and exhibit activation of Pink1/Parkin-mediated pathway of mitophagy. In hMSCs the GFP-labelled mitochondrial network co-localizes in close proximity with the RFP-LC3-labelled phagosome. Live cell imaging demonstrated that hMSC mitochondria are loaded in the cytoplasm into LC3-labelled vesicles and, subsequently, migrate towards the periphery of the cell where these MVs are incorporated into outward budding blebs in the plasma membrane where they are taken by nibbling macrophages. hMSCs use the release of arrestin domain-containing protein 1-mediated microvesicles (ARMMs) at the plasma membrane to secrete the LC3-labelled vesicles containing mitochondria. This process is characterized by the recruitment of the endosomal sorting complex required for transport (ESCRT)-associated protein tumour suppressor gene 101 (TSG101), and the arrestin domain-containing protein 1 (ARRDC1) to the cell membrane. Proteomic analysis confirms that hMSC-derived MVs contained TSG101 and ARRDC1 proteins. MSCs simultaneously shed miRNA-containing exosomes that inhibit Toll-like receptor (TLR) signalling, thereby de-sensitizing macrophages to the ingested mitochondria. *Summary/conclusion*: MSCs utilize extracellular vesicles to transfer mitochondria and shuttle micro-RNAs to optimize their survival. These studies mechanistically link immuno-modulatory activity with cell survival in MSCs and further demonstrate the important functional association between MSCs and macrophages.

O7A-258

The PI3K/mTORC1 signalling network regulates the cargo of exosomes secreted from HCT116 colorectal cancer cells

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Introduction: To survive adverse conditions, cancer cells produce a range of secreted signals to modulate their environment and co-ordinate their functions. Exosomes produced by the late endosomal multivesicular bodies represent a particularly complex signal that can reprogramme target cells via the action of multiple cargos. Late endosomes and lysosomes (LELs) are also classically implicated in degradation and recycling of cell contents, but more recently have emerged as an intracellular signalling hub, responding to micro-environmental and growth factor signalling to modulate cell growth via the LEL-associated, amino acid-sensitive, mechanistic target of rapamycin complex 1 (mTORC1). *Methods*: We investigated whether endolysosomal, amino acid-dependent mTORC1 activation and exosome secretion might be mechanistically linked using HCT116 colorectal cancer cells. We blocked different components of the PI3-kinase/mTORC1 growth regulatory network, including PI3-kinase (PI3K) and the putative amino acid-sensing intracellular transporter, Proton-Assisted Amino Acid Transporter 4 (PAT4), and analysed the composition of secreted exosomes. *Results*: A robust and substantive change in the relative levels of Caveolin-1 and the tetraspanin CD63 in exosomes was observed when PI3K/mTORC1 signalling was blocked. While both these proteins are highly concentrated within LEL compartments of HCT116 cells, they are selectively released within exosomes under different signalling conditions. HCT116 cells activate both a rapamycin-sensitive and rapamycin-insensitive complex, but only inhibition of the latter alters the exosome profile. *Summary/conclusion*: Our data highlight a previously unidentified cross-talk between amino acid-sensitive mTORC1-mediated growth regulation and exosome secretion in the endolysosomal system. This links environmentally induced changes in intracellular signalling with altered extracellular signalling and raises new issues concerning the effects of drugging the PI3K/mTORC1 network in cancer therapy.

O7A-259

Molecular mechanisms of miRNA sorting in extracellular vesicles

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Introduction: microRNAs (miRNAs) in extracellular vesicles (EVs) are important tools for intercellular communication; however, the proteins binding to miRNAs in EVs and the mechanisms involved in miRNA sorting are not fully understood. The purpose of this study was to identify these proteins and clarify the mechanisms responsible for the sorting of miRNAs in EVs. *Methods*: EVs were collected by conventional ultracentrifugation from serum-free conditioned media and resuspended in PBS. We performed proteomic analysis on the EVs derived from several cancer cell lines. To analyse the contribution of the candidate proteins on miRNA sorting in EVs, knockdown or overexpression experiments were performed. To confirm whether specific proteins bind to miRNAs, we carried out immunoprecipitation of protein-complex from EVs and checked the expression level of miRNAs by qRT-PCR. *Results*: Considering that EVs contain a large amount of miRNAs, we hypothesized that transport of miRNAs into EVs is regulated by RNA-binding protein highly enriched in EVs. Based on proteomic analysis, we decided to explore 8 candidate proteins with nucleic acid-binding properties. Using siRNA against these 2 candidate two proteins, we found that silencing of these 2 proteins decreased extracellular miRNAs. Conversely, the overexpression of these 2 proteins increased the amount of miRNAs in EVs.

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Finally, we detected miRNAs in the fractions isolated from EVs after immunoprecipitation of those 2 proteins. *Summary/conclusion*: These results indicate that 2 RNA-binding proteins are required for miRNA sorting in EVs.

O7A-260

BMP signalling controls a critical step after vesicle formation required for exosome release in *Drosophila*

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Introduction: Exosomes are initially formed in late endosomal multivesicular bodies (MVBs). Intriguingly, our previous work has highlighted a critical role for the surfaces of late endosomes and lysosomes (LELs) as conserved activation sites for the growth regulator, mechanistic Target of Rapamycin Complex 1 (mTORC1). We have recently characterized a powerful new in vivo system to dissect the role of the endolysosomal system in regulating growth and exosome biogenesis. It takes advantage of the *Drosophila* male accessory gland (AG), which shares similarities with the human prostate. A small subset of epithelial cells within the AG called secondary cells (SCs) specifically produces exosomes, which can be visualized within the AG lumen, and inside exceptionally large (>2 μm diameter) endolysosomal compartments. *Methods*: We have used the advanced molecular genetic methods and imaging approaches afforded by the fly to test whether a number of molecules implicated in mammalian exosome secretion in mammals, and several intracellular signalling cascades control exosome biogenesis and endolysosomal trafficking in SCs. *Results*: We show that known regulators of exosome secretion are required for SC exosome release and to produce a signal that blocks females from subsequent re-mating. Interestingly, the bone morphogenetic protein (BMP) signalling cascade, which drives the growth of SCs and also prevents female re-mating, is essential for exosome secretion, controlling a step after formation of vesicles in MVBs. When BMP signalling is blocked, SCs retain MVBs that do not appear to mature, explaining the effects on female reprogramming and potentially the associated SC growth defects. *Summary/conclusion*: Our data reveal an important new role for BMP signalling in endolysosomal trafficking events required for exosome secretion. We discuss the potential relevance of this finding to diseases such as prostate cancer, where BMP signalling is often misregulated.

O7A-261

Control of PTEN export in exosomes by P53

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Introduction: The 2 most important tumour supressors, p53 and PTEN (phosphatase and tensin homolog), have multiple layers of direct and indirect regulation. We have shown that PTEN can be exported in exosomes under the control of Ndfip1, an adaptor for Nedd4 ubiquitin ligases. Exosomal PTEN can be internalized by recipient cells with functional effects and strong implications for using exosomal PTEN as a mode of portable tumour suppression. Here, we provide evidence for a new layer of control of PTEN by p53, another tumour suppressor protein. *Methods*: We used supernatant from transfected 293T cells, wild type and p53 KO MEFs to harvest exosomes for electron microscopy, western blotting and uptake experiments. *Results*: We demonstrate that p53 can regulate PTEN and Ndfip1 in exosomes. Over-expression of p53 in 293T cells led to a reduction of PTEN and Ndfip1, but not other exosomal proteins, in exosomes. Removal of p53 in KO MEFs produced more PTEN and Ndfip1 in exosomes compared to wild-type MEFs. These results identify p53, the most commonly mutated tumour suppressor protein, to be an important regulator for exosomal export of PTEN and Ndfip1. This degree of molecular crosstalk between the 2 most prominent tumour suppressors in exosomal export of PTEN was unanticipated and suggests that intercellular communication via exosomes needs to be considered as an additional *modus vivendi* for tumour suppression. *Summary/conclusion*: The ability of p53 to regulate exosomal secretion of PTEN has significant implications on how tumour suppressors use exosomes to communicate between cells.

Jurriaanse room
Symposium Session 7B - EV in cancer metastasis
Chair: *David Lyden and Jacco van Rheenen* 10:30-12:00

O7B-262

RIG-I stimulation induces the release of tumour-suppressing exosomes from melanoma cells

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Introduction: Exosomes are involved in intercellular communication and have been demonstrated to promote tumour growth or to stimulate the immune system such as natural killer (NK) cells. Here, we investigate the effect of the innate immune receptor retinoic acid inducible gene-I (RIG-I) on the release and function of exosomes. RIG-I is a cytosolic pattern recognition receptor detecting viral nucleic acids by binding to 5′-triphosphorylated RNA (3p-dsRNA). Engagement of RIG-I can induce apoptosis in melanoma cells and suppress metastasis of melanoma in vivo in a type-I IFN and NK cell-dependent manner, but the underlying mechanisms are still unclear. *Methods*: We activated RIG-I in the primary human melanoma cell line D04mel by transfection of its ligand 3p-dsRNA, mimicking a viral infection. The released exosomes were analysed regarding the expression of ligands for the NK cell receptor NKG2D and the natural cytotoxicity receptors NKp30 (BAG6) and NKp46 (Vimentin). Furthermore, we analysed their functional capacity to induce an immune response in vitro and in vivo. *Results*: We found that activation of RIG-I in melanoma cells lead to an increased release of exosomes. Furthermore, the expression of the NK cell-activating ligand BAG6 (targeting NKp30) on the surface of released exosomes was induced. In addition, RIG-I and its ligand 3p-dsRNA were loaded into exosomes and transferred to immune- and tumour cells. Together, this led to induction of antiviral cytokines and Th1 chemokines, enhanced tumour cell lysis by NK cells and apoptosis in a human primary melanoma cell line in vitro. Intra-tumoural injection of RIG-I-induced melanoma exosomes led to the suppression of tumour growth in a subcutaneous melanoma model. *Summary/conclusion*: We show that tumour-derived exosomes released in response to RIG-I stimulation possess immune-stimulatory and anti-tumour capacity in vitro and in vivo. This pathway establishes a novel and unexpected link of a pattern recognition receptor and the release of exosomes.

O7B-263

Extracellular vesicles from renal cancer stem cells promote tumour phenotypic changes in mesenchymal stromal cells

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Introduction: Tumour cell interaction with cells of microenvironment, such as stromal, immune and endothelial cells, is associated with tumour progression. Mesenchymal stromal cells (MSC) are known to be recruited within the tumour and it is debated whether they support or inhibit its growth and spread. We previously found that renal cancer stem cells (RCSC) secrete EV capable to interact with cells of tumour microenvironment (Cancer Research, 2011). The aim of the present study was to analyse whether RCSC-EV can promote phenotypic changes in MSC that may support tumour progression. *Methods*: EV were obtained from supernatants of RCSC cultured overnight in RPMI with 0.1% of BSA and submitted to differential ultracentrifugation. MSC were incubated with RCSC-EV for 72 h (1 stimulus) or 2 weeks (3 stimuli). Incorporation of Vybrant™ Dil stained RCSC-EV within MSC was analysed by confocal microscopy. MSC migration studies were performed within transwells and visualized by crystal violent staining. Gene expression was evaluated by quantitative RT-PCR. Co-culture of EV-modified MSC with carcinoma cells were performed in a transwell system. *Results*: RCSC-EV incubation led to a 33% increase of MSC migration after 72 h and a 75% increase after 2 weeks. Gene expression analysis revealed an increase in the genes related to migration, invasion capacity (CXCR7, collagen IV, metalloproteinase 1, 2, 3 and 9) and cancer-associated fibroblast (CAF) phenotype (tenascin and fibroblast surface protein). When MSC were stimulated with EV for 2 weeks, the changes were persistent in the absence of additional stimulation. Renal carcinoma cells co-cultured with MSC modified by 2-week RCSC-EV-stimulation presented an increase of epithelial-mesenchymal transition (EMT) marker ZEB1. *Summary/conclusion*: These results show that RCSC-EV incorporated by MSC promote an increased migration and modulation of genes related to matrix remodelling and CAF phenotype. In addition, MSC modified by RCSC-EV were able to promote EMT in renal carcinoma cells, suggesting that the EV-mediated crosstalk between RCSC and MSC may play a role in tumour progression.

Financial Support: Associazione Italiana per la Ricerca sul Cancro (AIRC).

O7B-264

Cellular disposal of miR-23b by Rab27a/b-dependent exosome release is linked to acquisition of a bladder cancer metastatic state

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Introduction: Exosomes are small secreted vesicles that mediate intercellular signalling and sustain tumour growth and metastatic spread. It is unaddressed, however, how cellular disposal of molecules via exosomes may also influence such processes. *Methods*: Isogenic bladder carcinoma cell lines with different metastatic potential and clinical samples of bladder cancer (primary tumours and patient-matched lymph node metastases) were analysed. Vesicle characterization was conducted by EM, NTA and western blotting for exosomal markers and mRNA, and miRNA profiling of cells, tissue and secreted vesicles was conducted. *Results*: We identified increased relative secretion of miR23b, -224, and -921 in vesicles from metastatic cells. Parental cell enrichment for mRNA targets of exosome-exported miRNAs was observed. Rab27a and/or b gene silencing decreased the expression of kinesin motor proteins, exosome- and miRNA secretion, and led to intracellular rise in miR23b copies. The secretion of miR23b and -921 was primarily associated with an RNAse-resistant exosome fraction rather than a

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soluble fraction. Ectopic miR23b expression reduced cellular invasion, anoikis resistance and in vivo lung colonization of FL3 cells. In clinical samples of bladder cancer, high Rab27b expression correlated with disease progression (196 patients) and reduced cancer-specific survival (278 patients). Loss of miR23b and other vesicle-exported miRNAs was detected in lymph node metastases compared to primary tumours and accompanied by increased expression of their putative mRNA targets. *Summary/conclusion*: Our data suggest that exosome-mediated miRNA secretion may represent a route for selective tumour suppressor miRNA disposal during the acquisition of a metastatic state in bladder cancer.

O7B-265

The role of EMMPRIN for the pro-invasive function of tumour cell microvesicles and their identification in metastatic cancer patients

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Introduction: Tumour cells are known for their ability to create a favourable tumour microenvironment that is essential for tumour progression. Especially tumour cell microvesicles (T-MV, 100–1,000 nm) are known to drive stroma cells in a tumour-supporting phenotype. We hypothesized that T-MV also mediate intercellular communication between neighbouring tumour cells and facilitate tumour progression via a direct feedback loop. We aimed to investigate their mode of action and define MV-associated tumour markers that are involved in tumour–tumour crosstalk and allow identification of T-MV in cancer patients. *Methods*: MV were isolated from breast (cancer) cell lines in vitro and tested for their influence on tumour invasion. Western blotting, flow cytometry and proteomics approaches were employed to define MV-specific tumour markers, which were used to detect circulating T-MV in peripheral blood of metastatic cancer patients. *Results*: T-MV significantly enhanced breast cancer invasion in an autologous and heterologous manner through activation of p38/MAPK signalling, whereas benign MV did not have such an effect. Moreover, uptake of T-MV in a dynamin-dependent way was critical for their pro-invasive function. All T-MV were characterized by expression of a highly glycosylated isoform of the Extracellular Matrix Metalloproteinase Inducer (EMMPRIN) with N-glycosylation at N160 and N268. Deglycosylation or knockdown of EMMPRIN antagonized the observed pro-invasive function of T-MV. Moreover, we detected EMMPRIN-positive MV at very high levels in peripheral blood of metastatic cancer patients and identified EMMPRIN as a novel marker for circulating T-MV. *Summary/conclusion*: Breast cancer cells release EMMPRIN-positive T-MV into the local microenvironment as well as the bloodstream. The presence of glycosylated EMMPRIN on these T-MV not only increases the invasiveness of surrounding tumour cells in vitro but can also be used as a novel and highly specific marker for circulating breast cancer T-MV in metastatic cancer patients.

O7B-266

Metastasis promotion by Tspan8 and CD151 and the contribution of tumour exosomes

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Introduction: Metastasis formation is promoted by establishment of a premetastatic niche, which is accomplished by tumour-derived exosomes. As tetraspanins are constitutive exosome components, we asked whether exosomal tetraspanins, in particular the 2 metastasis-promoting tetraspanins CD151 and Tspan8, actively contribute to premetastatic niche formation. To further define whether Tspan8 and CD151 fulfil redundant or additive activities, Tspan8 or CD151 or both were stably knocked-down in highly metastatic rat pancreatic adenocarcinoma BSp73ASML cells (ASML^{wt}, ASML-Tspan8^{kd}, ASML-CD151^{kd}, ASML-Tspan8^{kd}/CD151^{kd}). *Methods*: Tspan8 and/or CD151 were stably knocked-down in ASML cells. Metastatic capacity was evaluated after intrafootpad tumour cell injection. Exosome were isolated by standard protocols, including sucrose density gradient purification. Exosome composition was analysed by MALDI-TOF and co-immunoprecipitation. Exosome activity was evaluated with respect to matrix degradation and modulation of target cell activity. *Results*: ASML-CD151^{kd} and ASML-Tspan8^{kd} cells metastasize via the lymphatics to the lung with a significant delay, whereas only one of five rats receiving ASML-Tspan8^{kd}/CD151^{kd} cells developed metastases, which confirms CD151 and Tspan8 contributing to metastasis and pointing towards distinct, but additive Tspan8 and CD151 activities. After demonstrating that the Tspan8 and CD151 complexes of ASML^{wt} cells are maintained in exosomes, we analysed the exosomal ASML-Tspan8^{kd}, ASML-CD151^{kd}, ASML-Tspan8^{kd}/CD151^{kd} tetraspanin complexes. Most striking has been the failure to recruit CD44v6 and the beta4 integrin chain as well as of the dipeptidase CD13 and of ADAM17 in the absence of Tspan8. In ASML-CD151^{kd} exosomes MMP9 was missing and MMP2 recruitment was reduced. These defects of ASML-Tspan8^{kd} and/or ASML-CD151^{kd} exosomes have severe consequences on modulation of the extracellular matrix such that binding of ASML-Tspan8^{kd} exosomes to LN332 is severely impaired and LN332 is hardly degraded. ASML-CD151^{kd} exosomes poorly degrade collagen I and IV and laminin111 and matrix degradation was not seen with ASML-Tspan8^{kd}/CD151^{kd} exosomes. The defective matrix degradation by ASML-Tspan8^{kd}, ASML-CD151^{kd} and ASML-Tspan8^{kd}/CD151^{kd} exosomes severely affects stroma cell and endothelial cell motility and invasiveness in vitro and in vivo. Due to the liberation of growth factors deposited in the ECM, ASML-Tspan8^{kd}, ASML-CD151^{kd}, ASML-Tspan8^{kd}/CD151^{kd} exosomes also distinctly influence stroma cell and hematopoietic cell proliferation and apoptosis resistance. *Summary/conclusion*: Due to the tetraspanin enrichment in exosomes, Tspan8 and CD151 play an important role in ECM modulation, thereby promote angiogenesis, premetastatic niche preparation and recruitment of hematopoietic cells. Taking the supplementing activities of exosomal Tspan8 and CD151, therapeutic concepts may profit from concomitantly attacking both tetraspanins.

O7B-267

Osteosarcoma-derived exosomes as a potential factor for pre-metastatic niche formation in the lung

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Introduction: The mechanism regulating lung metastasis of osteosarcoma (OS) is poorly understood. Recent studies have shown the involvement of tumour-derived exosomes in progression of malignancy. Here, we investigated the role of OS-derived exosomes in lung metastasis. *Methods*: We used a highly metastatic human OS cell line 143B cells expressing firefly luciferase and established a derivative cell line with shRNA knockdown of neutral sphyngomyelinase 2 (143B-KD). 143B cell-derived exosomes were isolated by ultracentrifugation. Original 143B cells were orthotopically transplanted to the right tibia of nude mice at 1.5×10^6 cells/mouse (group 1). Mice similarly transplanted with 143B-KD-nSMase2 cells were divided into 2 groups, and were intravenously administered 200 ml PBS (group 2) or 5 g-exosomes/200 ml PBS (group 3) twice a week for 3 weeks. Lung metastasis was monitored by IVIS system. *Results*: Three weeks after transplantation, lung metastasis was observed in 7/10 mice in group 1. In contrast, only 3/10 mice showed lung metastasis in group 2. Remarkably, however, 7/10 mice showed metastasis in group 3, indicating that systemically administered exosomes restored the metastatic ability. Luminescence measurements of lung regions confirmed a significant increase in

the luminescent signals in group 3 compared to group 2. On the other hand, in vitro we did not observe difference in proliferation rate or invasion capacity between original 143B cells and 143B-KD cells. Taken together, it was suggested that systemically administered exosomes did not act directly on the primary tumour cells, but acted on the lungs, thereby contributed to conditioning the pre-metastatic niche. *Summary/conclusion*: The results demonstrated that exosomes secreted by 143B cells into the circulation promoted lung metastasis. Thus, we hypothesize roles of exosomes in pre-metastatic niche formation in the lung, and are now exploring the underlying molecular mechanism.

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Van Weelde/Mees room
Symposium Session 7C1 - Late Breaking EV in cancer
Chair: *Jan Lötvall and Richard Simpson* 10:30-11:15

O7C1-5670

Double-stranded DNA in exosomes: a novel biomarker in cancer detection

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Introduction: Tumour-secreted exosomes are small membrane vesicles (30–100 nm) of endocytic origin secreted by most cell types and contain functional biomolecules like oncoproteins, retrotransposon RNA transcripts, single-stranded DNA (ssDNA), mitochondrial DNA and oncogene amplifications (i.e. c-myc). Here, we demonstrate for the first time that the majority of DNA inside the exosomes derived from different cell types is double-stranded. We provide evidences that exoDNA can be used as a novel surrogate marker of the mutational status of parental tumour cells representing the whole genome. *Methods*: The double-stranded nature of exoDNA was established using ssDNA-specific S1 nuclease (S1) or dsDNA-specific Shrimp dsDNase (dsDNase). Furthermore, atomic force microscopy imaging was used to confirm the presence of dsDNA in exosomes and characterize its structure. Deep sequencing of exoDNA and gDNA was performed using Illumina High Throughput DNA sequencing. AS-PCR assays were employed to detect mutations in *BRAF* and *EGFR* genes. *Results*: To characterize the nature of the DNA within exosomes, we eradicated extracellular and non-specific DNA present on the exosomal surface using ssDNA-specific S1 or dsDNA-specific dsDNase. Furthermore, to determine the nature of DNA present inside exosomes, we combined S1 and dsDNase enzymes to selectively digest ssDNA and dsDNA. We observed that dsDNA-specific dsDNase completely degraded the DNA associated with exosomes derived from leukaemia, colon cancer and mouse melanoma cells. Moreover, we observed that the size of the major population of dsDNA inside the exosomes ranges between 100 bp and 2,500 bp. We next determined the levels of dsDNA in exosomes from breast, lung, melanoma, pancreatic and leukaemia cancer cell lines. We could demonstrate that the level of dsDNA inside exosomes varies between different cancer models, with pancreatic cancer exosomes having relatively lower levels of dsDNA. We further confirmed the presence of dsDNA in exosomes using AFM imaging of exoDNA and control linearized dsDNA and further illustrated that the height of exoDNA was approximately up to 700 pm and similar to the linear dsDNA controls. Deep sequencing analysis revealed that exoDNA represents the entire genome coverage in an unbiased manner. Finally, we present evidences that oncogenic mutations in *BRAF*, *EGFR* and *K-RAS* can be detected in exoDNA derived from melanoma, lung and colon cancer cell lines, respectively. *Summary/conclusion*: Our data demonstrate for the first time the presence of dsDNA inside tumour-derived exosomes and representative of the whole genome. Our finding that exoDNA can be used to identify mutations present in parental tumour cells illustrates its significant translational potential as a potential novel biomarker which can be used for early detection of cancer and metastasis and to track the evolution of tumour mutations in response to therapy.

O7C1-5671

RNA contents of plasma and cerebrospinal fluid extracellular vesicles in glioblastoma patients

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Introduction: Analysis of EVs derived from plasma or cerebrospinal fluid (CSF) has emerged as a promising biomarker platform for therapeutic monitoring in glioblastoma patients. However, the genetic contents of the various subpopulations of EVs in these clinical specimens remain poorly defined. Here we characterize the relative abundance of select RNAs in EVs derived from the serum and CSF of glioblastoma patients and compared them to those derived from EVs of glioblastoma cell lines. *Methods*: EVs were isolated from the plasma and cerebrospinal fluids of nine glioblastoma patients as well as nine glioblastoma cell lines. The microvesicle subpopulation was isolated by pelleting at 10,000 × g for 30 min after cellular debris was cleared by a 2,000 × g (20 min) spin. The exosome subpopulation was isolated by pelleting the microvesicle supernatant at 120,000 × g (120 min). These EV subpopulations were characterized by Nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM). qRT-PCR was performed to examine the distribution of GAPDH, 18S rRNA, miR-21, miR-103, miR-24, and miR-125. *Results*: In plasma and cell line derived EVs, the relative abundance of the panel of RNAs in exosome and microvesicles were highly variable. In some specimens, the majority of the RNA species were found in exosomes while in other the majority of the RNA species were found in microvesicles. The one exception to this observation was that 18S rRNA was highly enriched in microvesicles derived from glioblastoma cell lines. In contrast, CSF exosomes were highly enriched for all RNAs tested relative to CSF microvesicles. *Summary/conclusion*: Our results indicate that the distribution of RNA species in plasma exosome or microvesicles is highly unpredictable. As such, EV RNA analysis in plasma should incorporate both subpopulations of EVs. In contrast, CSF exosomes constitute the major EV compartment that harbor RNA species and represent an excellent platform for glioblastoma biomarker development.

O7C1-5672

M-Trap: exosome-based capture of tumour cells as a new technology in peritoneal metastasis

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Introduction: The molecular and cellular bases that determine the stepwise process of metastasis suggest an intense dialogue of the primary tumour with the environment. Tissue-specific metastasis and pre-metastatic niches are concepts that are beginning to illustrate an active role of carcinomas in the determination of the most adequate sites to colonize: signals emitted and sensed both from the tumour and from the environment govern the remodelling of targeted tissues for a favoured reception of prone tumour cells disseminated from primary lesions. *Methods*: Human SKOV3 ovarian tumour cells intraperitoneally injected in SCID mice were used as model system for ovarian peritoneal dissemination. Nanotechnology-based 3D scaffolds decorated with exosomes purified from ascitic fluid from ovarian cancer patients were used for fabrication of M-Trap device. In vitro dynamic cell-adhesion assays and in vivo bioluminescence imaging systems were used to evaluate the effect of M-Trap

technology in peritoneal metastasis. *Results*: We here describe a novel approach in oncology trying to hamper the process of metastasis by interfering with this intense dialogue and a probe of concept in a model of ovarian cancer intraperitoneal dissemination: (a) we characterized exosomes as components within the ascitic fluid of ovarian cancer patients owning the ability to dialogue with tumour cells and modulate their localization; (b) we generated a nanotechnology-based capture device by embedding exosomes onto a 3D-scaffold (M-Trap) where metastatic tumour cells preferentially home; (c) we demonstrated that M-Trap completely remodelled the peritoneal pattern of metastasis in an ovarian cancer model; and (d) we finally evaluated the impact of M-Trap on the outcome of the animal model with a benefit on survival. *Summary/conclusion*: We present exosomes as key drivers of the crosstalk between metastatic tumour cells and their environment during massive peritoneal dissemination in ovarian cancer. We then created M-Trap device as an artificial pre-metastatic niche based on exosomes that might compete with natural niches for the capture of tumour cells metastasizing in the peritoneal cavity. In clinical setting, this would represent the capacity to modulate the pattern of metastatic dissemination, the alteration of the process of metastasis by preventing the generation of new foci, and the possibility to transform a systemic disease into a focalized disease where the therapeutic approaches would present again a reasonable effectiveness.

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Van Weelde/Mees room
Symposium Session 7C2 - Novel Developments in the EV field - Sponsored session
Chair: *Jan Lötvall and Richard Simpson* 11:15-11:45

O7C2-5673

Initial study of protein coated polystyrene beads as a model system for circulating microvesicles

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Introduction: Circulating microvesicles (cMVs) are membrane structures ranging from 50 nm to 1000 nm that are secreted by many cell types and have been found in blood, urine, saliva and other body fluids. These particles transfer information between cells by transporting selected mRNA and microRNA and contain specific subsets of proteins that are likely to correlate to their origin. Deciphering the protein profiles of particular vesicle populations and identifying their source is one of the main challenges in the field of cMV research and might be a way to identify and monitor diseases. In order to develop tools for cMV profiling, it is necessary to create controlled and reproducible test systems that mimic cMVs. *Methods*: We have generated such a system by conjugating proteins to polystyrene beads that are comparable to the size of released microvesicles. *Results*: Here, we present a model system showing specific interactions between the protein coated polystyrene beads and their corresponding antibodies. The specificity was confirmed by Luminex® assay and scanning electron microscopy (SEM). *Summary/conclusion*: These protein-coated beads allow us to optimize assay conditions in a defined environment, which could later be translated to biological systems. In addition, the protein profile of the polystyrene beads can be manipulated to study myriad proteomic patterns and disease indications where characterization of cMV interactions is essential.

O7C1-5674

A rapid and non-destructive magnetic bead-based exosome isolation and enrichment method

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Introduction: In order to isolate exosomes from various body fluids and cell culture supernatants, we have successfully developed ExoCap, which utilizes a magnetic bead-based isolation method. *Methods*: ExoCap consists of magnetic particles coupled with antibodies that recognize antigens on the exosome surface, an irrigation solution, and a reagent that releases the captured exosomes for analysis. The antibodies against CD9, CD63, CD81 and EpCAM were specifically selected for this kit. *Results/Conclusion*: ExoCap can separate easily exosomes within 30 minutes, without ultracentrifuge or any special equipment. A sample amount of 0.1 mL is sufficient. In addition, it is an animal-free system, which is superior to other methods for mass analysis. Moreover, this method enables non-destructive purification of exosomes. To confirm exosomal isolation from diverse body fluids (such as human serum, plasma, urine) and cell culture media, exosomes were examined by western blot, particle size distribution measurement and scanning transmission electron microscopy (TEM). Exosomes isolated by ExoCap had a lipid bilayer membrane, showed a particle size distribution around 100 nm and expressed tetraspanin molecules.

Networking lunch 12:00-13:00

Poster Viewing Sessions 7A, 7B, 8A, 8B, 8C, 9A, 10C: attended by authors Arcadis room 12:30-13:00

Poster Walk, by chairperson, Session 7A, 7B, 8A, 8B, 8C, 9A, 10C: attended by authors Arcadis room 13:00-14:00

JEV Editorial Meeting Schadee Room 12:00-13:00

Willem Burger room
Symposium Session 8A - EV in (cardio) vascular disorders
Chair: *Alison Goodall and Arne Trummer* 14:00-15:30

O8A-322

Prothrombin recycling and activation in vascular smooth cell-derived exosomes link coagulation and vascular calcification

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Introduction: Vascular calcification is a pathological deposition of hydroxyapatite (HA) in the vascular wall. HA precipitation is initiated by annexin–lipid complexes on exosomes secreted by vascular smooth muscle cells (VSMCs) and can be prevented by loading of exosomes with Matrix gamma-carboxyglutamic (Gla) residue-rich protein (MGP) or the exogenous, serum-derived glycoprotein, fetuin-A. Thrombosis is often associated with atherosclerotic calcification, and a pivotal coagulation factor, prothrombin (PT), is also enriched with Gla residues. Here we studied the effects of PT on VSMC calcification. *Methods*: Calcification of human VSMCs was induced by elevated calcium and phosphate. For uptake studies PT was labelled with Alexa488. Exosomes were isolated by differential ultracentrifugation. Thrombin generation was detected using S2238. *Results*: PT is not expressed by VSMCs but abundantly deposited in calcified arteries in vivo. PT effectively inhibits VSMC apoptosis and calcification in vitro and this effect is mediated by prothrombin fragment 1 (PTF1), which is enriched with Gla residues. Importantly, PT does not inhibit calcium phosphate precipitation in solution suggesting that it may act on the HA nucleation activity of exosomes. Notably, VSMCs rapidly uptake PT in a calcium- and phospholipid-dependant manner and load full-length PT into exosomes with sorting occurring via early and late endosomes. PT recycled in exosomes undergoes proteolytic activation upon exosome secretion as revealed by the presence of PTF1.2 and PTF1 on exosomes and an enhanced thrombin generation activity. Furthermore, binding of PT to phospholipids on exosomes prevented annexin A2 and A6 loading into exosomes which reduced the number of annexin–phospholipid nucleation sites and thus exosome pro-calcific activity. In turn, PT binding to exosomes and exosome pro-thrombotic activity was reduced in the presence of annexin A5. *Summary/conclusion*: PT is a novel circulating inhibitor of vascular calcification, which is recycled by VSMCs via an exosomal pathway. PT binds to phospholipids on the surface of exosomes via its PTF1 domain, and this binding causes thrombin generation and thrombosis. However, this also reduces the number of annexin–phospholipid nucleation sites preventing VSMC calcification.

O8A-323

Procoagulant extracellular vesicles in the patients with monoclonal gammopathy of undetermined significance and their impact on the thrombogenic profile

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Introduction: The most common plasma cell dyscrasia is monoclonal gammopathy of undetermined significance (MGUS) and is defined by the presence of a monoclonal protein on electrophoresis and absence of symptoms. On average, MGUS patients progress to multiple myeloma or a related disorder at a rate of 1% per year. Multiple myeloma is always preceded by MGUS. Both MGUS and multiple myeloma patients have an increased risk of developing venous thromboembolism (VTE), and the underlying mechanisms are unknown, which stresses the importance of discovering the underlying mechanisms essential for early intervention and treatment of these patients. Several biochemical risk factors have been suggested to be associated with the procoagulant state of these patients. One of these candidates is extracellular vesicles (EVs), carriers of procoagulant factors, for example, tissue factor (TF) and procoagulant phospholipids (PPL). Thus, we investigated whether increased levels of plasmatic EVs correlate to blood coagulability in a prospective study of MGUS patients. *Methods*: A total of 33 MGUS patients were included in the study. Plasma samples were taken and analysed at baseline and every 6 months. Plasma samples were also collected from matched control persons. Plasma particle content were analysed using nanoparticle tracking analysis (NTA). To measure hypercoagulability and thrombin generation of the patients, their plasma was analysed by means of calibrated automated thrombogram (CAT), and PPL activity was measured using a chronometric method. TF levels were detected through an enzyme-linked immunoassay. *Results*: Using NTA, we observe increased particle levels in MGUS patients when compared to control persons. Interestingly, patients exhibit significantly increased procoagulant activity and thrombin potential when compared to control persons. In addition, the levels of TF in MGUS differ from that of control persons, towards a more diseased profile. *Summary/conclusion*: In this prospective study on MGUS patients, we found elevated levels of plasmatic EVs and the hypercoagulable state in MGUS patients compared to matched controls. We stress the importance of identifying the EV subpopulations that may be responsible or contribute to the procoagulant state observed in MGUS. These findings might help explain the underlying mechanisms for thrombosis in multiple myeloma that will hopefully lead to prevention of thrombotic events at an earlier state.

O8A-324

Tissue factor is associated with two types of detergent-resistant membranes in extracellular vesicles

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Introduction: Tissue factor (TF), a transmembrane protein, is constitutively exposed on extravascular cells and their vesicles, and can be present in a non-coagulant and a coagulant form. Within cell membranes, microdomains of detergent-resistant membranes (DRM) are present, which are enriched in cholesterol and sphingolipids compared to their surroundings. DRM are resistant to non-ionic detergents, and cells contain both low-density DRM (DRM-L ρ1.09–1.13 g/ml) and high-density DRM (DRM-H ρ1.15–1.20 g/ml). We investigated whether vesicles contain DRM, whether vesicle-exposed TF is associated with DRM and whether the association of TF with DRM may affect the coagulant activity of TF. *Methods*: Vesicles were isolated from conditioned culture medium of human

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vascular smooth muscle cells (VSMC), human wound blood and human saliva. Vesicles were lysed in Triton X-100-containing buffer. DRM were isolated by OptiPrep gradient ultracentrifugation, and 9 fractions of 1 ml were isolated and analysed for density, TF antigen (ELISA, western blot), TF coagulant activity (fibrin generation assay), flotillin (DRM marker) and caveolin (marker of DRM containing caveolae) and tissue factor pathway inhibitor (TFPI; all western blot). *Results*: Vesicles of VSMC, wound blood and saliva contain coagulant and non-coagulant forms of TF, DRM-L and DRM-H. Coagulant TF is associated with DRM-L (fractions 2–5) for all 3 types of vesicles, but only DRM-L from wound blood vesicles contains detectable levels of flotillin and caveolin. Non-coagulant TF is associated with DRM-H (fractions 6–8) in all 3 types of vesicles, which all contain detectable levels of flotillin. In contrast, caveolin is detectable only in DRM-H of vesicles from VSMC and wound blood. *Summary/conclusion*: Our findings show that different forms of TF are associated with different types of DRM in vesicles. Thus, we hypothesize that the association of TF with the microenvironment may influence the coagulant activity of TF.

O8A-325

Extracellular vesicles carrying Sonic hedgehog activate a novel cardio-protective signalling pathway on adult rat cardiomyocytes

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Introduction: Extracellular vesicles (EVs) shed from T lymphocytes undergoing activation and apoptosis exert a beneficial potential effect on the vascular endothelium through their dual capacity to increase nitric oxide and reduce reactive oxygen species production via the activation of Sonic hedgehog pathway. However, little is known about the effects of EVs in adult cardiomyocytes. *Methods*: Thus, the goal of this study was to investigate the potential role of EV-associated Shh on adult rat cardiomyocytes and in vivo cardiac activity. *Results*: Here we show in adult cardiomyocytes that Shh can induce nitric oxide production, with subsequent activation of the ATP-dependent potassium current (IKATP). This reduces action potential duration and, subsequently, the amplitude of transient Ca^{2+} release and cardiomyocyte contraction. These effects are prevented by cyclopamine, an antagonist of the Shh receptor, the Patched–Smoothened complex and when cells were incubated with EVs lacking the Shh ligand. In addition, by measuring cardiac activity, we demonstrate that EVs reduce duration of QT interval of electrocardiogram leading to the shortening of ventricular cardiomyocyte action potential duration. *Summary/conclusion*: Besides its beneficial effect on both angiogenesis and endothelial function, we demonstrate here a novel effect of EVs carrying Shh acting directly on the cardiomyocytes and affecting cardiac activity participating to its cardio-protective properties. This study emphasizes the pleiotropic effect of EVs as potential therapeutic tools to improve cardiomyocyte function.

O8A-326

Functional transfer of selected miRNA and mRNA to mature heart cells by pluripotent stem cell-derived microvesicles – potential role in heart repair

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Introduction: Microvesicles (MVs) represent membrane-enclosed particles shed into the extracellular environment by direct budding from the cell plasma membrane or derived from the endosomal compartment. MVs carry bioactive components and may act as mediators of cell-to-cell communication by transferring such content from parent to target cells. In this study, we examined MVs derived from human-induced pluripotent stem cells (iPS) produced in our laboratory and investigated the transfer of selected miRNAs and mRNAs by iPS-MVs to mature cells. Moreover, we examined impact of the transferred components on selected functions of heart cells, including differentiation, metabolic activity, viability and survival in hypoxia. *Methods*: MVs were isolated by ultracentrifugation (100,000 g/1 h/twice). The level of selected transcripts related to pluripotency (OCT4, NANOG), angio- and cardiomyogenesis (GATA4, NKX2.5, GATA2, VE-CADHERIN) was examined in iPS cells and their MVs by real-time RT-PCR. Furthermore, multiantigenic profile of iPS cells and their MVs, including the expression of selected markers related to stemness (SSEA-1/3/4, Ter-1-80, Sca-1) as well as angio- and cardiomyogenesis (VSEGFR2, CD31, CD105), was examined by classical and imaging cytometry (ImageStream system). *Results*: We established that iPS-MVs are enriched in several miRNAs and mRNAs carried by their parental cell line. Similarly, most of iPS cell-derived surface molecules and receptors were detected on iPS-MVs. Moreover, we found that MVs may transfer the active content to primary mature cardiac cells. Importantly, we found significant impact of such transfer on selected functions of target cells. *Summary/conclusion*: The data indicate that iPS-MVs may represent natural nanocarriers of several proangiogenic, cardiomyogenic and antiapoptotic agents to mature heart cells enforcing both function and survival of mature heart cells, when delivered into ischemic myocardium. Thus, this report suggests the potential future role of iPS-MVs in heart regeneration.

O8A-327

Exosomes secreted by human cardiac progenitor cells inhibit cardiomyocyte apoptosis and improve cardiac function after myocardial infarction

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Introduction: Cardiac progenitor cell (CPC) transplantation improves cardiac function after myocardial infarction (MI). This effect is mediated, at least in part, by secreted factors. Exosomes (Exo) are secreted nano-sized membrane vesicles acting as intercellular carriers of proteins and nucleic acids, including microRNA (miRNA). Here, we investigated the role of Exo in the paracrine activity of human CPCs. *Methods*: Atrial appendage specimens were obtained from patients who underwent heart valve surgery. CPCs were derived from the cellular outgrowth of these specimens using the primary ex vivo tissue culture technique. Exo were purified from CPC-conditioned medium (CM-CPC) using ExoQuick and analysed by transmission electron microscopy (TEM). Functional effects of CM-CPC and purified Exo were assessed in vitro using angiogenesis and apoptosis models in human endothelial cells (HUVECs) and mouse HL-1 cardiomyocytes (CMC), respectively. Apoptosis was assessed by activated caspase 3/7 immunostaining. The effects of cryopreserved Exo derived from human CPCs were compared with those from normal human dermal fibroblasts (NHDF). In vivo studies were performed in a rat model of myocardial infarction (MI) induced by permanent left anterior coronary artery ligation. Exo were injected into the infarct border zone 1 h after coronary ligation. Cardiac function was assessed by echocardiography. Apoptosis was detected by TUNEL. *Results*: Culture medium conditioned by CPCs (CM-CPC) stimulated tube formation by endothelial cells (HUVECs) and inhibited apoptosis of mouse HL-1 cardiomyocytes after serum deprivation in vitro. Depletion of Exo abolished the proangiogenic and antiapoptotic activities of CM-CPC. Supplementation of Exo-depleted CM with Exo-CPC restored this activity. Exo-CPC inhibited cardiomyocyte apoptosis in a dose-dependent manner, whereas Exo secreted by normal human dermal fibroblasts (Exo-F) did not. miRNA analysis identified miR-146a-3p, miR-181a, miR-132 and miR-210-3p

among the most highly upregulated miRNAs in Exo-CPC versus Exo-F (15-, 11-, 5- and 4-fold, respectively). In a rat model of MI, Exo-CPC significantly reduced cardiomyocyte apoptosis and scar, enhanced angiogenesis and preserved LVEF 7 days post-MI ($0.8 \pm 6.8\%$) compared with controls ($-21.3 \pm 4.5\%$; $p < 0.05$) and Exo-F-injected hearts ($-12.0 \pm 6.3\%$). *Summary/conclusion*: Exo accounts for proangiogenic and antiapoptotic activities of the paracrine secretion of human CPCs, which preserve cardiac function post-MI. As a cell-free product, Exo-CPC may circumvent many of the limitations of cell transplantation for cardiac repair.

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Jurriaanse room
Symposium Session 8B - Standardization of EV measurements
Chair: *Michael Paulaitis and Paul Harrison* 14:00-15:30

O8B-328

Intra- and inter-individual variability in blood microvesicles measured by digital flow cytometry

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Introduction: This study calculated the intra-individual variability in absolute numbers of blood-borne microvesicles (MV, >0.2 μm in size) measured using standardized techniques by digital flow cytometry. *Methods*: MV were isolated by differential centrifugation from blood anti-coagulated with hidurin plus soybean trypsin inhibitor collected yearly for 5 years from healthy recently menopausal women (42–58 years of age, $n=118$) consented in the IRB-approved Kronos Early Estrogen Prevention Study at Mayo Clinic. Absolute numbers of MV of different cellular origin and positive for annexin-V were measured using fluorophore-conjugated annexin-V and cell surface-specific antibodies plus known count calibration beads. Within each MV parameter, coefficients of variation were computed within individuals using the standard deviation of their log-transformed serial data and averaged across participants. Time trends in the MV were tested using the general estimating equations method to account for repeated measures of data. $p<0.05$ was accepted as statistically significant. *Results*: Coefficient of variations for individual variability of numbers of MV (log transformed) were 20.4% for platelet positive, 48.8% for leukocyte positive, 43.3% for monocyte positive, 45.7% for endothelium positive and 17.6% for annexin-V positive MV. Significant time-dependent changes were observed for leukocyte- and endothelium-derived MV ($p=0.001$ for each). *Summary/conclusion*: This study provides the first global intra-individual variability in MV (>0.2 μm) by digital flow cytometry from healthy menopausal women. Knowing intra-individual variability is required if MV are to be used as biomarkers for diagnosis, prognostic assessment and management of individuals with suspected diseases.

O8B-329

Standardization of pre-analytical variables in the measurement of extracellular vesicles

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Introduction: The numbers and composition of extracellular vesicles (EV) in body fluids depend on collection and handling. Thus far, these variables have not been standardized, thereby hampering the comparison of EV measurement results between laboratories. In the context of the European Metrology Research Programme (EMRP), METVES (www.metves.eu), we aim to develop protocols to standardize collection, handling and storage of body fluids to improve the comparison of EV measurement results between laboratories. *Methods*: EV of erythrocytes and platelets were isolated at $18{,}890\times g$ or $100{,}000\times g$ for 0.5 and 2 h. Isolated EV were reconstituted in phosphate-buffered saline or in vesicle-depleted human normal pool plasma, and measured directly and after a single freeze/thaw cycle ($-20^{\circ}C$, $-80^{\circ}C$, liquid nitrogen). Vesicles were studied by resistive pulse sensing (RPS), nanoparticle tracking analysis (NTA), flow cytometry (FCM) and transmission electron microscopy (TEM). *Results*: Erythrocyte EV remain present as single vesicles under all experimental conditions, their diameter ranges between 100 and 200 nm (RPS, NTA, TEM) and the concentration decreases 2-fold during a single-freeze thaw cycle, irrespective of the freezing temperature and reconstitution solution. In contrast, platelet EV form clumps. The diameter of remaining single platelet EV is between 100 and 200 nm, but their concentration increases 2-fold during a single freeze-thaw cycle. Generally, concentration of EV quantified by FCM is 10^3–10^4-fold lower than those by RPS and NTA. *Summary/conclusion*: The effects of pre-analytical conditions markedly depend on the cellular origin of EV. Because human plasma contains both types of EV, analysis and interpretation of results should take collection and handling conditions into account.

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O8B-330

Improved characterization of EV preparations based on protein/lipid ratio and lipid properties

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Introduction: In the rapidly growing field of extracellular vesicles, there is much debate about classification of these structures. Furthermore, standardization and quantification solely based on particle enumeration or determination of protein content is inconclusive. The goals of the present study were to identify novel parameters that enable improved characterization of EV preparation subtype and quality. *Methods*: EVs secreted by cells lines (bv-2, Jurkat, THP-1, U937) as well as those found in human whole blood plasma and released by platelets were used in this study. The protein content of EVs was determined by the microBCA assay. For determination of the lipid content we used a simple and inexpensive lipid microassay. EV subpopulations were isolated by differential centrifugation and gravity-driven filtration as described earlier (*J. Extracell Vesicles* 2013;2:20677). Vesicle number and quality were assessed by using qNano and tEM, respectively. Quantitative fluorescence microscopy and flow cytometry were used to assess the membrane lipid properties of the different preparations of vesicles. Furthermore, we tested the sensitivity of EV preparations to detergent lysis. *Results*: We found characteristic protein/lipid ratios for different subpopulations of EVs. Importantly, damaged or poor quality EV preparations (as confirmed by tEM) had significantly altered protein/lipid ratios. Furthermore, different lipid membrane orders (based on spectroratiometric fluorescence) allowed for the distinction of the different EV populations in fluorescent microscopy and flow cytometry. *Summary/conclusion*: Our data suggest that vesicle composition

and quality of different EV preparations can be better characterized by determining protein/lipid ratio and lipid properties.

O8B-331

Standardization of vesicle detection by flow cytometry using traceable beads and optical scattering theory

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Introduction: Flow cytometry is currently the most used method for single vesicle detection in the clinic. At present, most laboratories select vesicles by setting an inclusion gate based on the scatter signal from 2 polystyrene bead sizes, resulting in a reproducibility of 15–91% in a previous multicentre study. Comparison of flow cytometry results of vesicle measurements between laboratories, however, is not straightforward because (a) the refractive index of beads and vesicles are not matched and (b) the relation between the size ranges of beads and vesicles is affected by the optical configuration of the flow cytometer. *Methods*: We analytically describe the relation between the diameter, refractive index and light scattering of traceable polystyrene beads (www.metves.eu) with Mie theory to find the relationship between scatter and size of vesicles. Assuming a vesicle refractive index of 1.40, this procedure is expected to minimize the impact of the refractive index mismatch and eliminate differences between optical configurations. Therefore, we initiated a study including 20 centres worldwide to determine whether this procedure improves the reproducibility of vesicle concentration measurements between laboratories. The International Society on Thrombosis and Haemostasis is sponsoring this activity, which will take place between February 2014 and June 2014. Each centre measures PMP and erythrocyte vesicle samples with lactadherin and CD61-PE or CD235a-PE staining, respectively. *Results*: Based on theoretical simulations we expect this procedure to reduce the coefficient of variation on the measured concentrations by 20% compared to the current standard. We will present preliminary data from the study. *Summary/conclusion*: This method will allow a closer approximation of the size of vesicles and improve the reproducibility of concentration measurements with different flow cytometers. There may be a difference between assumed and true refractive index, which would result in a difference between the desired and actual vesicle size range. However, this refractive index difference is small compared to the error made by assuming the refractive index of polystyrene to be equal to vesicles and will not affect the concentration reproducibility.

O8B-332

Fluorescently labelled liposomes for defining instrument detection sensitivity for measuring microparticles by flow cytometry with and without imaging

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Introduction: Research groups propose use of polystyrene beads (PSB) to standardize flow cytometers for microparticle (MP) detection and to size MPs. Biological vesicles such as MPs and liposomes have a lower refractive index and scatter light approximately 10-fold less than PSB. Therefore, use of PSB measures particles of a much larger size than most MPs. We propose the use of lipid-based vesicles such as liposomes to define the level of detection sensitivity of flow cytometers (FCMs) with and without imaging. *Methods*: Liposomes were prepared by a modification of the Szoka/Papahadjopoulos filtration technique. Avanti Polar lipids were dissolved with cholesterol and lipophilic dye (DiI and DiO) in chloroform. Sizing of liposomes was performed by the repeated filtration through different sizes of polycarbonate filter. Different sizes of liposomes (0.200 μm labelled with DiI; 0.2, 0.4 and 0.6 μm labelled with DiO) were confirmed by dynamic light scattering (DLS) and detected by imaging flow cytometry (Imagestream MKII, ISX) and conventional FCM. Geometric means (GM) of the fluorescence intensity of the DiO were calculated for the 3 different sizes of liposomes in diluted and undiluted samples (1:1, 1:100, 1:1000). *Results*: ISX can clearly resolve all liposomes above background (buffer only), whereas only a small portion is detected by FCM. In comparison to polystyrene beads, liposomes have significantly lower side scatter intensity. Calculation of the fluorescence geometric means of 3 different sizes of liposomes (approx. 0.2 μm, 0.4 μm and 0.6 μm) showed a linear increase in fluorescence means. Thus, a calibration curve for sizing of MP could be established using the fluorescence intensity. Dilution of liposomes demonstrated swarm detection by FCM as indicated by a decrease in fluorescence GM with FCM but not with ISX. *Summary/conclusion*: Lipid-based vesicles such as fluorescently labelled liposomes can be clearly detected and visualized by imaging flow cytometry, whereas FCM was only able to detect swarms of liposomes. As their scatter profile is close to MPs they offer a more accurate way to define the lower level of detection sensitivity of the instruments. The use of multiple sizes of fluorescently labelled liposomes provides a potential method for the sizing of MPs.

O8B-333

Separation of dendritic cell-derived extracellular vesicles by means of density gradients

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Introduction: Extracellular vesicle (EV) secretion by antigen-presenting cells has been described for decades. Our studies were initially focused on endosomal-derived EVs called exosomes, but it becomes now clear that other types of EVs can be secreted simultaneously. A widely used protocol of exosome purification involves removing large EVs by 10,000 g centrifugation, followed by pelleting small EVs at 100,000 g. The resulting pellet qualifies as exosomes if enriched in endosomal markers. However, recent studies started to question the specificity of some established protein markers. In this study, we reassessed the distribution of proteins classically used as exosome markers in different subpopulations of EVs and performed quantitative proteomic analyses to identify new specific markers. *Methods*: EVs were purified by differential ultracentrifugation and sucrose or iodixanol gradient floatation from conditioned medium of human monocyte-derived dendritic cells (DC). EVs were analysed by western blotting, electron microscopy and NTA. Moreover, LC-MS/MS analyses were performed and proteomic composition was quantitatively analysed in 4 different fractions recovered after iodixanol separation. *Results*: In our culture conditions, DC secrete EVs pelleting at either 10,000 g or 100,000 g that bear exosome markers. In both pellets, iodixanol gradient separates vesicles in 2 major subpopulations. Proteomic analyses showed a large overlap of protein components in these 4 fractions, but a ''label-free'' quantitative analysis unravelled differential enrichment of some families of proteins (e.g. mitochondrial vs endosomal) in the different EV subpopulations. Contrary to iodixanol, floatation into sucrose showed a different behaviour of the 10,000 g and 100,000 g pellets, with respective densities of the major fractions of 1.17–1.19 g/ml vs. 1.14 g/ml. *Summary/conclusion*: Our data highlight the heterogeneity of exosome preparations obtained by differential ultracentrifugation and the need to examine separately each sucrose gradient fraction within the previously defined 1.13–1.19 g/ml exosome densities.

Van Weelde/Mees room
Symposium Session 8C - EV in body fluids
Chair: *Damien Gheldof and Juan-Manuel Falcon-Perez* 14:00-15:30

O8C-334

Detection of platelet-derived extracellular vesicles using flow cytometry and tunable resistance pulse sensing

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Introduction: Flow cytometry has traditionally been used to detect extracellular vesicles; however, this work has typically been limited by the detection capabilities of the machine. Tunable Resistance Pulse Sensing (TRPS) is capable of estimating EV size and count, based on change in electrical resistance following the passage of EVs through a nanopore. In this study, we aimed to compare the detection capabilities of both systems. *Methods*: Platelet-poor plasma was obtained from 10 normal donors and frozen at -80°C until use. Extracellular vesicle (EV) counting was performed using an LSR-II flow cytometer equipped with a forward scatter PMT detector for CD41+/Lactadherin+ EVs. TRPS for EVs was performed using the Izon qNano. EV procoagulant activity was assessed using the Stago Procoag-PL assay. *Results*: Intra-assay CVs of the TRPS for the detection of mean EV size and count were 1.01 and 6.20%, respectively. Using the smallest nanopore (NP100) for TRPS, the smallest EVs that could be detected were 67.4 nm. Using this nanopore, the mean EV size was 104.7 nm; however, our flow cytometer could only detect beads of >110 nm. EV counts were significantly higher using TRPS when compared to flow cytometric EV counts ($4.1 \times 10\text{^}7 \pm 1.8 \times 10\text{^}7$ vs. $3.0 \times 10\text{^}3 \pm 0.5 \times 10\text{^}3$, $p = 0.0039$). There was no significant correlation between EV counts measured using TRPS and flow cytometry ($p = 0.95$) or with the Procoag-PL assay ($p = 0.31$), but there was a significant correlation between flow cytometry and the Procoag-PL assay ($p = 0.013$). *Summary/conclusion*: TRPS is capable of detecting greater numbers of EVs than is possible with flow cytometry, and these represent smaller EVs. The lack of correlation between these smaller EVs with the Procoag-PL assay suggests these EVs are not procoagulant. Flow cytometric EV counts are capable of providing information about EV procoagulant activity.

O8C-335

Profiling the microRNA content of platelets, platelet-derived exosomes and plasma

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Introduction: microRNAs are regulators of protein expression, affecting ~90% of mRNA translation. Anucleate platelets contain an abundant array of microRNAs which may modulate protein expression during thrombopoesis or within the platelets. Alternatively platelet microRNAs are packaged into extracellular vesicles (EVs) for release into the circulation where they may modulate protein expression in target cells. On activation platelets release both procoagulant microparticles (MPs) and exosomes. To confirm that microRNA is predominantly released in the form of exosomes, we compared the effect of different platelet agonists and then compared the microRNA profile of platelets with that of the released exosomes, and with the microRNA profile in plasma from healthy subjects. *Methods*: Washed platelets from healthy volunteers were maximally stimulated with agonists specific for GPVI (collagen-mimetic peptide CRP-XL), PAR-1 (SFFLRN), PAR-4 (AYGPKF) or thrombin (PAR1 and PAR4). Exosomes were identified by western blotting (using the CD63 MAb RFAC4) and procoagulant MPs were detected by flow cytometry (annexin V binding) and support of thrombin generation (CAT assay; Stago Laboratories). Exosomes were then isolated from thrombin-stimulated platelets from 4 healthy subjects using ExoQuick (Cambridge Bioscience). RNA was reverse-transcribed and amplified, and the microRNA profile was characterized by RT-PCR (TaqMan microRNA microarray cards). *Results*: Different agonists generated different EV populations. Only the GPVI agonist CRP-XL produced procoagulant MPs while all agonists generated $CD63^{+ve}$ exosomes. The microRNA profiles of paired samples from platelets and thrombin-generated exosomes were highly correlated ($r = 0.8666$; $p \leq 0.0001$). However, while 271 microRNAs were expressed in all platelet and exosome samples 82 were observed only in platelets. The correlation between the platelet and exosomes microRNA profiles with that in normal plasma was also significant ($r = 0.8186$ and $r = 0.7426$ respectively; $p \leq 0.0001$). The most highly expressed miRNA in all 3 samples was hsa-miR-223. *Summary/conclusion*: These results demonstrate that different agonists release different EV populations from platelets, with PAR agonists generating predominantly exosomes. microRNA profiling showed that the majority of platelet microRNA is released into their exosomes, and this microRNA represents a significant proportion of the microRNA seen in the normal circulation. microRNAs retained in platelets may have specific regulatory roles within platelets and those released may be involved in cell–cell communication.

O8C-336

Detection of bovine bioactive milk-derived microvesicles in human serum

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Introduction: Bovine and human breast milk contain microvesicles (MVs) carrying immune-related miRNAs; however, it is unknown whether these MVs are present in commercial milk we consume. In this study, we confirm the presence of MVs in commercial milk and show in vitro and in vivo uptake of vesicles. *Methods*: By differential ultracentrifugation, followed by ExoQuick isolation, we isolated MVs from milk. NanoSight analysis was performed to estimate vesicles size (Ø120 nm) and concentration ($\sim 1*10^{10}$ MVs/ml). The expression of milk-derived mRNA and miRNA was confirmed by RT-qPCR. TGFbeta levels were measured with a CAGA-fLuc reporter construct. MVs were acidified to test stability. Cellular uptake of PKH-67 labelled MVs was analysed by either confocal microscopy or flow cytometry. Human serum samples were purchased from the blood bank (2010-REU-Loo). Serum MVs were isolated by ultracentrifugation. The expression of bovine milk-derived mRNA in serum MVs was analysed by RT-qPCR. *Results*: We found clear levels of mRNA, miRNAs (e.g. miR-let-7a, 124a) and active TGFbeta in milk MVs. To test the stability of these vesicles, we used a luciferase reporter assay to measure in vitro NFkB-activation. Acidification, up to gastric acid level, did not alter the inhibitory

effect that MVs had on NFkB-activation. In vitro, we showed cellular uptake of MVs in murine macrophages, splenic antigen presenting cells, non-phagocytic fibroblasts and even murine ileum explants. Interestingly, we also confirmed the presence of mRNA specific for bovine milk protein in MVs obtained from serum in 4 out of 15 blood donors. *Summary/conclusion*: We clearly showed that commercial milk contains stable MVs, which are resistant to acidification. To our knowledge, this is the first study to show that bovine milk-derived MVs are present in circulation. Our data suggest that bovine milk-derived MVs might pass the stomach and intestinal wall, and this warrants further research to determine their biological effect in humans.

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O8C-337

Human breast milk contains various subpopulations of extracellular vesicles carrying immune modulatory proteins

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Introduction: Extracellular vesicles (EV) with immune modulatory properties in human breast milk may be involved in instruction of the neonatal immune system. Previously, we developed a density gradient-based protocol for efficient and reliable recovery of EV from breast milk. Here, we established a milk storage protocol allowing isolation of naturally occurring EV, while avoiding the generation of contaminating EV induced by storage. In addition, we identified and characterized various subpopulations of breast milk EV. *Methods*: EV were isolated from fresh human milk or stored cell-free milk supernatant. EV subsets and non-floating complexes were characterized by high-resolution flow cytometry, cryo EM, proteomics and western blotting. *Results*: Cold storage at $\leq 4^{\circ}$C of unprocessed milk led to milk cell death. By spiking human milk with murine cells, we found that cell death during cold storage led to contamination of the milk EV population with vesicles generated during storage. Storage of fresh milk samples after immediate removal of cells and fat prevented this contamination, whereas the EV recovery was comparable to fresh milk. Analysis by high-resolution flow cytometry revealed that quantities of EV and their distribution over the density gradient were comparable in milk of different donors. The predominant milk EV subpopulations resided in the 1.12–1.18 and 1.18–1.21 g/ml density fractions. Analysis by high-resolution flow cytometry and cryo EM showed that both EV populations were heterogeneous in forward light scattering, size, and annexin V labelling. Additionally, long tubular structures were identified in the 1.12–1.18 g/ml fraction. By comparative proteomic analysis of EV and non-floating complexes, we found several immune modulatory molecules, such as CD59, to be exclusively present in EV, while MFG-E8, butyrophilin 1A1 and MUC-1 were enriched in EV compared to non-floating structures. *Summary/conclusion*: We developed an efficient storage method for breast milk samples for reliable recovery of naturally occurring EV. Several subpopulations of EV were discriminated in breast milk and we identified immune modulatory proteins (selectively) present in these EV.

O8C-338

Circulating vesicular microRNA-31 levels as marker for impaired osteogenesis

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Introduction: A major driving force of ageing is the struggle between the accumulation of damage in cells and tissues and the counteracting repair systems of organisms. Mesenchymal stem cells (MSCs) counteract this decline but their regenerative power decreases with age. In particular osteogenic differentiation potential of MSCs has been shown to decrease with age thereby contributing to slowed down bone formation and osteoporosis. While much is known about cellular ageing of MSCs, little is known about factors of the aged systemic environment influencing their functionality and serving as diagnostic marker for impaired osteogenesis. *Methods*: Isolation of extracellular vesicles by differential centrifugation followed my immunopurification. Determination of vesicular miRNA-31 levels by quantitative real-time PCR. *Results*: While searching for extrinsic factors that influence osteogenesis of MSCs extracellular vesicles (EVs) were found. Exposition of MSCs to CD63-positive EVs secreted by senescent endothelial cells (senECs), which were shown to accumulate with age in vivo, or isolated from plasma of human elderly donors failed to induce osteogenesis compared to MSCs incubated with secreted EVs of young endothelial cells or plasma-derived EVs of young donors. We attributed the age-dependent impairment of osteogenesis by CD63-positive EVs to vesicular miR-31 which was shown to be enriched within EVs of senECs and within plasma-derived EVs of elderly donors but also in EVs of patients suffering from osteoporosis. Overexpression of miR-31 in MSCs reduced osteogenic differentiation capacity while inhibiting miR-31 enhanced osteogenesis in vitro. MiR-31s underlying molecular inhibitory effect was illuminated by demonstrating that miRNA-31 targets FZD3, a factor necessary for osteogenic differentiation. Finally, we were able to rescue MSCs from the inhibitory effect of EVs isolated from senECs or from plasma of elderly donors by transfecting them with a miR-31 inhibitor. *Summary/conclusion*: Summarizing our data suggest that changes in vesicular miR-31 plasma levels correlate with the age of the donors as well as with the presence of osteoporosis and that vesicular miR-31 it is able to inhibit osteogenesis of MSCs. Thus, it might serve as a diagnostic and therapeutic target whenever osteogenesis is a limiting factor.

O8C-339

Characterization of CD133+ vesicles from normal human urine

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Introduction: Extracellular vesicles (EVs) present in urine mainly derive from cells of the nephron, thus representing an interesting tool to mirror the kidney physiological state. We identified a population of $CD133^{+}$ progenitors, shown to be involved in renal repair after injury. The aim of the present study was to identify and isolate $CD133^{+}$ EVs in normal urine (uEVs) and characterize their proteic and microRNA (miRNA) content. *Methods*: uEVs were isolated from the urine of healthy subjects with a protocol consisting of a sequence of centrifugation, filtration and ultracentrifugation. uEVs were magnetically sorted using the MACS Miltenyi system. Proteins

were extracted with Ripa Lysis Buffer and analysed by western blot analysis; microRNAs were extracted with mirVana kit (Ambion) and analysed by quantitative RT-PCR; surface markers were analysed through cytofluorimetric analysis after adsorption on 4 μm latex beads. *Results*: The cytofluorimetric analysis of total uEVs showed the presence of CD24, CD81, CD9 and CD133 in normal subject uEVs. The sorting protocol was successfully set up as confirmed with western blot and cytofluorimetric assays. Compared to the $CD133^-$uEVs, the $CD133^+$uEVs were also positive to Megalin, Aquaporin1 and CD2AP suggesting their origin from glomerular and tubular structures, with a low expression of exosomal markers. qRT-PCR showed the presence of several microRNAs involved in stem cell survival (mir210, mir34a), proliferation (mir191, mir222), self-renewal and differentiation (mir1225-5p, mir21). The positive fraction was enriched in mir34a (related to cell cycle arrest and apoptosis) and mir1225-5p (related to the regulation of CD133 expression). *Summary/conclusion*: In the present study, we identified and characterized a population of $CD133^+$uEVs. Their presence in normal urine may reflect a homeostatic activity of CD133+ progenitors. These findings can be exploited for further analysis of CD133+ EVs in renal pathology and diagnostics.

Networking coffee — Arcadis room — 15:30-16:00

Poster Viewing Sessions 7A, 7B, 8A, 8B, 8C, 9A, 10C posters not-attended by authors — Arcadis room — 15:30-16:00

Willem Burger room
Symposium Session 9A - Isolation of EV
Chair: *Chris Gardiner and Kenneth Witwer* 16:00-18:00

Introduction (15 min) by Chris Gardiner

O9A-340

Single-step isolation of extracellular vesicles from plasma by size-exclusion chromatography

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Introduction: Isolation of extracellular vesicles from plasma is difficult due to the presence of proteins and lipoproteins. In most studies, vesicles are isolated using differential centrifugation or density gradient ultracentrifugation. Differential centrifugation results in co-isolation of contaminants, such as protein aggregates, and density gradient ultracentrifugation cannot separate vesicles from high-density lipoproteins (HDL). In this study, we developed a fast, easy and single-step protocol to isolate vesicles from human plasma by using size-exclusion chromatography (SEC). *Methods*: Platelet-depleted plasma was loaded on a Sepharose CL-2B column (n = 3). Fractions of 0.5 ml were collected and analysed by nanoparticle tracking analysis (NTA), resistive pulse sensing (RPS), flow cytometry (FCM), and transmission electron microscopy (TEM). The concentrations of HDL and protein were measured in each fraction. All results are presented as % of total recovered in all fractions. *Results*: Fractions 9–12 contain 46% ± 6 of particles larger than 70 nm (NTA), 61% ± 2 of the CD61+ vesicles present in all fractions (FCM), <5% of HDL and <1% of protein. Highest quantities of HDL and protein are present in fractions 18–20 (32% ± 2) and 19–21 (36% ± 2), respectively. SEC results in a recovery of CD61+ vesicles in fractions 9–12 of 43% ± 23 of the starting material, with an 8- and 70-fold enrichment of HDL and protein, respectively. Enrichment improves to 20- and 330-fold, respectively, when only fraction 9 is collected. *Summary/conclusion*: Single-step SEC efficiently isolates extracellular vesicles with a diameter larger than 70 nm from human plasma. Application of SEC will improve the reliability of studies on size distribution, proteomics, functional properties and TEM imaging of extracellular vesicles.

O9A-341

Removal of lipoproteins from human plasma by anti-Apolipoprotein B antibody-coated magnetic beads

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Introduction: Optical single particle tracking (OSPT) is a method which allows for quick size and concentration measurements of submicron particles. However, being a non-phenotypic detection method it holds the inherent limitation of not enabling clear distinction between extracellular membrane vesicles (EV) and other types of particles in the same size range. It has been stated that over 98% of the particles detected in human platelet-free plasma (PFP) appear to be lipoproteins (Gardiner et al. JEV, 2013). This challenge may be met by ultracentrifugation of PFP for isolation of EV by pelleting and subsequent re-suspension, but this approach may damage the EV and in any case entails removing them from their physiological medium which is plasma (Witwer et al. JEV, 2013). The aim of this study was to evaluate a method potentially removing lipoproteins from human PFP samples by use of Protein G magnetic beads coated with antibodies against Apolipoprotein B-48/100 (ApoB), which is present in chylomicrons, VLDL, IDL and LDL. *Methods*: Following incubation of 11 PFP samples with ApoB antibody-coated beads, the beads were separated from the PFP samples by application of a magnetic field. The samples were analysed with OSPT both before and after the addition and removal of magnetic beads to determine the percentage of submicron particles removed by this procedure. In addition, 1 sample was analysed with an ELISA assay specific for human ApoB and an EV Array designed for capturing exosomes in order to estimate the degree of removal of ApoB-carrying lipoproteins and EV, respectively. *Results*: Applying the magnetic bead procedure on the PFP samples resulted in a mean reduction by 58% of the total particle concentration measured by OSPT but with a wide variation. The mean size of the remaining particles generally increased. Preliminary measurements with ELISA and EV Microarray showed a reduction of ApoB to a level not distinguishable from background signal in the ELISA assay but also a 16% reduction of the EV Array signal. *Summary/conclusion*: Anti-ApoB antibody-coated magnetic beads may hold potential for removal of lipoproteins from human PFP prior to non-phenotypic EV measurement but may also induce other changes of the EV population.

O9A-342

Placental perfusion as a model of syncytiotrophoblast vesicle release

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Introduction: Different vesicle types exhibit diverse functions in health and disease. To study placental vesicles, ex vivo dual placental lobe perfusion is used to prepare syncytiotrophoblast (STB)-derived vesicles (STBV). However, these preparations contain vesicles of varying types (i.e. microvesicles (MV) and exosomes) and origin (i.e. STB, red blood cells (RBC) and platelets). Therefore, improved purification and fractionation of STB-derived vesicles is required to facilitate research in this field. *Methods*: A protocol using sequential centrifugation and filtration was developed to produce highly pure STBV preparations from normal term placental perfusate (n = 5) that were then enriched for either exosomes or MV. Five colour flow cytometry, nanoparticle tracking analysis (NTA), western blotting and transmission electron microscopy (TEM) were used to assess purity and size distribution of vesicles throughout the procedure. The Oxfordshire Research Ethics Committee approved this study and informed written consent was obtained from recruits. *Results*: Freshly collected placental perfusates contained high levels of contaminating RBC/vesicles (CD235a/b +ve; 62%) and platelets/vesicles (CD41 +ve; 14%). Centrifugation at 1,500 × g (2 × 10 min) removed platelets/vesicles (44%) and RBC/vesicles (92%) giving a resultant supernatant (SN) containing 77% STB vesicles (placental alkaline phosphatase (PLAP) +ve). Subsequent centrifugation at 10,000 × g

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(30 min) enriched STB MV (PLAP +ve; >85%) in the pellet (modal size 392 ± 17 nm), with low levels of RBC (3.0%), platelets (5.7%) and leucocyte (2.3%) derived vesicle contamination. By TEM, the MV-enriched fraction contained a heterogeneous population of vesicles in both size and appearance. The MV-depleted SN was then passed through a 0.22 mm filter before centrifugation at 150,000 × g (2 h). The resultant pellet gave an exosome fraction (modal size 145 ± 8 nm), highly enriched for Alix and CD63 and homogeneous in size and appearance by TEM. *Summary/conclusion*: A robust protocol was developed for isolation of highly pure STBV preparations and fractionation to give enriched preparations of STB exosomes and MV from placental lobe perfusate. This technique will improve characterization and investigation of the role of STB-derived vesicles in normal and pathological pregnancies.

O9A-343

The impact of disparate isolation methods for extracellular vesicles on downstream RNA profiling

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Introduction: Despite an enormous interest in the role of exosomes in cancer progression, there is currently no clear consensus on reliable protocols for their isolation. We performed a comparative study to identify an optimal method to isolate pure exosomes for proteomics and transcriptomics. *Methods*: Conditioned medium was prepared from breast cancer cells that were grown for 24 h in DMEM supplemented with 0.5% exosome-depleted foetal bovine serum. After general filtration steps and concentration, exosomes were isolated using ultracentrifugation (UC), OptiPrep™ Density Centrifugation (ODG) and commercial precipitation-based methods ExoQuick™ (EQ) and Total Exosome Isolation™ (TEI). Yield and purity of isolated exosomes were assessed by complementary methods, including nanoparticle tracking analysis (NTA), immunoelectron microscopy (IEM), western blot analysis, flow cytometry, and proteome and microarray-based RNA expression profiling. *Results*: The highest protein concentration was obtained by EQ and TEI; however, typical exosome markers were more abundantly detected in UC and ODG preparations by IEM (CD63) and western blot (CD63, Alix, Tsg101, HSP70 and 90α). NTA revealed that EQ is able to extract the highest numbers of particles; however, given the western blot data, these are presumably not all exosomes. Coomassie brilliant blue staining and mass spectrometry analysis of selected protein bands identified significant serum contamination in all preparations except for ODG. RNA concentration was significantly lower in ODG preparations. Messenger RNA and long non-coding RNA expression profiling revealed the highest technical and biological reproducibility for ODG preparations. Heat map analysis differentially clustered RNA expression profiles from UC and EQ preparations and ODG samples. *Summary/conclusion*: All methods are able to isolate exosomes but with different yield and purity. The purest and most reproducible preparations of exosomes, containing a unique RNA expression signature, are obtained by ODG.

O9A-344

Ultrafiltration and size exclusion liquid chromatography enable high yield isolation of pure exosomes that retain their biophysical properties

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Introduction: Extracellular vesicles (EVs) have gained increasing attention as mediators of intercellular communication, as a source of novel biomarkers and as therapeutic agents. However, a major obstacle has been the lack of methods permitting the isolation of high yields of pure exosomes that retain their biophysical and biological properties. Here we describe a purification method based on ultrafiltration (UF) with subsequent liquid chromatography (LC) and compare it with the current standard ultracentrifugation (UC) protocol. *Methods*: Conditioned media is spun at 300 g, 1,500 g and filtered through a 0.2 μm filter. Samples are divided and either purified using classical UC, including PBS wash or by ultrafiltration with subsequent LC (Sephacryl S-300 or 500-HR columns). Samples are analysed by western blot, NTA, fluorescence correlation spectroscopy (FCS), electron microscopy (EM) and LC-MS/MS-based proteomics. The biodistribution of near-infrared-ladled exosomes are assessed in mice. *Results*: UF-LC yields approximately 5- to 10-fold more EVs than UC with contaminating proteins efficiently eliminated by size-exclusion LC. Proteomics analysis show very similar protein profiles with nearly 2,000 proteins identified; however, EM and FCS analysis indicate that EVs purified by UF-LC retain their biophysical properties whereas UC purification compromise vesicle shape and integrity, and leads to vesicle aggregation. The retention of the inherent biophysical and biological properties of EVs by UF-LC results in an in vivo biodistribution profile that is devoid of particle accumulation in the lungs. Additionally, we show that UF-LC is highly applicable to EV isolation from complex cell culture media and human biological fluids. *Summary/conclusion*: UF-LC is a robust and scalable methodology that results in high yields of biophysically and biologically intact exosomes that will prove highly suitable for biological studies and future clinical applications.

O9A-345

Biologic impact of different extracellular vesicle populations on cells *in-vitro*

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Introduction: Previous studies showed that Pulmonary derived vesicles entered murine marrow cells and induced expression of surfactant B and C. Initially, this was due to transfer of mRNA and a transcriptional active agent from lung cells but at longer time intervals the altered marrow phenotype was due to transcriptional modulation. In these studies exosomes were studied isolated by differential centrifugation; 300 g, 10,000 g and then 100,000 g. In this isolation, larger vesicles are discarded in the 10,000 g separation. *Methods*: Lung tissue from C57BL/6J male mice and Fischer rats were cultured for 1 week in Bronchial Epithelial Growth Media and conditioned media harvested. We then evaluated vesicles from each step of the differential centrifugation. These fractions were cultured with murine marrow for 1 week, then established in cytokine-supported liquid cultures, and RNA extracted and characterized as to origin (rat or mouse) at 2-week intervals of culture out to 8 weeks. *Results*: Mouse marrow cultured with rat vesicles isolated as exosomes (10,000 g, 100,000 g) or vesicles from the 10,000 g spin or vesicles from both the 10,000 and 100,000 g spins were analysed for surfactant B and C mRNA expression. Expression was maintained out to 8 weeks and was moderately increased with the exosome fraction (10,000 and 100,000 g) as compared to the other 2 fractions. This expression was initially both of mouse and rat mRNA, but in later time points only mouse mRNA was expressed, thus confirming our previous data showing that the lung vesicles induced a stable long-term epigenetic change in marrow cells. However, in experiments where mouse vesicles were incubated with mouse marrow

the population of vesicle with both exosomes and larger microvesicles (100,000 g) was most potent in inducing surfactants B and C. *Summary/conclusion*: Different centrifugation fractions of vesicles induced different biologic effects on marrow cells in these experiments. Our previous data showing that lung vesicle interaction with murine marrow cells induced a long-term epigenetic effect was confirmed. Vesicle studies need to be carried out with a realization that different centrifugations may give varying results depending on different experimental conditions such as mouse lung-derived vesicles versus mouse marrow and rat-derived vesicles versus mouse marrow.

O9A-346

Cancer-derived extracellular vesicle isolation by aqueous two-phase system

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Introduction: Extracellular vesicles are released by various cell types, particularly tumour cells, and may be potential targets for blood-based cancer diagnosis. However, studies performed on blood-borne vesicles to date have been limited by time-consuming process in isolating extracellular vesicles, and lack of effective purification strategies. To overcome the limitations, we develop a simple strategy employing aqueous two-phase system (ATPS) to isolate vesicles from whole blood sample. This method can save more than 5 h by comparison to classical extracellular vesicle isolation method using ultra-centrifugation and gets well-purified extracellular vesicles by separating protein and extracellular vesicles. *Methods*: Six-week-old C57BL6/j mice were grown with melanoma after subcutaneous injection of a million B16BL6 cells into the basal body. Blood was collected by cardiac puncture 3 weeks after initial injection. Extracellular vesicle isolation is processed from whole blood sample by 2 methods: a classical method and a new method. A classical method is using ultra-centrifuge and a new method is using aqueous two-phase system which is made by PEG (35 kDa) and DEXTRAN (500 kDa). In the new method, dissolve PEG and Dextran in blood, and make PEG (4.5%, weight/volume) and Dextran (1.6%, weight/volume). Next, make the sample into PH 12 using NaOH. The sample is centrifuged for 5 minutes to separate phases. Extract bottom phase and top phase from sample, and carry out Bradford assay, western blot, RNA Isolation and PCR with the sample from the classical method. *Results*: In the new method, extracellular vesicle is effectively isolated and trapped into dextran phase. Through Bradford assay and RNA measurement, we identify that extracellular vesicles enrich in bottom phase and are highly purified because protein enrich into top phase. According to the western blot and PCR with the sample from the classical method and the new method, strength of PCR and western band in samples from two methods is very similar. *Summary/conclusion*: Using the new method, we detect cancer faster, compared to the classical method. And also, aqueous two-phase method can be applicable to purification of extracellular vesicle from protein mixture.

Jurriaanse room
Symposium Session 9B - Release and function of EV RNA
Chair: *Andy Hill and Esther Nolte-'t Hoen* 16:00-18:00

O9B-347

Exosomes provide a protective and enriched source of miRNA for biomarker profiling by next-generation deep sequencing

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Introduction: microRNAs (miRNA) are small non-coding RNA that have been shown to circulate in biological fluids and are enclosed in exosomes. Next-generation deep sequencing has provided the ability to profile miRNA in biological fluids. This technique presents a viable screening tool to detect miRNA biomarkers. However, collection and handling of biological fluids needs to be greatly improved for miRNA analysis in order to reliably detect differences between healthy and disease patients. The low abundance of RNA in biological fluids creates difficulties in its isolation, of which exosomal miRNA is a small fraction, making downstream analysis challenging. *Methods*: Several methods were investigated to maximize exosomal and RNA yield from a number of biological fluids: urine, whole blood, plasma, serum, synovial fluid and breast milk. We then profiled baseline miRNA expression using deep sequencing to compare miRNA profiles obtained from non-exosomal samples versus exosomes prepared by ultracentrifugation methods and commercial exosome isolation kits. *Results*: Overall, miRNA were found to be significantly enriched and intact in exosomes isolated from biological fluids compared to non-exosomal preparations. In particular, while RNA was found to be degraded in cell-free urine, we found the RNA to be intact within exosomes. Exosomes isolated from plasma and serum were found to contain an enriched percentage and profile of miRNA species compared to non-exosomal samples. In addition, the presence of other non-coding RNA and coding RNA suggest exosomes carry specific genetic information that represents the origin of the fluid. *Summary/conclusion*: We have established a robust platform for the isolation and deep sequencing of exosomal RNA from a number of biological fluids. This workflow provides a reliable method of identifying miRNA biomarkers associated with a number of human diseases. The enrichment of miRNA in exosomes may increase sensitivity and specificity of miRNA disease biomarker assays.

O9B-348

Post-transcriptional processing and modifications define small RNA sorting into exosomes

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Introduction: Demonstrating selective sorting of small RNA molecules into exosomes is highly relevant for understanding how their function and mobility within and between cells is regulated. Currently, selective incorporation of small RNA molecules into exosomes and the mechanisms involved are poorly understood. Here we report the existence of deterministic sorting of small RNAs through the endo-exosomal pathway adding to the emerging concept that exosomes functioning as RNA-based communication devices. *Methods*: We performed a comprehensive analysis of small RNA sequencing data from 6 paired cells/exosomes samples of B-cell origin and from human biofluid microvesicles in combination with publically available small RNA sequence data. For data analysis, custom-designed computational algorithms and statistical approaches supported by RT-PCR-based experiments were used to show that small RNA molecules can be both selectively incorporated into exosomes and strongly retained in the cells. *Results*: While microRNAs (miRNAs) are, in general, less frequent in exosomes compared to the cellular content, a subset of miRNAs exists that is strongly and consistently released. Specifically, diversification of mature miRNA sequences correlate significantly with the tendency to be retained or released and depends, in part, on the number of post-transcriptionally added nucleotides. The latter was also observed for other exosome-incorporated small RNA molecules. *Summary/conclusion*: The combined results indicate that sorting of miRNAs into exosomes is associated with their biogenesis, functional activity and stability, which significantly advances our understanding of exosomal small RNA cargo selection and provides a rationale for studying non-coding RNA processing, modification and miRNA turnover in combination with exosome biology.

O9B-349

Macrophage regulation of tumour angiogenesis: role of exosome-derived microRNAs

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Introduction: Macrophages are known to infiltrate tumours in large numbers, and it is now well established that they promote angiogenesis (the formation of new blood vessels). Macrophages modulate endothelial cell (EC) behaviour by secreting a variety of soluble mediators. Recent studies have also suggested that exosomes are able to mediate cell-to-cell communication via microRNA (miR) transfer, suggesting a new mechanism whereby macrophages could communicate to ECs to promote tumour angiogenesis. However, the mechanisms by which miRs are loaded into exosomes and the functional consequences of miR transfer to ECs are little understood. Here, we investigated the significance of macrophage-derived exosomes (''exo-macs'') and their miR cargo (''exo-miRs'') for macrophage-EC communication. *Methods*: We performed qPCR-based miR arrays of macrophages and their exo-macs, as well as deep sequencing of the cells' transcriptomes. We also employed lentiviral vectors to either overexpress or squelch individual miRs in the macrophages. To assess transfer and activity of miRs in recipient cells, we used a panel of reporter lentiviral vectors, along with flow cytometry, immunofluorescence and qPCR. *Results*: By these strategies, we discovered that miR sorting to exo-macs is in part controlled by the abundance of miR targets, which is modulated by cell activation. By biologically or artificially increasing target gene-transcripts, selected miRs are retained in the cell's cytoplasm (site of miR activity) and are excluded from multivesicular-bodies (sites of exosome biogenesis), thus limiting exo-miR production. We then

asked whether functional exo-miRs are transferred from exo-macs to ECs. To this aim, we isolated exo-macs from both *Dicer*-knockout and wild-type macrophages, and analysed miR transfer to *Dicer*-knockout ECs. We found that selected exo-miRs are transferred from macrophages to ECs to modulate their gene expression, both in vitro and in vivo. *Summary/conclusion*: These findings suggest a model for exo-miR biogenesis whereby cell activation-dependent fluctuations in target-gene abundance finely regulate miR output to exosomes. Furthermore, exo-miRs can then be transferred from macrophages to ECs and, therefore, have the potential to regulate vascular biology.

O9B-350

Exosome-associated microRNA signatures are differentially enriched in the extracellular milieu of human CD4+ T-cell subsets

Paola De Candia[1], Anna Torri[1], Mariacristina Crosti[2], Monica Moro[2], Jens Geginat[3], Massimiliano Pagani[4] and Sergio Abrignani[1]
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Introduction: $CD4^+$ T lymphocytes orchestrate adaptive immune responses by differentiating into various subsets of effector cells, traditionally characterized by the release of different cytokines, which ultimately define their biological functions. However, it has recently been described that upon activation lymphocytes also release a large amount of exosomes containing regulatory RNAs, including microRNAs (miRNAs). We have previously provided an ''atlas of intracellular microRNA expression'' in human lymphocytes and demonstrated that the extra-cellular miRNome does not mirror the intra-cellular one; thus, we here wanted to thoroughly analyse exosome-associated miRNAs released by effector T cells, to identify subset-specific extra-cellular miRNA signatures. *Methods*: To this aim, human $CD4^+$ effector T-cell subsets (Th1, Th2, Th17 and regulatory T cells) were purified from blood by sorting through various combinations of surface markers, then cultured separately and stimulated with anti-CD3 and anti-CD28 antibodies. Exosomes present in lymphocyte extracellular milieu were isolated by microfiltration, and both intracellular and exosome-associated whole miRNome profiled. *Results*: Our results indicate that different T-cell subsets show specific enrichment of different miRNAs in released exosomes. Moreover, the relation between intracellular regulation and extracellular disposal of miRNAs clearly suggests that exosome release works as an additional layer of post-transcriptional regulation with very rapid effects on target genes of the discarded miRNAs. *Summary/conclusion*: In conclusion, the integrated study of all the nodes forming both intra- and extra-cellular miRNA networks may provide a comprehensive view of the molecular mechanisms underlying T-cell functions in health and disease and may profoundly change the knowledge we have on how these lymphocytes affect the extracellular environment.

O9B-351

Subpopulations of extracellular vesicles released from the human LIM1863 colon cancer cell line reveal distinct miRNA-enrichment profiles

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Introduction: EVs are nanometer-sized membranous vesicles released from most cell types, and due to their multifaceted cargo of mRNAs, miRNAs, non-coding RNAs, proteins and lipids have emerged as important mediators of cell–cell communication. EVs comprise 2 subtypes originating by distinct biogenesis pathways: shed microvesicle (sMVs) by plasma membrane blebbing and exosomes via the intracellular endosomal–exosomal trafficking pathway. To date it has been difficult ascribing specific biological functions to EV subtypes due to heterogeneity issues. Previously, we compared traditional methods for isolating human colorectal cancer (CRC) cell line LIM1863-derived exosomes (Tauro Methods 2012 PMID 22285593) and using immunoaffinity capture we characterized proteome profiles of 2 distinct exosomal populations and purified sMVs (Tauro 2013 MCP PMID 23230278). We have extended this study and report here miRNA-enrichment profiles for these 3 EV subtypes. *Methods*: Here we provide an integrated analysis of the genome-wide miRNA expression profiles, using the illumina HiSeq 2000 platform and bioinformatics tools, of 3 EV subtypes released from the LIM1863 CRC cell line. A standard workflow has been developed for isolating and purifying these EVs (2 distinct exosome populations and sMVs). An orthogonal method (quantitative real-time PCR, qRT-PCR) was employed for validating miRNA expression levels. *Results*: Our results highlight general or cancer-specific roles of many genes and miRs (oncogenes and tumour suppressor genes) implicated in CRC biology. While many miRs are common to all 3 EV populations (e.g. miR-320 family, miR-200c, miR-211) and are implicated in CRC, the 2 exosome populations were clearly distinguishable, from each other and from sMVs based on abundant miR-enrichment profiles (i.e. whole cell miR/EV ratios). Unique miRs (albeit of low abundance), and hitherto not ascribed in CRC biology, were revealed in all 3 EV subtypes. To understand the potential functions of EV subtype miRs we next performed extensive bioinformatic analysis. miR Target genes were predicted using TargetScan software and KEGG pathway databases used to annotate target genes. Enriched signalling pathways were identified by a p-value (t-test) and q-value (Benjamini correlation): including Wnt signalling pathway, pathways in cancer, apoptosis, various types of N-glycan biosynthesis and folate biosynthesis. A salient finding was the presence of lower-abundance miRs unique to each EV subtype. *Summary/conclusion*: This systematic analysis revealed 3 different EV subtypes released from LIM1863 CRC cells with distinct proteome and miRNA profiles. Our findings demonstrate that miRs could be utilized as signatures to distinguish CRC subtypes. These data provide a valuable source for future CRC biology studies directed towards EV function and highlight promising genes, signalling pathways/processes, and miR signatures that might have useful diagnostic or therapeutic applications.

O9B-352

Exosomes, RNA and genomic wrecking balls

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Introduction: Treatment of cells with ionizing radiation causes DNA damage. Neighbouring cells that are not directly hit with radiation can nevertheless exhibit DNA damage as well, a curious phenomenon known as the bystander effect. Bystander effects can also lead to chromosomal/genomic instability within the progeny of bystander cells, similar to the progeny of directly irradiated cells. The factors that mediate this cellular communication have not been fully characterized. In this study, we tested the hypothesis that the bystander effect mediator contains an RNA molecule that may be carried by exosomes. *Methods*: Human MCF-7 cells were irradiated with 2 Gy of X-rays, and the extracellular media was harvested. Exosomes were isolated from the conditioned media and transferred onto fresh cells. Levels of DNA damage were measured by counting chromosomal aberrations and using the comet assay. *Results*: Exosomes isolated from media conditioned by irradiated cells were able to induce significant levels of DNA damage in fresh cells. Treatment of exosomes with RNase abrogated their ability to induce this observed bystander effect. The genomic damage induced by radiation-induced exosomes was propagated for several generations, suggesting these exosomes can induce long-term genomic instability. *Summary/conclusion*: These results suggest that the bystander effect is at least in part mediated by exosomes and that an RNA

component is involved. The data also imply that exosomes may induce a long-term program of genomic instability that may have an epigenetic basis. This work widens the repertoire of roles for exosomes and has implications for cancer radiotherapy.

O9B-353

EVpedia 2.0-based systematic analysis of vesicular mRNAs and miRNAs

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Introduction: Mammalian cells release extracellular vesicles (EVs), which are spherical bilayered proteolipids containing various molecules, including RNAs. Many transcriptomic analyses on mammalian EVs have been performed, but the studies have employed different platforms to identify RNAs associated with EVs isolated from different species. Here, we systematically analyse the datasets of vesicular mRNAs and miRNAs based on EVpedia 2.0 (www.evpedia.info), an integrated database of high-throughput data for systematic analyses of EVs. *Methods*: Transcriptomic datasets comparing mRNA and miRNA expressions of EVs and their originating cells were selected from EVpedia 2.0, and a virtual array was built using ortholog mapping of RNA databases. The normalized expression ratios between vesicular and cellular RNAs were calculated, mapped onto the virtual array and depicted as scatter plots. The plots were used to find the correlation between vesicular and cellular RNAs, and to define EV-enriched RNAs. EV-enriched RNAs were classified into common EV- and specific EV-enriched RNAs using statistical analyses. These common EV- and specific EV-enriched RNAs were subjected to systematic analyses to explore their biological significance. *Results*: A total of 11 mRNA and 20 miRNA datasets were selected from EVpedia 2.0. The scatter plots of vesicular and cellular RNAs revealed that vesicular RNAs were positively correlated with cellular RNAs. In addition, we found that EVs harboured EV-specific mRNAs and miRNAs. Systematic analyses on these EV-enriched RNAs suggested that common EV-enriched mRNAs and miRNAs may contribute to several pathophysiological functions of EVs, and some specific EV-enriched RNAs could serve as novel biomarkers for human diseases, including cancer. *Summary/conclusion*: EVpedia 2.0-based systematic analysis of vesicular RNAs suggests a novel strategy to understand pathophysiological functions of EV-associated RNAs and identify novel biomarker candidates for human diseases.

O9B-354

Comparison of circulating microRNA detection methods and feature of microRNAs in healthy volunteer's serum

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Introduction: Serum miRNA profiles are widely reported by many research groups, but they do not always coincide well. The reason of this difference is suggested to be, at least partly, due to a difference in the RNA detection method. In this presentation, the miRNA expression data obtained by different detection tools and also miRNA profiles of more than 150 healthy volunteers' serum have been discussed. *Methods*: We used same serum miRNA fraction for (a) ''TaqMan'' Array MicroRNA Card, (b) QIAGEN miScript miRNA PCR Array, (c) ''3D-Gene'' Human miRNA oligo chip, (d) ''Hi-seq'' Next Generation Sequencer, (e) Nanostring ''nCounter'' Analysis system and compared the resulting serum miRNA profiles. We also did the mass (more than 150) analysis of healthy human volunteers' serum miRNA profiles detected by ''3D-Gene'' miRNA microarray, which includes whole miRNAs in miRBase release 20. *Results*: Serum miRNA profiles were detected differently by tools with and without ''pre-amplification'' protocol. The ''pre-amplification'' caused some bias in the miRNA detection. ''3D-Gene'' microarray detection system, which does not use ''pre-amplification'' process, could detect miRNAs even with low expression. With ''3D-Gene'' microarray system, we studied the circulating miRNAs in more than 150 healthy volunteers' serum. This mass data would be a strong base of searching new biomarkers of several diseases. *Summary/conclusion*: We will show the solution for research tools for circulating miRNA analysis, and the results of its effects.

Van Weelde/Mees room
Symposium Session 9C - EV Biomarkers in urological cancers
Chair: *Guido Jenster and Suresh Mathivanan* 16:00-18:00

O9C-355

Detection and characterization of prostate cancer-derived exosomes with a sensitive ELISA and a phage display

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Introduction: Exosomal proteins and RNAs reflect cellular conditions of the cell of origin. Isolation and characterizing of cancer-derived exosomes from body fluids will enable us to diagnose diseases and determine prognosis without the burden and complications of invasive techniques, such as biopsies. In this study, we developed a highly sensitive ELISA for characterizing prostate cancer (PCa)-derived exosomes from urine. Furthermore, a nanobody phage display was created to select phages that bind to prostate exosomes. *Methods*: An ELISA was developed using streptavidin plates and biotinylated capture antibodies (CD9/CD63) and Europium-labelled anti-CD9/CD63 detection antibodies. Urine samples from women, 135 men with or without PCa and men who underwent radical prostatectomy were collected after digital rectal exam (DRE). Urinary creatinine and PSA were measured for normalization. Also, exosomes from 40 cancerous and non-cancerous cell lines were collected. A nanobody phage display library created from a Llama immunized with human PCa cells was used to select phages that specifically bind to prostate exosomes. *Results*: Our ELISA measures exosomes with high sensitivity directly from cell culture medium, urine and serum. DRE increased the signal of exosomal CD9/CD63 in urine from men. CD63 expression in urine from women and from men after radical prostatectomy was very low. After normalization of expression levels for urinary PSA, CD63 was higher expressed in urine from PCa patients ($p = 0.0006$). Five phage-expressing nanobodies binding to exosomes were selected. ELISA and cell FACS showed their binding specificity. *Summary/conclusion*: An ELISA with CD9 and/or CD63 detection antibody enables us to measure exosomes from urine with a very high sensitivity. Urinary CD63 signals are mainly derived from prostate exosomes and are increased in patients with PCa. Nanobodies recognizing urinary exosomes were selected that have prostate and PCa-specific binding characteristics to explicitly capture and detect PCa-derived exosomes.

O9C-356

ExoScreen as a novel ultra-sensitive liquid biopsy of circulating extracellular vesicles

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Introduction: Cancer cells secrete small membranous extracellular vesicles (EVs), including exosomes, into their microenvironment and circulation. Although their potential as cancer biomarkers has been promising, the identification and quantification of EVs in clinical samples remain challenging. In this study, we established a highly sensitive and rapid analytical technique for profiling circulating EVs directly from serum samples of patients with colorectal cancer. *Methods*: We have developed a bead-based proximity assay named *ExoScreen*, which is based on AlphaLISA technique. In this assay, EVs are captured by 2 antibodies modified in distinct ways. One is a biotinylated antibody, and the other is an antibody conjugated to AlphaLISA acceptor beads. To identify the component of cancer-derived EVs, EVs derived from the colorectal cancer cell line HCT116 cells and colon fibroblast cell line CCD-18Co cells were subjected to proteomic analysis. Next, we used *ExoScreen* to detect cancer-derived EVs in human clinical samples (colorectal cancer patients' serum; 194 samples, healthy donors' serum; 191 samples). *Results*: *ExoScreen* detecting CD9 and CD63 enabled to identify the EVs in 5 μl of healthy donor serum. Furthermore, our proteomic analysis revealed that the amount of CD147 was significantly high in the EVs from HCT116 cells, whereas the expression could not be observed in CCD-18Co cells derived EVs. Most importantly, we found that CD147 and CD9 double-positive EVs were significantly higher in serum from cancer patients than in serum from healthy donors. We are now investigating the utility of EVs in urine for bladder cancer diagnosis. *Summary/conclusion*: These works describe a new liquid biopsy technique to detect disease-specific circulating EVs and provides novel perspectives in translational medicine from the standpoint of diagnosis and therapy.

O9C-357

AGR2 wt and splice variants as a novel biomarker for the non-invasive exosome-based diagnosis of prostate cancer

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Introduction: Prostate cancer is the most common malignancy in men and the second most common cause of cancer-related death in men. For 2012, the incidence was estimated to be 67,600 patients but 5- and 10-year survival rates of 100 and 98% are achieved under state-of-the-art treatment (RKI and GEKID 2012; SEER, June 2013). However, still ca. 12,000 men die of prostate cancer every year (RKI and GEKID) in Germany. Diagnostic gold standard is the histological confirmation by invasive needle biopsies with partly severe side effects. The most common indication for histological confirmation is given by elevated levels of the widely used blood-based test for the prostate-specific antigen (PSA). Sensitivity and specificity of the PSA test are reported to be 76–100% and 6–66%, respectively. Low sensitivity leads to high rates of delayed or missed diagnosis of relevant prostate tumours. Low specificity produces high rates of false-positive test results, which lead to substantial numbers of unnecessary invasive confirmatory biopsies considering the incidence of prostate cancer. It is evident that clinically applicable non-invasive biomarkers with higher sensitivity and specificity than PSA are still urgently needed to further reduce cancer-related mortality but also reduce unnecessary invasive diagnostic procedures. *Methods*: In this study, we assessed the diagnostic potential of new splice variants of anterior gradient 2 (AGR2) gene, using urine exosomes as a biomarker source. Exosomes were isolated from the urine of 27 patients with prostate carcinoma and 14 patients with be benign hypoplasia (BHP). The isolated vesicles were characterized by electron microscopy and dynamic light scattering. Additionally, by

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using western blotting their prostate origin and protein levels of exosome markers were verified. Then, using quantitative RT-PCR, the levels of 5 AGR2 splice variants (sv C, E, H, G and H) in addition to wild-type AGR2 (AGR2wt) were assessed in the exosomes. Further, to verify if the AGR2 transcripts are located inside the vesicles, RNase treatment was applied prior to the RNA isolation. *Results*: The AGR2wt and the splice variants were significantly distinguished between benign and prostate tumour in tissue biopsies ($p \leq 0.05$, $n = 41$). However two of the splice variants (svG and svH) but not AGR2wt proved to be particularly selective as single diagnostic markers for prostate cancer in urine exosomes outperforming the currently used serum PSA. Receiver operating characteristic (ROC) curves showed svG and svH (AUC 0.94, $p = 0.044$ and AUC 0.96, $p = 0.026$) but not AGR2wt (AUC 0.91, $p = 0.12$) as having the greatest discriminatory power in predicting prostate cancer compared to serum or exosomal PSA. *Summary/conclusion*: AGR2 svG and svH pre-present new powerful diagnostic markers for the non-invasive determination of prostate cancer using urine exosomes.

O9C-358

Protein biomarkers for prostate cancer in urinary exosome-like vesicles

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Introduction: The existing clinical biomarkers for prostate cancer (PCa) are not ideal, since they cannot specifically differentiate between those patients who should be treated immediately and those who should avoid over-treatment. Therefore, new biomarkers for fast and reliable diagnosis of PCa are highly desirable. Because of the location of the prostate in the body, its secreted products, including exosome-like vesicles (ELVs), can be detected in urine. The aim of this project is to identify new protein biomarkers from urinary ELVs to improve PCa detection. *Methods*: We standardized the ultracentrifugation-based method for isolation of ELVs from urine samples. The obtained vesicles were characterized by electronic microscopy and western blot, and quantified by nanoparticle tracking analysis. Next, we carried out a discovery phase by label-free LC-MS/MS. We selected 24 samples: 8 benign samples, 8 low-risk PCa samples (Gleason = 7(3 + 4)) and 8 high-risk PCa samples (Gleason > 7). Protein of 20 μg was digested following the method called Filter-Aided Sample Preparation. Peptides were analysed using an Orbitrap Velos mass spectrometer. Analysis of the data was carried out measuring the ion peak intensities and applying several different statistic approaches, in order to increase the reliability of the results. *Results*: We have identified a profile of differentially expressed proteins between the 3 study groups. The next step will be the validation of these protein candidate markers, by targeted techniques such as selected reaction monitoring in a new set of samples. These validated proteins will be further characterized using in vitro models. *Summary/conclusion*: In the future, this could improve current PCa detection, distinguishing aggressive from clinically insignificant PCa and other benign conditions and, therefore, avoiding PCa-related over-diagnosis and over-treatment.

O9C-359

Small RNA sequencing of urine microvesicles reveals miRNAs that identify prostate cancer patients

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Introduction: Prostate cancer (PCa) diagnosis and follow up is primarily based on invasive (repeated) biopsies. Since biopsies can miss tumour cells, cause infections and discomfort, development of non-invasive tests that are sensitive and specific for PCa is warranted. Therefore, we profiled the small RNA content of urine microvesicles from patients with RNA-seq to identify PCa-associated biomarkers. *Methods*: Urine was collected after signed informed consent and was approved by the local medical ethical committee. Microvesicles were isolated from urine by ultracentrifugation and treated with RNAse A. A total of 100 ng small RNA was used for preparation of the miRNA libraries, which were sequenced on PE100 (Illumina). miRNA expression was validated by qRT-PCR. *Results*: About 200 miRNAs were associated with urine microvesicles from all patients. The most abundant miRNA we identified was hsa-miR-10b-5p, at >140,000 reads per million (RPM; >15% of all reads) in all patient samples. Comparing results from patients without cancer, with cancer and metastatic cancer, we observed a gradual shift from 3p (guide) to 5p (passenger) arm usage for miR-30e and miR-1307 in patients with cancer, and was more pronounced in metastatic patients. By RT-PCR, the arm shift of miR-30e could be validated ($p < 0.05$). In addition, in urine microvesicles from prostate cancer patients we identified a distinguishing miRNA profile that was validated in a second set of 15 patients ($p < 0.002$). *Summary/conclusion*: RNAseq of urinary microvesicles yields miRNA-based biomarkers that may distinguish healthy from cancer patients. Further validation in large cohorts will provide proof whether urinary microvesicles are useful for low-invasive PCa diagnostics.

O9C-360

miR-34a is an intracellular and exosomal predictive biomarker for response to docetaxel with clinical relevance to prostate cancer progression

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Introduction: Resistance to docetaxel (RD) can limit the success of treatment for castration-resistant prostate cancer (CRPC). We have previously demonstrated that exosomes derived from conditioned media (CM) of resistant cell lines can induce resistance when applied to secondary cells. Here we investigated the intracellular and extracellular miRNA expression in a panel of cell line models of RD *versus* their parent, sensitive cell lines. *Methods*: We performed global miRNA profiling on the cells and corresponding extracellular vesicles (EVs) from 3 cell line models of RD. The expression of several identified miRNAs was assessed in 4 publicly available clinical datasets, representing tissues and urine from prostate cancer *versus* benign patients, as well as prostate tissues from patients with biochemical recurrence versus non-recurrence and also from patients experiencing metastatic disease *versus* primary disease and benign tissue. Transfection of miR-34a mimics and inhibitors was used to manipulate miR-34a levels in cells to assess effects on response to docetaxel and target protein expression. *Results*: TEM and western blotting suggested that the vast population of EVs isolated were exosomes. On average 76.5% of miRNAs detected in cells was also present in EVs while on average 9.8% were detected in EVs only. Linear regression analysis demonstrated a strong correlation in the detection of miRNAs in EVs and their corresponding cells of origin. Decreased miR-34a expression showed substantial clinical relevance and so was chosen for further functional assessment. Our knockdown and over expression studies confirmed that miR-34a directly regulates BCL-2 and may, in part, regulate response to docetaxel. *Summary/conclusion*: This study confirms that EVs derived from the media conditioned by a panel of prostate cancer cell lines

do represent the cells of origin. Furthermore, our functional assessment of miR-34a supports its role as a predictive biomarker for RD in CRPC.

O9C-361

Exosomal RNA in prostate cancer: diagnostic/prognostic biomarkers and potential effectors of PCa progression

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Introduction: Although the prostate-specific antigen test (PSA) can be used to non-invasively diagnose and monitor prostate cancer (PCa), it offers no predictive information on patient outcome. Given the exponential interest in circulating exosomal RNA content for the development of non-invasive tests for various cancers, we explored the potential utility of exosomal RNA in PCa diagnosis and prognosis. *Methods*: We used prostate cancer cell lines (LNCaP, PC3, DU145 and VCaP) and a control transformed prostate epithelial line (PNT2) as a source of exosomes. Exosomes were isolated by differential ultra-centrifugation and confirmed by transmission electron microscopy (TEM). RNA was extracted using RNAzol RT, and RNA quality and concentration were analysed on an Agilent Bioanalyzer 2100 prior to profiling on both Affymetrix and ArrayStar microarray platforms. Differential expression was assessed using the Partek Genomics Suite software package. Several of the most highly overexpressed RNAs in PCa exosomes versus normal prostate exosomes were validated by qPCR. *Results*: PCa exosomes were highly enriched for miRNAs. We have defined a panel of exosomal microRNAs (exomiRs) that differentiate cancerous from normal prostate cells and, importantly, can define the androgen dependence status of our cells. We are currently translating these findings to the clinic by testing our unique exomiR profile on exosomes isolated from PCa patients' blood/urine (obtained with informed consent, approved by St Vincent's Hospital Human Research Ethics Committee). Furthermore, in silico analyses (Partek Genomics Suite, Cytoscape) predicted that many upregulated PCa exomiRs may target components of the TGFb-signalling pathway. We examined the differentiation of fibroblasts to myofibroblasts, a phenomenon shown to be exosomal-TGFb dependent. RNA extracts taken at various time points following exosome stimulation were examined for exomiR suppression of predicted target mRNA. Surprisingly, we found no role for the tested exomiRs in this differentiation process. *Summary/conclusion*: Exosomal RNAs show great promise as diagnostic/prognostic biomarkers for PCa and will hopefully fill a significant gap in the clinical care of PCa patients. However, understanding the biological role of PCa exomiRs remains a challenge.

O9C-362

Prostate cancer-derived exosomes as predictors of response to therapy

Aris Panaretakis, Pedram Kharaziha and Sten Nilsson
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Introduction: Prostate cancer (PrCa) is the most frequently diagnosed cancer and a leading cause of cancer-related deaths in men. Therapies against PrCa are highly effective for localized diseases. However, treatment efficacy is greatly diminished once PrCa cells develop resistance to androgen deprivation and other conventional prostate cancer therapies. Importantly, there are currently no biomarkers that can predict which patients will benefit from a particular treatment. One promising source of novel predictive biomarkers are extracellular vesicles (EV), such as exosomes and microvesicles, since they may be used (a) as a non-invasive source of biomarkers and (b) for real-time prognosis and monitoring of disease progression. *Methods*: For the in vitro studies, exosomes have been isolated from human PrCa cell lines sensitive or resistant to commonly used anti-cancer agents such as taxotere and abiraterone. The purity and quality of the isolated exosomes is confirmed by electron microscopy, immunophenotyping for exosomal markers, density gradient centrifugation and western blotting. Quantitative targeted LC/MS analysis was performed on these preparations and their parental cell lines. For the in vivo studies, EVs were isolated from plasma of prostate cancer patients that are sensitive or resistant to treatment. The isolated EVs were examined by electron microscopy and by western blotting. *Results*: Bioinformatics analysis of isolated exosomes from the prostate cancer cell lines sensitive or resistant to therapy revealed a specific signature of proteins that may predict the primary or secondary resistance to a particular therapy. Importantly, this molecular signature was verified in the EV isolated from the plasma of a pilot set of prostate cancer patients sensitive or resistant to the particular therapy. *Summary/conclusion*: Prostate cancer cell-derived extracellular vesicles may be a valuable resource of predictive biomarkers. There are currently no such predictive biomarkers available in the clinic and their identification will allow clinicians to select for the most effective treatment modalities for patients in a personalized manner.

Caris Life Sciences – sponsored session — Willem Burger room — 18:00-19:00

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Poster Presentations

Arcadis room

Poster Session 7A - EV cargo selection and release mechanisms

Chair: *Emanuele Coccuci and Guillaume van Niel* 13:00-14:00

P7A-268

Potential role of mRNA fragments secreted by cancer cells in functional deregulation of recipient cells

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Introduction: Exosomal mRNAs were shown to be translated into proteins in target cells. However, we demonstrated recently that the majority of mRNAs carried by human cancer cell-derived exosomes is fragmented. These mRNA fragments are derived largely from the 3′ untranslated regions (UTRs), thus precluding their translation into proteins. Since the 3′-UTRs of transcripts are rich in miRNA-binding sites, exosomal RNA may act as competing RNA to regulate stability and translation activity of mRNAs in recipient cells. Here we examined potential cellular targets of competing exosomal RNA fragments to evaluate possible functional effects on recipient cells. *Methods*: To assess possible functional effects of exosomal mRNA fragments, we performed the analysis of gene ontologies (GOs) associated with the protein products of mRNAs from which the exosomal mRNA fragments are derived. GOs were analysed using Panther database and statistical model using official gene symbols as primary entries. Bonferroni correction for multiple comparisons was applied to the p-values. *Results*: The protein products of the potential mRNAs targeted by exosomal mRNA fragments were found to be significantly enriched in enzyme modulation and in proteins participating in extracellular transport, cell adhesion, establishment of chromatin architecture and protein phosphorylation. On the contrary, the proteins encoded by the full-length secreted mRNAs are specialized in cell communication, cell surface receptor-linked signal transduction and system development. A large number of the products of these transcripts are localized in the extracellular matrix. *Summary/conclusion*: Genetic information carried by cancer cell-derived exosomes consists of not only full-length mRNAs but also a large number of mRNA fragments. Exosomal mRNA fragments acting as competing RNA may potentially lead to upregulation of proteins involved in cell adhesion and protein phosphorylation and as a result deregulate cell motility, adhesion and other functions in recipient cells.

P7A-269=OP3-133

Regulation of exosome RNA cargo

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P7A-270

Effect of Gsk3βknockdown on human mast cell exosome release and RNA profiles

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Introduction: Exosomes are vesicles of endocytic origin released by many cells. The mechanism of exosome release is very complicated and unclear. Glycogen synthase kinase 3β is a key factor for diverse cellular response. But the effect of GSK3βknockdown on human mast cell exosome release and RNA profiles is still unknown. *Methods*: In this study, we use the cells, which were knockdown of GSK3βprotein following transduction, with GSK3β-targeted shRNA. The exosome was characterized by electron microscopy. The total RNA of exosomes was analysed by Bioanalyzer and proteins were analysed by western blot. *Results*: Our results show that the protein and RNA of exosome release from human mast cells with GSK3β knockdown is increased compared with the scramble RNA transducted HMC-1 cells. Our results also show that the proteins of downstream of the GSK3βcell signal pathway increased. *Summary/conclusion*: Glycogen synthase kinase 3β plays a vital role in the release of exosome from human mast cells.

P7A-271

MicroRNAs in PMN-ectosomes result from a specific sorting

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Introduction: Extracellular vesicles, in particular exosomes, have been shown to harbour messenger RNAs (mRNAs) and microRNAs (miRNAs) that can be delivered to other cells. miRNAs are a class of evolutionarily conserved, small (22–24 nucleotides long), non-coding RNA molecules that play important regulatory roles in various biological processes. They regulate post-transcriptional gene expression by affecting the degradation and translation of target mRNAs. Ectosomes shed from the cell surface by polymorphonuclear neutrophils (PMN-Ecto) have a size between 50 and 300 nm, express phosphatidylserine as well as a specific selection of cell membrane proteins. Here we analysed whether they contain miRNAs that could be conveyed to recipient cells. *Methods*: We isolated PMN and PMN-Ecto from 6 healthy donors and extracted total RNA. The quality was verified by an Agilent 2100 Bioanalyzer profile. miRNA array profiling was conducted at Exiqon Services, Denmark, using the miRCURY LNA™ microRNA Array 7th Gen. The quantified signals were background corrected and normalized using quantile normalization method. We validated the results obtained in the array analysis by real-time RT-PCR. *Results*: miRNA was present in PMN-Ecto. The principal component analysis and the hierarchical clustering showed that the miRNA profile of PMN-Ecto was significantly different compared to the PMN. Many miRNAs were differentially expressed either in PMN or in PMN-Ecto. We present in this work our preliminary results showing that a specific sorting of miRNAs exist between the mother cells and their ectosomes. *Summary/conclusion*: Further investigations are necessary to understand the mechanism responsible for the miRNA sorting, and after transfer of the ectosome to a recipient cell, to define their role in the new cellular environment.

P7A-272

Qualitative analysis of small RNAs in human endothelial cells and derived exosomes identified by deep-sequencing

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Introduction: Exosomes contain a variety of RNA molecules, including mRNA, miRNA and sncRNA, which can be functionally transferred to target cells. The RNA content of exosomes differs from that of their cells of origin, indicating that RNA molecules can be selectively incorporated into or excluded from exosomes at their biosynthesis. *Methods*: To gain further insight in the RNA-mediated functional properties of endothelial cell-derived exosomes, we analysed the small RNA content of endothelial cells and exosomes secreted by these cells by deep-sequencing. Differences in identity and quantity of RNA classes were determined and qualitative differences in the coverage of sequences in cells and exosomes were analysed. *Results*: We identified several classes of RNA, including mRNA (fragments), miRNA, snRNA and mtRNA, confirming the presence of a broad spectrum of non-coding RNAs in cells and exosomes. Quantitative analysis revealed the enrichment of numerous miRNAs, mtRNA and, in particular, yRNA and vault RNA. Qualitative analyses to investigate the distribution of particular RNA strands or fragments between cells and exosomes indicated that for miRNAs, the distribution of 3p and 5p strands is mostly similar in cells and exosomes, though in cells, additional fragments of the pre-miRNA stem-loop could be identified. Furthermore, in exosomes identified were generally shorter than in cells, especially considering mRNA, mtRNA and snoRNAs, suggesting that exosomes contain degraded fragments these RNAs. *Summary/conclusion*: Overall, our analysis shows that not only quantitative but also qualitative differences in RNA content between cells and exosomes can be observed, providing information about the function and biogenesis of exosomes.

Funding: This research was supported by the Netherlands Organization for Scientific Research.

P7A-273

Benzene DNA/RNA adducts in microvesicles of cigarette smokers' saliva, clotted blood and urine detected by spectroscopy in support of CHEN'S 1968 Hypothesis of Genetic Exchange

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Introduction: In 1968, the author CHEN first demonstrated that DNA, a molecule of genetic material moved from cell to cell and is associated with normal and abnormal cellular growth and differentiation in mammalian skin autotransplants. The corollary of his MD thesis ''Intercellular Exchange of Genetic Material (DNA/RNA) in the control of growth and differentiation'' states that any disturbances of the exchanging system or the genetic messages between cells can result in abnormal growth, even cancer. Accordingly, cigarette carcinogens exert their carcinogenic effects by altering the genetic messages within intercellular information carrier microvesicles from oral and pulmonary sites transported by blood and body fluids to distant receptor cells. In a paper to the Int CNAPS VIII Conf John Hopkins 2013, the author demonstrated that toluene, a cigarette smoke carcinogen, formed DNA/RNA adducts in microvesicles of smokers' saliva, clotted blood and urine. The present paper to determine whether benzene, another cigarette smoke carcinogen, also formed DNA/RNA adducts is part of continuing studies to investigate the 40% association of cigarette smoke and bladder cancer found in a Trinidad study. *Methods*: Microvesicles in saliva, clotted blood and urine of cigarette smokers and non-cigarette smokers were examined by spectroscopic analysis. *Results*: In non-smokers' DNA/RNA 15 nucleotides and more In smokers' benzene DNA/RNA adducts 15 nucleotides and more were present in microvesicles of saliva clotted blood and urine. *Summary/conclusion*: This first demonstration of benzene adducts in microvesicles in cigarette smokers' saliva, clotted blood and urine provides a means by which benzene is transported and act by intercellular transfer of altered genetic messages benzene DNA RNA adducts inducing mutagenesis in sensitive recipient cells.

Funding: This supports CHEN'S 1968 Hypothesis of Genetic Exchange.

P7A-274

Release of P-selectin-exposing microparticles from platelets requires binding of fibrinogen to activated αIIbβ3

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Introduction: Whereas circulating microparticles (MP) from megakaryoctes and platelets expose αIIbβ3 (glycoprotein IIb-IIIa), only platelet microparticles (PMP) expose P-selectin (CD62p; *Blood* 2009; 113: 1112), and CD62p+ PMP have been associated with platelet activation in cardiovascular disease (*Clin Chem* 2006; 52(4): 757−664). Here we investigated the mechanisms underlying the release of CD62p+ PMP. *Methods*: Citrate-anticoagulated blood was collected from healthy subjects (n = 4–6). Platelet-rich plasma was activated by ADP (10 mmol/L) in the absence and presence of abciximab, in a multiplate aggregometer (30 minutes, stirring conditions, 37°C). Thereafter, supernatant was collected and analysed by flow cytometry. *Results*: In fresh plasma, on average14,000 αIIbβ3-exposing MP (mean; n = 6) were detected, of which 5.6% stained for CD62p. Addition of ADP-induced platelet activation and fibrinogen binding, resulting in platelet aggregation. After 30 minutes, the number of αIIbβ3-exposing MP increased to 21,624 ± 5,310, and all newly formed PMP exposed P-selectin. In the presence of abciximab, which inhibits the binding of fibrinogen to αIIbβ3 but does not abrogate platelet activation, the release of PMP was completely inhibited. *Summary/conclusion*: The release of PMP exposing P-selectin requires not only platelet activation but also binding of fibrinogen to αIIbβ3. Therefore, we hypothesize that the occurrence of true (CD62p+) platelet-derived MP in human plasma is not only a marker of platelet activation but rather mirrors on-going (basal) thrombus formation and degradation, which may explain their increased presence in the blood of patients with cardiovascular disease.

P7A-275

Analysing the effects of circadian rhythm on the ability of lung-derived extracellular vesicles to modulate bone marrow cellular phenotype

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Introduction: Extracellular vesicles (EVs) have tremendous therapeutic potential. As such, it is becoming increasingly important to fully delineate the factors influencing their biogenesis and function to help standardize isolation techniques and establish normative controls. Numerous factors are thought to influence EVs, but little is known about how circadian oscillations alter EV function. We report here our preliminary studies exploring the effects of circadian rhythm on lung-derived EV modulation of bone marrow target cell phenotype. *Methods*: C57BL/6J mice were housed on a controlled 12 h light/dark schedule. First, at 4, 8, 12, 16, 20 and 24 h after light onset (HALO), lungs were harvested (2 mice/time point) and co-cultured with murine WBM (all from mice at HALO 9), separated by a

cell-impermeable membrane. After 24 h co-culture, the WBM was analysed for pulmonary-specific mRNA expression by RT-PCR. WBM cultured alone served as a negative control. Second, lungs harvested from mice (all at HALO 2) were cultured for 48 h. EVs were isolated from the lung-conditioned media by ultracentrifugation. At 4, 8, 12, 16, 20, and 24 HALO, WBM was harvested (3 mice/time point), cultured with or without the lung-derived EVs for 24 h and analysed for pulmonary-specific mRNAs by RT-PCR. Finally, at HALO 12 and HALO 24, lungs were harvested (3 mice/time), cultured for 24 h, and EVs isolated from the lung-conditioned media were analysed for number (Nano-Cyte®) and protein content. *Results*: There were clear oscillations in the expression patterns of pulmonary-specific mRNA in co-cultured marrow cells when the circadian time point of the lung donor was altered. Compared to WBM cultured alone, WBM co-cultured with HALO 12 lungs across a cell-impermeable barrier showed a 1,100- to 2,500-fold increase in both surfactant A and surfactant C mRNA expression. No surfactant A or C expression was detected in WBM co-cultured with HALO 16 or HALO 24 lungs. There was a trend toward increased numbers of EVs generated from HALO 12 lungs when compared to HALO 24 lungs, suggesting that differences in the phenotype change may in part be related to circadian influences on vesiculation. In contrast, no statistically significant differences in the level of pulmonary mRNA expression were found when the circadian time point of the marrow at the time of EV exposure was altered, suggesting that it may be the originator tissue of EVs, rather than target bone marrow cells, that is most susceptible to circadian variation. *Summary/conclusion*: Based on our preliminary data, we conclude that circadian rhythm is likely an important modulator of EV-mediated intercellular communication and warrants more detailed study to define its role in EV biogenesis and function.

P7A-276

A novel regulatory pathway for exosome release from mammary epithelial and breast cancer cells

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Introduction: Extracellular vesicles, such as exosomes, are released from both normal and tumour cells, display a wide range of biological functions and represent a novel intercellular communication pathway about which much still remains to be understood. In particular, what regulates overall release and homeostatic levels in both healthy and diseased states remain essentially unknown. In this study, we used the human mammary epithelial cell line HMEC B42 and a breast cancer line derived from it (B42 clone 16) to investigate the regulation of exosome release. *Methods*: Exosome numbers were quantified using nanoparticle tracking analysis in cultures before and after supplementation with concentrated suspensions of exosomes purified from both the normal and tumour cells. *Results*: Exosome release into the culture medium was regulated by the presence of exosomes derived from the original cell type. Exosomes from normal mammary epithelial cells were also observed to inhibit exosome secretion from breast cancer cells, whereas added exosomes from a bladder cancer cell line displayed only a minor effect, indicating a degree of tissue-specific regulation. *Summary/conclusion*: These data highlight a previously undescribed novel feedback regulatory mechanism for controlling the release of exosomes. This pathway may indicate a new therapeutic approach for influencing the release of potentially harmful exosomes from tumour cells.

P7A-277

Identification of distinct subpopulations of EVs secreted by human skeletal myotubes

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Introduction: Cells are known to secrete many types of extracellular vesicles (EVs) and the types and compositions depend on the cell type, cellular microenvironment, physiological and pathophysiological conditions of the cell. It was reported that skeletal muscles secrete EVs mainly in the 50–100 nm size range that are released upon muscle differentiation. However, little is known about the biological functions of these EVs, and whether any heterogeneity exists in this population. *Methods*: In this study, we employed a clonal MycHSMM cell line derived by *MYC* immortalization of primary human skeletal muscle myoblasts. EVs secreted by differentiated myotubes were enriched using membrane filtration. Vesicles with GM1 ganglioside-enriched membranes or exposed phosphotidylserine (PS) were isolated by their affinity to cholera toxin B or annexin V, respectively, using magnetic bead technology. *Results*: The immortalized myoblast cell line has a normal karyotype, an accelerated rate of proliferation, and retains its ability to elongate and fuse with adjacent cells to form multinucleated myotubes. These myotubes stained positively for markers of skeletal muscle differentiation such as myogenin, desmin, dystrophin and myosin heavy chain 2. Differentiated myotubes secrete EVs with a modal diameter of 100 nm as analysed by nanoparticle tracking analysis (NTA). This EV preparation also contained vesicles with a density of 1.10–1.18 g/ml and positive for exosome markers such as ALIX, TSG101 and CD9. We further profiled these vesicles by identifying subpopulations with GM1 ganglioside-enriched membranes or exposed PS, and these subpopulations contained distinct protein cargoes. *Summary/conclusion*: Skeletal muscles secrete a wide repertoire of EVs, and these include vesicles with GM1-enriched membranes, suggesting that they originated from lipid raft microdomains in the plasma membrane. Further work to explore the biological significance of these subpopulations of muscle EVs is underway.

P7A-278

The glycosphingolipid synthesis inhibitor PDMP modulates the composition of exosomes released from PC-3 cells

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Introduction: The mechanism of exosome biogenesis and release is not yet fully understood. The interplay and the involvement of several proteins, such as Rab- and ESCRT- proteins, in the biogenesis and release of exosomes have been investigated to some extent. However, the role of lipids in exosome formation and release is less studied. We are now aiming to investigate whether glycosphingolipids play a role in the release of exosomes from the human prostate cancer cell line PC-3. *Methods*: Exosomes were isolated from the conditioned media of PC-3 cells by ultracentrifugation. The relative amounts of exosomes were estimated by: (a) quantifying the levels of the exosomal proteins CD81, CD29, flotillin-1, vinculin, annexin A2 and caveolin-1 from western blots; (b) nanoparticle tracking analysis using nanosight; and (c) measuring total exosomal protein using the BCA assay. *Results*: Western blot analysis of exosomes and cells showed that the inhibition of glycosphingolipid synthesis by PDMP increased the levels of caveolin-1 and annexin A2, but caused variable effects on the levels of other proteins such as CD81, CD29, flotillin-1 and vinculin in exosomes. Moreover, nanoparticle tracking analysis using the Nanosight instrument NS500 showed no difference or even a small decrease in the number of released exosomes per cell between control and PDMP-treated cells. Furthermore, no difference in total protein content in exosomal fraction from PDMP-treated and untreated cells was observed. *Summary/conclusion*: Interestingly, these results suggest that the reduction in the endogenous levels of glycosphingolipids modulates the composition of exosomes, rather than changing the exosome release *per se*.

Arcadis room
Poster Session 7B - EV in cancer 2
Chair: *Olivier de Wever and Dolores di Vizio* 13:00-14:00

P7B-279

Inhibition of EGFR signalling in tumour cells affects extracellular vesicle secretion and content

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Introduction: The epidermal growth factor receptor (EGFR) is a well-established target for anti-cancer therapy, and several approved inhibitors are used in the clinic. EGFR-positive tumour cells secrete extracellular vesicles (EVs) that can transfer functional EGFR to surrounding endothelial cells, thereby promoting angiogenesis and tumour development (1). We have investigated the effects of EGFR inhibitors on characteristics and EGFR status of EVs secreted by tumour cells. *Methods*: The effects on tumour cell proliferation of the monoclonal antibody Erbitux® (cetuximab) and the kinase inhibitor Tarceva® (erlotinib) were determined by exposure to EGFR-positive cell lines followed by MTS assay. EVs were isolated from tumour cells after activation by EGF and exposure to EGFR inhibitors. Nanoparticle tracking analysis was used to determine the number of secreted EVs after treatment. Western blotting was performed to assess treatment effects on (phosphorylated) EGFR in the isolated EVs. Effects of endothelial signal transduction after exposure to tumour cell-derived EVs were determined by western blotting. *Results*: Stimulation of EGFR-positive cell lines with EGF results in secretion of EVs that contain phosphorylated EGFR. Exposure of tumour cell lines to EGFR inhibitors resulted in a decrease of secreted EVs, reduction of total EV EGFR content and amount of phosphorylated EGFR. *Summary/conclusion*: Erbitux® and Tarceva® have different modes of action but comparable effects on the amount and activation of EGFR in tumour cell-derived EVs. Inhibition of (activated) EGFR in tumour cell-derived EVs may be a mechanism by which molecular inhibitors retard tumour development and angiogenesis.

Reference

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P7B-280

Association between YM155 resistance and extracellular survivin splice variants in acute myeloid leukaemia

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Introduction: Survivin, a member of the inhibitor of apoptosis family, is one of the most highly expressed transcripts in cancer and virtually undetectable in most normal adult tissues. Survivin expression in acute myeloid leukaemia (AML) has been found to have anti-apoptotic functions and linked to poor survival outcomes. Tumour cells have the ability to release survivin in exosomes, which is involved in intracellular signalling and appears to modulate the tumour microenvironment. The novel survivin inhibitor, YM155, potently inhibits the growth of various cancers, including AML, showing a promising potential for development of an alternative cancer therapy. Survivin has many splice variants (SSVs), including 2B and DeltaEx3, which share many of the anti-apoptotic properties of wild-type survivin, yet the functions of these variants has not been well characterized. We hypothesized that differences in extracellular SSVs might be associated with response to YM155. *Methods*: Two human AML cell lines, HL60 and U937, were exposed to different concentrations of YM155 and the cell viability was measured using the MTT assay. Total RNA was isolated from exosomes precipitated from supernatant with ExoQuick-TC. Extracellular SSV transcripts from distinct culture conditions were detected using PCR amplification techniques. *Results*: HL60 and U937 cell lines have very different levels of sensitivity to YM155 exposure with IC_{50} values of <1 nM and 50 nM, respectively. Intriguingly, we found that exosomes isolated from HL60 cells contain wild-type survivin transcripts, which disappeared when these cells were cultured in IC_{50} concentrations of YM155. Conversely, exosomes isolated from U937 cells were not found to contain any survivin transcripts until these cells were exposed to YM155. Exosomes isolated from U937 cells cultured with YM155 contained wild-type, 2B, and DeltaEx3 survivin transcripts in quantities that appeared to increase in a dose-dependent manner. *Summary/conclusion*: The ability to produce exosomes containing survivin transcripts upon exposure to YM155 may be a mechanism which helps promote the tumourigenic environment and resistance to YM155. Monitoring extracellular SSVs in clinical samples from AML patients treated with YM155 could be relevant to clinical outcomes and helpful in treatment decisions.

P7B-281

Role of exosomes in the radiation-induced bystander effect

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Introduction: It was once assumed that radiation produced genetic damage and other biological responses only in cells where the radiation directly traversed the nucleus. However, it is now understood that the surrounding non-irradiated cells are also affected through intercellular communication with nearby irradiated cells. The repercussions of this bystander effect impact both radiation health risks and the therapeutic efficacy of radiation treatment. Several mechanisms involving gap junctions and secreted molecules have been implicated in this bystander phenomenon but the role of exosomes has yet to be clearly demonstrated. However, given the diverse protein and nucleic acid payload of exosomes, there is reason to believe exosomes may be involved. *Methods*: Using glioblastoma cells (T98G, U87) as a model system, the contents of exosomes harvested 24 h after exposure to an absorbed dose of 8 Gy of ^{137}Cs gamma rays are being studied. Liquid chromatography and

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mass spectrometry are being used to compare exosomes produced by irradiated cells to those produced by sham-treated cells. The effect of exosomes shed from the irradiated tumour cells on the induction of stressful effects in recipient bystander cells will be examined using cells normally present in the glioma microenvironment (normal astrocytes, microglia and neural stem cells). *Results*: Data will be presented on survival, induction of DNA damage, modulation of stress-inducible signalling pathways, and activation and differentiation of recipient bystander cells. Based on proteomic analysis, and guided by data in the literature, the effect of candidate molecules contained in exosomes that could be implicated in a bystander response will be explored. *Summary/conclusion*: Together, these results will proffer potential mechanisms for the degenerative effects that arise following cancer radiotherapy and may lead to formulation of novel protective strategies.

P7B-282=OP1-108

Exosomes mediate drug resistance in melanoma

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P7B-283

Dendrogenin A promotes secretion of anti-tumour exosomes from aggressive melanoma cells

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Introduction: Dendrogenin A (DDA) was identified as a natural metabolite derived from cholesterol that induces in vitro and in vivo differentiation and death of various tumour cells (de Medina et al., *J Med Chem*. 2009 and *Nature Commun*. 2013). We observed by electron microscopy that tumour cells treated with concentrations of DDA that induced cell differentiation contained more vesicles with a morphology typical of multivesicular bodies (MVBs) than control cells, and secreted small vesicles. The studies realized with human (SKMEL28) and murine (B16F10) melanoma cells treated by DDA indicated that these effects were associated with an increase in intracellular levels of cholesterol and lyso-bis phosphatidic acid (LBPA or BMP), components of MVBs and exosomes. *Methods*: Secreted vesicles isolated from DDA- and vehicle-treated melanoma cells for 24 h were analysed for their size, density and exosomal markers as well as markers associated with melanoma differentiation and exosome biogenesis. *Results*: Our data established that DDA-secreted vesicles were exosomes. Tetraspanins were found in equal amount in DDA and control exosomes. In contrast, the levels of heat-shock protein (Hsp70), MHCII, tyrosinase, tyrosinase-related protein 2, MART1/melan, Rab27a and BMP were increased in DDA exosomes compared with control exosomes. A single intradermal injection of DDA exosomes, isolated from DDA-treated B16F10 cells, into immunocompetent mice grafted with B16F10 tumour, inhibited the growth of the tumours by 80% after 3 weeks of treatment and significantly enhanced the survival of mice compared with the vehicle-treated group. In contrast, no anti-tumour effect was observed with exosomes isolated from vehicle-treated cells. *Summary/conclusion*: Our results indicate that DDA modified these aggressive tumour cells to produce exosomes with anti-tumour activities in vivo. To date, no compound has been described that enhances the anti-tumour activity of tumour exosomes. These properties define DDA as an innovative drug-candidate against cancer.

P7B-284

Oncogenic PML-RARa regulates biological activity of extracellular vesicles produced by leukaemic cells

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Introduction: Acute promyelocytic leukaemia (APL) is driven by a balanced reciprocal translocation between chromosomes 15 and 17, which results in the transforming fusion between promyelocytic leukaemia gene (PML) and retinoic acid receptor alpha (RARa). The oncogenic effect of PML-RARa relies on RARa-dependent inhibition of cellular differentiation and can be selectively obliterated by all-*trans* retinoic acid (ATRA) therapy. In combination with arsenic trioxide (ATO) and chemotherapy, ATRA triggers complete remission in the majority of APL cases. ATRA also alters interactions between APL cells and the vascular system, including the reversal of tissue factor (TF)-related coagulopathy. We hypothesize that at least some of these effects include the impact of PML-RARa on qualitative and quantitative aspects of cellular vesiculation. In the present study, we interrogate the impact of PML-RARa on the profile of extracellular vesicle (EV) emission by APL cells. We also explore the role of PML-RARa-regulated EVs in the course of interactions between APL cells and some of the key elements of the vascular system, such as coagulation cascade and endothelium. *Methods*: We employed human NB4 cells harbouring t(15;17) translocation and responsive to ATRA as a model of APL. Vesiculation of these cells was profiled by nanoparticle tracking analysis (NTA) and the vesicle cargo analysed using mRNA profiling, RT-PCR, western blotting and ELISA assays. Uptake of EV-related material by endothelial cells (HUVEC) was measured using PHK-26 staining and flow cytometry, while the resulting changes in cellular phenotype were monitored by tissue factor procoagulant assays, growth and migration bioassays, and molecular profiling. *Results*: ATRA treatment reveals a parked regulatory impact of PML-RARa on the sizes and abundance of EVs produced by NB4 cells. Leukaemic EVs contain transcripts for both, PML-RARa oncogene, and several of its targets, such as tissue factor and a series of key regulators of angiogenesis. In line with these findings, the uptake of NB4-derived EVs confers functional changes on endothelial cells, including procoagulant phenotype and modulation of growth, survival and migration. *Summary/conclusion*: Oncogenic PML-RARa regulates at least some aspects of the web of interactions that occur between leukaemic (APL) cells and their vascular niches. This process involves oncogene-driven emission of EVs and transfer of their cargo between leukaemic and endothelial cells.

P7B-285

Tumour-derived exosomes from bladder cancer patients induce epithelial to mesenchymal transition

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Introduction: Exosomes are nano-sized membrane vesicles with endosomal origin that are released from a variety of cell types and serve as means of communication between cells. They have been studied in many cancer forms and are believed to be of great importance in the tumour microenvironment. To be able to understand their function/s in tumourigenesis may lead to new strategies for cancer therapy. Epithelial to mesenchymal transition (EMT) is an important event in tumour metastasis, as it allows cancer cells to move to new sites. Epithelial cells lose their epithelial feature and acquire a more mesenchymal phenotype, which is characterized by the expression of different markers such as vimentin.

Methods: Tumour tissue and non-tumour tissue were disrupted by gentle MACS by using collagenase. Cells and exosomes from lymph nodes were collected after passing tissue though a cell strainer. Exosomes were isolated from the supernatant by different ultracentrifugation and filtration steps. *Results*: We found that exosomes isolated from tumour tissue from urinary bladder cancer patients express more inflammatory molecules compared to exosomes from non-malignant tissue. In addition, exosomes from malignant tissue express EMT markers on their surface. The same pattern is seen in the expression of inflammatory markers on exosomes from draining lymph nodes compared to non-draining nodes. In addition, the induction of IL-6 in a bladder cancer cell line is shown to be higher after stimulation by exosomes from normal tissue compared to tumorous tissue. This might lead to downregulation of EMT markers and thus to a lower EMT response, compared to the exosomes from tumour tissue. *Summary/conclusion*: These results suggest a role for exosomes in EMT induction of neighbouring cancer cells, which may be of importance when designing new cancer therapeutics.

P7B-286

Mesenchymal transition-like phenotype induced in lung epithelial cells by mast cell exosomes

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Introduction: The most common type (85%) of lung cancer is non-small cell lung cancer (NSLC) and it is usually detected in its last stages (III, IV) of cancer. Activated mast cells surrounding the lung epithelial cells and other cancer have been implicated in cancer progression. Mast cells activation is associated with secretion of various factors, including exosomes. The aim of this study was to determine whether mast cell-derived exosomes may induce a metastatic phenotype and protection against oxidative stress, in a lung epithelial cancer cell line. *Methods*: Exosomes were isolated by differential centrifugation. The uptake of human mast cells (HMC1)-derived exosomes by A549 cells was detected using fluorescent microscopy. Morphological and inverse migration assays were performed to evaluate the migratory phenotype in the lung epithelial cells after receiving mast cells-derived exosomes. To determine if any changes were associated with classical epithelial to mesenchymal transition (EMT), we measured the protein expression of N-cadherin and E-cadherin in the recipient lung cancer A549 cell line. *Results*: Mast cell-derived exosome were taken up by A549 cells and localized preferential to one end of the nuclear periphery. Lung epithelial cells showed fibroblast-like morphology and a migratory phenotype when exposed to mast cell-derived exosomes. This phenotype was related to enhanced surface expression of N-cadherin and reduced expression of E-cadherin. *Summary/conclusion*: Mast cell-derived exosomes induce EMT-like phenotype in lung epithelial cells which could be related to lung cancer progression.

P7B-2870

The potential role of exosomes in conferring phenotypic traits between cell lines

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Introduction: Metastatic melanoma represents a complex and aggressive disease, with high ability to develop resistance towards commonly used drugs. To study melanoma biology, we have previously established 2 cell lines from biopsies of metastatic melanoma. Melmet 1 displays an invasive phenotype while Melmet 5 fits better with the proliferative phenotype. The cell lines have been well characterized with respect to growth rates, invasive potential, drug resistance and growth pattern in animal metastasis models. Interestingly, they differ in all of these phenotypic traits. For example, although both cell lines carry mutated BRAF, they respond differently to the mutated BRAF inhibitor vemurafenib. Exosomes are vehicles trafficking biological information across membranes. Their cargo is known to reflect, but not equal, the cell of origin. To investigate whether exosomes secreted by the Melmet 1 and Melmet 5 cells may reflect, and possibly transfer the cell-specific phenotype, we have studied both cell lines and their corresponding exosomes. *Methods*: To educate the cells we have grown the Melmet 1 cells in the presence of Melmet 5 exosomes, and vice versa. The educated cells are then analysed with respect to invasive and migratory abilities and sensitivity to drugs. To explore the proteomics of the exosomes and possibly identify candidate proteins that may help explain some of the phenotypic differences, we have performed mass spectrometry and western analyses on the exosomal proteins. *Results*: Preliminary results indicate that exosomes from the more drug-resistant Melmet 1 cells are able to reduce the sensitivity of Melmet 5 cells to both vemurafenib and dacarbazine treatment. Differences in the content of several proteins, for example, apoptosis-related proteins such as survivin were found. We also detect the membrane proteins CSPG4 (chondroitin sulphate proteoglycan 4) and B7H3 (CD276), and can with immunomagnetic selection isolate populations of exosomes expressing these markers for further characterization. *Summary/conclusion*: Phenotypic differences in cell behaviour are reflected in the exosome population.

P7B-2871

Exosomal microRNAs as potential indicators of an anti-tumour response in melanoma

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Introduction: Exosomes can influence target cells via their protein, mRNA, or microRNA load. Our aim is to investigate microRNA profiles of melanoma exosomes and to reveal potential markers indicating an anti-tumour response. *Methods*: After exosome isolation and RNA extraction, Agilent miRNA-Microarrays were used for analysis. *Results*: In melanoma cell culture, we identified 5 microRNAs selectively exported via exosomes ($p < 0.01$). In plasma from melanoma patients, we found an additional 103 exosomal microRNAs, indicating that microRNAs originate also from other somatic cells. Plasma samples from healthy donors and melanoma patients showed minor variation (coefficient of variation < 1). However, on average, microRNA signal intensities were significantly increased in melanoma samples ($p < 0.01$), which might be explained by a higher microRNA load per exosome or a higher total number of exosomes in cancer patients. After tumour removal, the signal intensities across all exosomal microRNAs were lower in comparison to melanoma patients. This difference was significant for the group of high-risk ($p = 0.01$) and low-risk patients ($p < 0.01$). Two plasma samples from presumed healthy donors had elevated exosomal microRNA levels. Subsequent analysis revealed dysplastic nevi. After surgery, microRNA signals returned to normal. A signal reduction was also detected in exosomes of a melanoma patient 2 weeks after the treatment with a BRAF inhibitor. *Summary/conclusion*: We hypothesize that an increase of exosomal microRNAs in patient plasma might not directly be caused by the tumour. Since the elevated microRNAs were also found in healthy donors, we suggest that an increased exosomal microRNA level indicates an immune response against the tumour. Decreased microRNA signals might reflect a diminished immune response, for example, after tumour removal. However, persisting high miRNA signals like in high-risk patients could indicate residual tumour cells. Exosomes might be potential cancer markers but also an indicator for an immune response against the tumour.

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Arcadis room
Poster Session 8A - EV in (cardio) vascular disorders
Chair: *Alexander Kapustin and Douglas Losordo* 13:00-14:00

P8A-288

The bio-distribution of endothelial-derived extracellular vesicles in mice

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Introduction: The release of extracellular vesicles (EVs) has been implicated in cardiovascular disease (CVD). EVs can be generated by the damaged endothelium. Endothelial damage can predispose to atherosclerosis, the underlying pathology in CVD. However, the biological distribution of endothelial-derived EVs remains poorly understood. The aim of this study was to label and track the tissue distribution of endothelial EVs injected into mice at an early time point of 30 minutes. *Methods*: Mouse endothelial cells (sEND-1) were treated with tumour necrosis factor-alpha (TNF-α) (10 ng/ml) in EV-free Dulbecco-modified eagle medium (DMEM) for 18 h. Supernatants were collected and EVs isolated using centrifugation, filtering and ultra-centrifugation techniques as recommended by the International Society of Extracellular Vesicles (ISEV). Isolated EVs were analysed using nanoparticle tracking analysis (NTA) for size and concentration. EVs were labelled with the green fluorescent dye PKH67 (Sigma-Aldrich). Labelled EVs were injected into CD-1 wild-type mice by tail vein injection at a concentration of 8.5×10^8 and sterile saline used as a control. Animals were euthanized 30 minutes post-injection and tissues analysed for the presence of EV using histological techniques. *Results*: Isolated endothelial-derived EVs had a narrow size distribution (<200 nm). PKH67-labelled EVs were easily detected in liver, lung and spleen. EVs were most abundant in sections of liver followed by lung and spleen. PKH67-labelled EVs remained stable after paraformaldehyde (4%) treatment for up to 2 months. *Summary/conclusion*: PKH67-labelled EVs were detected in tissues of mice 30 minutes post-injection and most readily detected in the liver. This study highlights the utility of cell dyes for in vivo tracking of EVs. Further work is needed to accurately quantify the bio-distribution and the sub-cellular compartmentalization of endothelial-derived EVs.

P8A-289

In vitro characterization of endothelial-derived microparticles stimulated from pathologically relevant cellular insults

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Introduction: Elevated circulating concentrations of endothelial-derived microparticles (EMPs) have been found in patients with cardiometabolic diseases. EMPs have emerged as potential biomarkers for endothelial dysfunction. Causes of elevated EMPs remain unclear. The aim of this study was to assess the effect of pathologically relevant insults on EMP characteristics. *Methods*: EMPs were derived from human vascular endothelial cells (HECV) following a 24-h incubation with either culture medium alone (non-stimulated) or a cellular insult (H_2O_2, 10 mM; hypoxia, 1% O_2; glucose naive; insulin, 2.5 nM; or testosterone, 1 μM). EMP morphology was assessed using confocal microscopy. EMP concentration and size was analysed using nanoparticle tracking analysis. Flow cytometry was used to assess annexin V positivity and surface adhesion molecules on EMPs. *Results*: Cellular treatments did not affect EMP size ($n=4$) (non-stimulated, 134 ± 8 nm; hypoxia, 131 ± 8 nm; H_2O_2, 138 ± 4 nm; testosterone, 112 ± 16 nm; insulin, 168 ± 50 nm; glucose naive, 119 ± 88 nm, $p > 0.05$). Hypoxia, H_2O_2 and glucose-naive treatments induced an increase in EMP production (4.3 ± 1.1^{e4}, 9.2 ± 1.8^{e8}, 6.5 ± 3.8^{e4} MPs/cell, respectively) compared to non-stimulated EMPS (4 ± 4.3^{e4} MPs/cell, $p < 0.0001$). Treatments did not affect cell viability. ICAM-1, VCAM-1, P-selectin, E-selectin and PE-CAM were detectable on all EMPs; however, no difference in expression levels was observed between EMPs. Compared to non-stimulated EMPs ($11 \pm 11\%$), a greater apportion of EMPs derived from insulin ($57 \pm 4\%$), testosterone ($59 \pm 4\%$) and glucose-naive ($64 \pm 5\%$) treatments were annexin V positive ($p < 0.0001$). *Summary/conclusion*: Hypoxia, H_2O_2 and glucose-naive treatments increased EMP production in HECV cells. Cellular stimuli did not affect EMP surface adhesion molecule profiles. EMPs derived from insulin, testosterone and glucose-naive treatments resulted in an increased production of annexin V positive EMPs.

P8A-290

Phenotype of endothelial-derived microparticles in arterial hypertension might depend on pathophysiologic mechanism

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Introduction: Hypertension (HTN) is a leading risk factor for cardiovascular diseases. Endothelial dysfunction and damage is an early event in these complications. Nevertheless, the pathogenesis of HTN is poorly understood. Factors such as increased angiotensin II (AII) activity and aldosterone excess are implicated. We hypothesize that circulating microparticles (MPs) of endothelial origin are elevated in HTN, but their phenotypic characteristics depend on the pathophysiology of HTN. *Methods*: Mice were treated with AII (400 ng/kg/min) via mini-osmotic pumps or nitro-L-arginine methyl ester (L-NAME) 30 mg/kg/day in drinking water for 14 days. Systolic blood pressure (SBP) was measured using tail-cuff manometry. Enumeration and phenotyping of endothelial MPs were determined in platelet poor plasma using imaging flow cytometry and the following surface markers: E-selectin (CD62E), endoglin (CD105), VE cadherin (CD144), annexin 5 (AV). *Results*: Compared to untreated controls ($n=3$) AII-treated mice ($n=4$) had an increase in SBP by 30 mmHg and significantly elevated total counts of AV-negative CD105-positive MPs ($p=0.048$). On the other hand, L-NAME-treated mice ($n=3$) had an elevation of SBP of 15 mmHg on average. Interestingly, total number of MPs, including AV-negative MPs were reduced in L-NAME-treated mice ($n=3$) compared to controls ($n=5$) ($p=0.022$), whereas several different markers for endothelial MPs were elevated in these mice [AV+CD144+ ($p=0.023$), AV+CD62E+ ($p=0.023$), AV+CD105+ (0.023), AV-CD144+ ($p=0.04$)]. *Summary/conclusion*: Different subsets of endothelial-derived MPs are elevated in AII- and L-NAME-treated mice. This might suggest different mechanisms of endothelial response and injury to different forms of HTN. Further studies are needed to confirm these findings and to examine the effects of severity and duration of different forms of HTN on circulating endothelial MPs. Early detection of endothelial injury could lead to early intervention of HTN and could potentially result in reduction in cardiovascular diseases.

P8A-291

The dynamic release of extracellular vesicles from endothelial cells following an inflammatory stimuli

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Introduction: Endothelial cell-derived extra-cellular vesicles (EVs) are released under basal conditions as well as in response to inflammatory stimuli. Their abundance in the circulation is associated with endothelial dysfunction and arterial stiffness. The aims of this study were to characterize the release of EVs over time and measure size distribution following an inflammatory stimulus. *Methods*: Human umbilical endothelial cells (HUVEC) were cultured in exosome-depleted growth medium. Cells were stimulated with TNF-α (1 ng/ml) over a time course of 24 h. EVs were isolated from tissue culture supernatants using differential centrifugation as recommended by ISEV guidelines. The size and concentration of EVs were measured using Nanosight nanoparticle tracking analysis system (LM10) calibrated with 100 and 300 nm silica beads, with camera settings adjusted accordingly. *Results*: EV release from HUVEC over 24 h increased from 4.4×10^8 to 1.7×10^9/ml under basal conditions and from 5×10^8 to 3.8×10^9/ml with TNF-α treatment. There was a significant increase in TNF-α stimulated EV release compared to basal after 1 h; this difference was maintained at 3, 6 and 24 h. The rate of EV release was greatest at 1 h following TNF-α treatment (1.1×10^9/ml). Vesicles from the basal fractions had a modal size distribution of 90–130 nm. However, in TNF-α-stimulated cells, there was significant increase in the proportion of vesicles >200 nm at the later time points of 6 and 24 h. *Summary/conclusion*: This study demonstrates distinct differences in the dynamics of EV release from HUVEC following an inflammatory stimulus. The size distribution was consistent with exosomes, shedding microparticles and larger apoptotic bodies, with 90% or more falling into the exosome size range. These data point to a controlled and orchestrated release of vesicles with inflammatory stimuli and provide the basis for further investigation into the dynamics of EV generation with inflammation.

P8A-292

Exosomes released from endothelial cells are cardioprotective

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Introduction: Cardiac vascular endothelium can communicate with the underlying myocardium and modulate its activity. Exosomes are nanosized vesicles that carry proteins and miRNAs and can transmit protective signals. The aim of this study is to characterize exosomes released from endothelial cells (HUVEC) and determine whether they can protect cardiomyocytes against simulated IR injury. *Methods*: Exosomes were purified from HUVEC using differential centrifugation and characterized by nanoparticle tracking analysis, electron microscopy and flow cytometry. *Results*: Co-culture of cardiomyocytes with normoxic HUVEC cells prior to in vitro simulated IR reduced the percentage of myocyte death from $80 \pm 11\%$ to $51 \pm 4\%$ ($p < 0.05$; $n = 3$), respectively, indicating that a diffusible factor from endothelial cells can confer protection in cardiomyocytes. Pre-incubation of cardiomyocytes with exosomes purified from HUVEC reduced the percentage of cell death after simulated IR from $88 \pm 4\%$ to $55 \pm 3\%$ ($p < 0.05$; $n = 3$). Interestingly, preconditioning of HUVEC cells by exposure to 30 minutes simulated IR stimulated exosome release by ~2-fold. Incubation of cardiomyocytes with exosomes from preconditioned HUVEC prior to in vitro simulated IR further reduced cell death to $45 \pm 10\%$ ($p < 0.05$ vs control; NS vs. normoxic exosomes; $n = 3$). Using fluorescence microscopy of fluorescently labelled HUVEC exosomes and human plasma exosomes, endocytosis was demonstrated to occur into HUVEC cells, but surprisingly, not into primary adult rat cardiomyocytes. However, exosomes from both preconditioned and control HUVEC cells rapidly activated ERK1/2 phosphorylation in cardiomyocytes, which is known to be cardioprotective. *Summary/conclusion*: Our data suggest that exosomes released from endothelial cells can confer resistance to simulated IR in cardiomyocytes. Further work is under way to investigate the mechanisms of protection by endothelial cells.

P8A-293

Potential role of translationally controlled tumour protein in PAH

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Introduction: Pulmonary arterial hypertension (PAH) is characterized by extensive vascular remodelling. The mechanisms mediating the hyper-proliferation of endothelial cells (ECs) and smooth muscle cells (SMCs) in PAH remain unclear. *Methods*: Blood outgrowth endothelial cells (BOECs) were isolated from healthy volunteers and PAH patients. Extracellular vesicles (EVs) (microparticles and exosomes) were isolated by sequential centrifugations and ultracentrifugations, respectively. *Results*: As the result of a proteomic screen in patient-derived endothelial cells, we identified that the anti-apoptotic protein translationally controlled tumour protein (TCTP) and EVs may be implicated. We found that BOECs from PAH patients with mutations in BMPR-II (PAH-BOECs) expressed higher levels of TCTP and released more EVs than control-BOECs. Moreover, TCTP was detected within the EV released from BOECs. We also performed siRNA knockdown of BMPR-II in control-BOECs and found an increase in TCTP expression, indicating that the enhanced TCTP expression observed by the PAH-BOECs is likely to be a result of impaired BMPR-II expression. Interestingly knockdown of BMPR-II also showed a dramatic reduction in miR-27b, a microRNA described to regulate TCTP expression. Similar results were found in PAH patients bearing BMPR-II mutation showing a significant reduction in miR-27b expression compared to control subjects. We also found that loss of TCTP in control-BOECs led to changes in morphology and cytoskeletal organization, increased apoptosis and reduced proliferation. Furthermore, in an animal model of PAH, rats treated with monocrotaline (MCT) demonstrate an increase in lung TCTP from day 2 and dramatically increase after 3 weeks, when pulmonary arterial pressure rises. *Summary/conclusion*: Therefore, we propose that the balance between TCTP production and release into EV from the endothelium could be critical for the prevention of the vascular remodelling.

P8A-294

Number and origin of microparticles are correlated with fibrin clot properties in patients with coronary artery disease

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Introduction: Increased levels of circulating microparticles (MPs) reflect platelet activation and endothelial dysfunction in both stable coronary artery disease (CAD) and acute coronary syndrome (ACS). Although the physiological role of platelet-derived microparticles (PMPs) and endothelial-derived microparticles (EMPs) remains to be established, their procoagulant and fibrinolytic activities have been attributed to them. Endothelial-derived microparticles are carriers of

different endothelial origin coagulation factors, that is, tissue factor (TF), which contribute to the formation of clots. *Objective*: As MPs have coagulation properties, we investigated the contribution of PMPs and EMPs in fibrin clots permeability and resistance to lysis in patients with advanced artery disease. *Methods*: Ten patients with uncomplicated ACS and positive test for myocardial necrosis (high sensitivity cardiac troponin T – hs-TnT) and classified as ST-elevation myocardial infarction (STEMI) admitted within 24 h after the chest pain onset and 10 patients with non-symptomatic stable CAD were enrolled. MPs were collected immediately and prepared using ultracentrifugation method from platelets poor plasma (PPP). MPs were analysed by flow cytometry method. Fibrin clot permeability was determined in vitro in clots formed from citrated plasma samples, activated 20 mmol/L calcium chloride and 1 U/ml human thrombin, and the permeation coefficient (Ks) was calculated. Clot lysis time was determined as the time required for a 50% decrease in clot turbidity (time to half-lysis, $t_{50\%}$) in presence of 1 U/ml human thrombin and 1 μg/ml recombinant tissue plasminogen activator, rtPA. *Results*: Both groups were similar with age and other epidemiologic and biochemical risk factors. There were no differences between median number of total MPs in ACS and CAD groups: 22.2 (12.5–31.5) kMP/ml vs. 15.8 (12.7–18.1) kMP/ml ($p = 0.029$), respectively. Total number of CD42 positive PMPs tends to be higher in ACS patients than in CAD ones: 2.2 (1.1–3.4) kMP/ml vs. 1.1 (0.5–1.3) kMP/ml ($p = 0.08$). Interestingly, endothelial CD31positive MPs were significantly higher in ACS than in CAD patients: 1.7 (0.8–7.2) kMP/ml vs. 0.7 (0.4–1.2) kMp/ml ($p = 0.035$). Median Ks and $t_{50\%}$ value were higher in ACS patients [8.4 (7.9–9.0) dyne/cm^2 and 81.5 (68–89) s] compared to CAD patients [9.3 (8.9–9.6) dyne/cm^2 and 65.5 (62–94) s] ($p = 0.011$ and $p = 0.035$), respectively. In ACS group only total MPs significantly correlated with Ks ($r = -0.64$; $p = 0.044$) and $t_{50\%}$ ($r = 0.67$; $p = 0.033$). The same relationship was observed for PMPs and EMPs. In CAD group, Ks and $t_{50\%}$ correlated significantly with EMPs only: $r = -0.80$; $p = 0.005$ and $r = 0.85$; $p = 0.002$, respectively. Interestingly, there were no relationships between TF-bearing MPs and clot properties in both groups. *Summary/conclusion*: Our study shows that both PMPs and EMPs are the important contributors to the clot properties in patients with advanced cardiovascular disease.

P8A-295

Association between oxidized low-density lipoproteins and circulating microparticles in familial hypercholesterolemia

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Introduction: Microparticles (MPs) are elevated in a variety of pathological conditions, including cardiovascular disease. Familial hypercholesterolemia (FH), particularly when associated with Achilles tendon xanthomas (ATX), predisposes to atherosclerosis. The aim of the present study is to investigate MPs in FH subjects and healthy controls. In addition, the association between oxLDL-C and various MP subpopulations is explored in FH subjects with ATX. *Methods*: Thirty subjects with FH and 24 age- and gender-matched normolipidemic controls were included. FH subjects were selected on the presence or absence of ATX, according to medical records and confirmed by ultrasonographic measurements. Before study entry, FH subjects underwent an 8-week washout period of lipid-lowering medication. Plasma levels of circulating MPs derived from platelets (PMPs), erythrocytes (ErytMPs), endothelial cells (EMPs), and tissue factor-positive cells (TF+ MPs) were measured by a new generation BD FACSAria™ III digital flow cytometer. The research protocol was approved by the local Scientific Ethics Committee, and written informed consent from participants was provided before enrolment. *Results*: Total MP numbers and levels of EMPs and TF+ MPs were all significantly increased in FH subjects, compared to the normolipidemic controls. No differences in plasma levels of ErytMPs or PMPs were observed. The presence of ATX did not affect MP levels. However, the increased plasma levels of oxLDL-C in FH subjects with ATX were strongly associated to plasma levels of EMPs and PMPs. *Summary/conclusion*: Increased plasma levels of total MPs, EMPs and tissue factor-positive MPs may contribute to elevated atherothrombosis risk in patients with FH. The association between oxLDL and levels of EMPs and PMPs in FH subjects with tendon xanthomas may support that increased oxidative stress is involved in release of these MPs.

P8A-296

Serum purified extracellular vesicles from rats trained under different intensities of aerobic exercise show different counting, size and physical properties

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Introduction: Extracellular vesicles (EVs) are heterogeneous sized bilipid vesicles secreted by a large number of cells and tissues first described in the early 80s. They raised a huge increase in interest since 2007, when it was shown for the first time their ability to carry functional genetic information among cells, such as mRNAs and microRNAs, being able to change proteomic profile in the recipient cell. The aim of this work was to characterize EVs purified from the serum of rats trained at different intensities of exercise using tunable resistive pulse sensing (TRPS), dynamic light scattering (DLS) and atomic force microscopy (AFM). *Methods*: Three groups of rats were exercised in a treadmill under different intensities [low intensity (L), moderate intensity (M), high intensity (H)]. Non-exercised group was used as control [non-exercised (NE)]. After the exercise, blood was collected and EVs were purified from 250 μL of serum using Exoquick (System Biosciences). We evaluated the EV diameter and estimated the particle/ml using TRPS (qNANO). Furthermore we analysed turbidity for 595 nm, electrical conductivity, zeta potential, Z-size and polydispersity index by DLS and EV height, volume and surface using AFM in dynamic mode. *Results*: TRPS showed 147.6 nm(NE); 154.5 nm(M); 166.3 nm(H) mode for particle diameter and a concentration of 1.1E+009(NE); 1.2E+009(M) and 1.4E+009(H) particles/ml. Electrical conductivity was 0.087(NE), 0.086(L), 0.046(M) and 0.088(H). Zeta potential measured by DLS showed −18.7(NE), −15(L), −20.5(M), and −176(H). Polydispersity index showed 0.528(NE), 0.699(L), 1(M) and 1(H). Z-average from size particles measured by DLS showed 201.8 nm(NE), 130.5 nm(L), 242.8 nm(M), 245.9 nm(H). AFM curve description size (height × average number of vesicles in nm) showed 45.38 nm(NE), 23.30 nm(L), 11.17 nm(M), and 16.70 nm(H). *Summary/conclusion*: The results from TRPS and DLS for particles size showed an increase in EV size associated to the increase in exercise intensity instead of the results obtained by AFM that showed decrease of EV size associated to the increase of exercise intensity. Sizing, counting and physical characterization are important aspects of EV investigation. However, as shown here the characterization strongly depends on the method used.

P8A-297=OP2-119

Human tears and sweat trigger clotting of blood: the role of tissue factor-exposing vesicles

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Arcadis room
Poster Session 8B - Standardization of EV measurements
Chair: *Jeroen de Vrij and Hinrich P. Hansen* 13:00-14:00

P8B-298

Contribution of FBS-derived RNA in cell cultures and comparison of different FBS depletion protocols

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Introduction: The RNA content of extracellular vesicles (EVs) is an important mediator of cell-to-cell communication. Foetal bovine serum (FBS) is usually supplemented in in vitro system; hence FBS-derived EVs can contaminate the isolated exosomes. They are readily taken up by cells to in recipient cells. Therefore, it is important to eliminate these vesicles. Different ultracentrifugation protocols are available but there is no comparative study available that describe total FBS EVs elimination. The aim of this study was to determine the total EV-associated RNA depletion efficiency of different ultracentrifugation protocols. *Methods*: FBS was either diluted (30%) or further processed undiluted before it was centrifuged for 0, 1.5 or 18 h to deplete EVs. Exosome isolation was then made from media supplemented with the different EV-depleted FBS obtained from the procedure described above. RNA was used to measure the efficiency in removing FBS EVs. Isolation and profiling of exosomal RNA were performed using miRCURY and Bioanalyzer. FBS-derived vesicles/particles were characterized for their structure (electron microscopy), protein markers (western blot) and their ability to protect RNA (RNAase/proteinase K treatment). We also evaluated the biological functionality of FBS-derived exosomes to study the relevance of the FBS exosome depletion step. *Results*: The 18 h centrifugation protocol was most efficient (~98%) in eliminating RNA-containing EVs/particles compared to the 1.5 h centrifugation (~30%). Both protocols that use undiluted and diluted FBS during the depletion step were found to be equally efficient in depleting RNA-containing vesicles. FBS contains vesicles that are positive for exosomal marker, and their RNA cargo is protected from RNAase. FBS-derived RNA containing vesicles induces epithelial mesenchymal transition (EMT) like phenotypic changes in recipient cells. *Summary/conclusion*: It is very important to evaluate the efficiency of FBS EV depletions from the media used in cell cultures and consider their effect on cells, as bovine EVs can be taken up by cultured cells and could contaminate the exosomes preparation from cell culture system.

P8B-299

Pre-analytical parameters affecting exosome research in biomarker discovery

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Introduction: Extracellular vesicle (EV) research needs standardization of sample handling, collection and processing for future diagnostic application. Pre-analytical studies are often contradictory or address microvesicles (MVs) rather than exosomes from complex biofluids. In this study, we evaluated pre-analytical parameters affecting the quantity and typology of exosomal population. *Methods*: Blood was withdrawn from healthy volunteer donors (who signed an informed consent) and processed following strict SOPs to obtain different biological matrices: serum, platelet rich-plasma, platelet-free plasma and plasma in the presence of protease inhibitors (P100). The impact of delayed processing, centrifuge parameters, anticoagulants (EDTA or CTAD), the platelets presence and protease inhibitors on the number of EVs and their distribution was investigated through nanotracking analysis (NTA) and immune-based assays. Since platelet-derived EVs are suggested to account for a large fraction of serum EVs, blood matrices were pre-cleared and analysed to evaluate the expression of common vesicular and cell type-specific biomarkers on EV subpopulations. *Results*: Our study confirms the importance of a pre-clearing step to separate exosomes from microvesicles prior any downstream analysis. Delayed processing of samples increases the expression of platelet-derived markers in exosomal fractions. Anticoagulants and protease inhibitors have low impact on EV number and distribution. Vesicles positive for canonical exosome markers were abundant in serum samples while signal in plasma samples was dependent on platelets contamination. *Summary/conclusion*: Our study provides results relative to pre-analytical factors that impact on the composition of EV and that could affect immune and molecular tests downstream steps following their isolation. Our findings may contribute to the development of unified SOPs for exosome biomarker research and clinical applications.

P8B-300

Microparticle analysis by flow cytometry depends on well-defined pre-analytical conditions

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Introduction: Microparticles (MPs) are small vesicles budded from the membrane of blood cells and endothelial cells. These particles are known to be procoagulant, and increased numbers of MPs are found in various diseases. Currently, there is no standardized method for MP measurement available; therefore, it is not possible to compare MP studies. In the present study the relation between different pre-analytical and analytical methods and the outcome is investigated. *Methods*: The numbers and cellular origin of MPs in citrated blood samples from healthy volunteers were determined with the BD FACSCalibur using standard methods as published. Fluorescein isothiocyanate (FITC) labelled lactadherin (Haematologic Technologies inc.) was used to label phosphatidylserine on MPs. The effects of freezing/thawing and washing on the number of circulating MPs were explored, as well as the effect of different buffers on MP counts. *Results*: Good reproducibility of numbers of MPs in repetitive measurements of frozen plasma samples over several months was achieved. Lactadherin was shown to be suitable for MP identification in a calcium-free environment, allowing MP measurement in whole blood samples. We investigated the number of MPs in fresh and frozen/thawed MP samples, which revealed that freezing leads to a 10-fold increase of the number of MPs compared to fresh material. In addition, washing MP samples leads to either an increase of MP numbers in fresh samples, or a decrease of MP counts in frozen samples. *Summary/conclusion*: In the current study, we show that it is possible to measure MPs in whole-blood. All additional steps to obtain MP samples lead to different MP counts. MPs are preferably measured in fresh samples without additional washing steps, to prevent degradation processes and platelet activation leading to false-positive characterization of MPs. Further studies concerning standardization of instrument settings and to investigate the sensitivity of the flow cytometric measurements with different flow cytometers are undertaken.

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P8B-301

Do conditions of freezing and time-in-freezer really matter?

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Introduction: Extracellular vesicles (EVs) are important biological markers in a number of pathological conditions. Often, EV concentration is altered in disease compared to healthy controls. In large studies it is usually impractical to immediately analyse samples, requiring samples to be stored. Few data exist detailing the effect of freezing on EV size and concentration, yet cells are known to be extremely sensitive to the method of freezing. Moreover several studies have shown freezing causes alterations in EV characteristics such as increasing phosphatidylserine exposure and reduced antibody-based detection of surface antigens. We aimed to quantify the effect of freezing on EV size and concentration hypothesizing that freezing damages EVs, resulting in a decreased overall size but increased concentration. *Methods*: EV samples were isolated from the blood of four healthy volunteers collected in citrate vacutainers. EV samples were then aliquoted and frozen by five different methods. The size and concentration of EVs were analysed fresh, after 1 day, 1 week, 1 month and 3 months of storage by each method using tuneable resistive pulse sensing (TRPS). *Results*: No significant difference in median EV counts was observed when the individual freezing techniques were compared with each other. Overall time of freezing significantly increased EV concentration ($p = 0.014$) when data from the combined methods were analysed. Subsequent analysis using a general linear model further suggested that time was a significant factor in increasing EV count ($p = 0.009$). EV mode size showed a decreasing trend with time of freezing though this was not significant. *Summary/conclusion*: Freezing may cause gradual disintegration of EVs over time regardless of method, accounting for the increased concentration and tendency towards a decreased mode particle size. A larger sample size would strengthen the data and help to confirm the findings.

P8B-302

Pre-analytical treatment of blood samples prior to phenotyping by EV Array

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Introduction: Exosomes are endosome-derived vesicles between 40 and 100 nm in diameter that are secreted by many cell types. The quantity and molecular composition of exosomes shed from various cell types differ considerably. It is therefore expected that plasma will contain a wide range of exosomes with different phenotypes, reflecting the phenotype of the cells that produced them. As blood samples are biologically active and since most Vacuette tubes used for blood drawing contain agents that alter the biological composition in the sample, it is likely that the changed biology will have some effect on the extracellular vesicles in the samples. In order to analyse the extracellular vesicles from blood samples and to be able to compare the results with, for example, the results from other groups, it is essential to know the effect of the pre-analytical treatment. *Methods*: In this study, the pre-analytical treatment of blood samples was investigated. Blood was drawn from five individuals in Vacuette tubes containing either ACD, CPDA, K3 EDTA, Z serum clot activator or lithium heparin. The samples were subsequently exposed to different centrifugation procedures, freeze-thaw cycles or Exoquick purification. The samples were then stored at either RT, 4°C, −20°C, −40°C, −80°C or −160°C for 2 months. The exosome contents of the samples were analysed for 39 different protein surface markers using the EV Array (Jørgensen et al., 2013, *JEV*). *Results*: It was possible to perform a successful EV Array analysis of the exosomal contents of all the blood sample types, but when using some of the tubes an increased background signal was observed, giving a higher signal-to-noise ratio. *Summary/conclusion*: In general, this investigation establishes the fact that the EV Array can analyse all types of blood samples. However, it is important that the pre-analytical treatment is kept constant when comparing samples.

P8B-303=OP2-122

Towards traceable size determination of extracellular vesicles

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P8B-3031

Small particle flow cytometry to quantify MVs in complex biologic samples

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Introduction: The aim of these studies was to reliably quantify the number of MVs in complex biologic samples using the small particle flow cytometer Apogee A50 and fluorescent dyes for MV components. Light scatter properties and staining of specific MV components determined true MVs for quantification. *Methods*: Dyes used in initial studies included PE-conjugated CD9/CD63/CD81 Abs and SYTO12 (nucleic acid stain). Titration studies were performed for CD9/CD63/CD81 and SYTO12 double positive results. Reproducibility studies were performed to determine consistency for day to day and interplate comparisons. Additionally, exosome-associated esterase was detected in ExoQuick isolated MVs using CFDA-SE and a ViiA7 machine. Finally, prostate cancer cell-derived exosomes were stained with CFDA-SE and quantified in the flow cytometer. *Results*: Standard curves for tetraspanin$^+$ and STYO12$^+$ gave average values for normal plasma of 108.5̂6 MV/ml ($R^2 = 0.9957$), benign prostate patient 354.4̂6 MV/ml ($R^2 = 0.9961$), inflammatory prostate disease 348.2̂6 ($R^2 = 0.9955$), HGPIN 296.1̂6 MV/ml ($R^2 = 0.9986$) and prostate cancer 260.1̂6 ($R^2 = 0.9934$). Reproducibility studies showed good day to day reproducibility but differences were noted between aliquots of plasma. There was good correlation for ViiA7 generated CFDA-SE standard curves for 18 samples with CVs between 0.55 and 0.75. Flow studies confirmed CFDA-SE staining of MVs with optimal staining between 5and 25 μM. *Summary/conclusion*: We show here that small particle flow cytometry can be used to reliably quantify MVs in human plasma. Future studies will incorporate additional dyes such as fluorescent lipid-intercalating dyes, labelled lectins and anti-PS Abs. Additionally other platforms will be evaluated and compared with flow cytometry including DLS and qNano. Using this platform additional biomarkers can simultaneously be evaluated on individual MVs.

Arcadis room
Poster Session 8C - EV in body fluids
Chair: *Cecillia Lässer and Emma Guns* 13:00-14:00

P8C-304

Tissue factor bearing microparticles: optimization of flow cytometric detection

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Introduction: Tissue factor microparticles (TF-MPs) are highly procoagulant and likely linked to thrombotic risk. Flow cytometric (FCM) detection of TF-MPs in plasma samples (PFPs) is challenging and complicated by the weak expression of TF antigen and high procoagulant potential. Our aim was to optimize FCM-based detection protocols for TF-MPs. *Methods*: A high-sensitivity flow cytometer (Gallios, Beckman-Coulter) was set-up for a FSC-based threshold of 0.3 μm bead-eq. using Megamix-Plus beads. TF detection on MPs used PE conjugate of a new, high-affinity antibody against the upper, most accessible, part of TF (SBTF1-PE) and phosphatidylserine was probed using either annexin V-FITC or lactadherin-FITC $\pm Ca^{2+}$ in the dilution buffer, respectively. *Results*: False TF-positive events, initially observed in MP-free plasma (MPFP), disappeared when reagents were pre-treated by centrifugation (24,000 g, 5 minutes), as suggested (1). When purified TF-MPs derived from highly TF+ pancreatic tumour cells (bxpc-3) were spiked into MPFP and analysed by FCM after AnnV-FITC staining using Ca^{2+}-binding buffer, MPs were not detected as expected and replaced in the MP gate by new non-fluorescent events whose apparent size was related to the level of spiking. At the highest levels of TF-MPs, clot formation was clearly visible in the tube in <5 minutes, impeding FCM analysis. Thus, despite final 1:10 dilution of pure plasma, the use of Ca^{2+} binding buffer can induce short-time clotting in TF-MP-rich PFPs. For lower amounts of TF-MPs, delayed clotting time allowed FCM but micro-aggregates were visible in dual scatter plots, suggesting micro-clots formation. Addition of direct thrombin inhibitors (DTIs) such as hirudin or dabigatran avoided this artefact of premature coagulation. Alternatively, PS staining using lactadherin-FITC with Ca^{2+}-free buffer was also effective. *Summary/conclusion*: The following tips may optimize detection of TF-MPs in PFPs: (a) use optimal anti-TF PE-MAb conjugates, (b) remove aggregates in reagents by quick-spin centrifugation, and (c) avoid micro-clots by blocking thrombin in AnnV-stained PFPs. Since this artefact, falsing MP counts, remains most often visibly undetected and since TF-MP content in PFPs is a priori unknown, we recommend the systematic addition of a DTI.

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P8C-305

Profiling of urinary proteases and protease inhibitors associated with extracellular vesicles in patients with diabetic nephropathy

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Introduction: Urinary extracellular vesicles (UEVs) have attracted increasing research attention as found to be a precious source of diagnostic and prognostic disease biomarkers. We have recently devised a new and simple method to enrich vesicles from urine and used it to detect the variety of proteases and protease inhibitors associated with UEVs in patients with type 2 diabetic nephropathy. *Methods*: Urine samples from 16 healthy volunteers were collected among the laboratory staff and 36 representative patient samples from the Finnish Diabetic Nephropathy (FinnDiane) Study Group and divided into three groups based on the level of albuminuria. Isolated UEVs were screened on a nitrocellulose membrane blot array to detect simultaneously the relative changes of 34 different proteases and 32 protease inhibitors, respectively. Protease and protease inhibitor profiles and quantitation were established from the pixel average of fluorescent density changes of spots using an infrared Odyssey scanner and plotted with its image analysis software. Quantitations with more than 1.5-fold difference were considered. *Results*: Arrays showed a progressive increase of cathepsin-C, -D, and -X/Z/P in the samples from patients with macroalbuminuria while no appreciable changes were observed in the array for kallikreins. Further, the array showed a moderate altered expression of metalloproteases with a progressive decrease of MMP-2 and bimodal trend for MMP-9 which increased in the normoalbuminuric cohort while a decrease in the micro- and macroalbuminuric groups were observed. No major variations were observed for the set of tissue inhibitors for metalloproteases (TIMPs). On the other hand, substantial variation was found for the cystatins. *Summary/conclusion*: This study shows for the first time characteristic alterations in protease and protease inhibitor profiles associated with UEVs in DN. These results suggest that the underlying mechanisms may reveal important mechanistic, prognostic, and diagnostic features in advancing kidney damage.

P8C-306

RNA extraction from urinary extracellular vesicles isolated by hydrostatic dialysis

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Introduction: Urinary Extracellular vesicles (UEVs) carry proteins and nucleic acids, which are characteristic for the cell of origin located in the kidney and urogenital tract. The presence of small RNAs, in particular, in UEVs has gained interest for their importance as disease biomarkers. This highlights urine as ideal biofluid also to discover biomarkers of early kidney damage and to combat the increasing number of people suffering of chronic kidney diseases. Although urine can be easily obtained in large quantities, UEV enrichment from large cohorts of patients is a challenging task. The differential centrifugation method is considered as the ''gold'' standard, but depending on the size of the rotor available and sample volumes, forces applied to sediment vesicles may vary in each run leading to unsatisfactory recovery of UEVs. Aim of this study was to investigate if novel hydrostatic dialysis-based method of UEV enrichment yields high-quality RNA from samples. *Methods*: Our team has recently presented a hydrostatic dialysis-based method for vesicle isolation. Briefly, urine is centrifuged on low speed to remove debris (cells, bacteria) and poured into a funnel connected with 1,000 kDa molecular weight cutoff dialysis membrane. After primary sample concentration, 200 ml of milliQ water is poured into the funnel to force removal of remaining components with molecular weight below 1,000 kDa. This approach concentrates and dialyses the sample with a one-step protocol. Here we compared RNA isolation from the fraction above 1,000 kDa and a pellet 200,000 g coming from ''gold'' standard technique using three different methods of RNA extraction.

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TRIzol method has been used according to the Chomczynski and Sacchi protocol. To obtain large and small RNA fractions separately, combined miRNeasy Micro and Mini Kit have been used according to Qiagen protocol. Urine Exosome RNA Isolation Kit from Norgen Biotek has been presented as a specific method for UEVs and was used as the third method here. RNA quality was checked in Nanodrop Spectrophotometer ND-1000 and Agilent Bioanalyzer capillary electrophoresis. *Results*: Concentrations and quality of samples were analysed with particular focus on OD value from Nanodrop usually not reported in publications. Our results showed that introduction of hydrostatic dialysis method allows to: (a) avoid carry-over of the problematic yellow urinary pigments into RNA sample; (b) value of OD 260/280 around 2.0 for RNA coming from large volumes of urine (~250 ml) suggesting high purity of RNA; and (3) comparable profile in Agilent Bioanalyzer from all three extraction techniques. High-quality material can be used for further analysis like qPCR with minimized risk of carrying over artefacts coming from extraction steps. *Summary/conclusion*: Hydrostatic dialysis protocol yields concentrated sample and avoids interference of pigments and soluble protein and thus favours extraction of high-quality RNA.

P8C-308

Exploration of microRNAs in porcine milk exosomes

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Introduction: Breast milk contains complex nutrients and facilitates the maturation of various biological systems in infants. Exosomes, membranous vesicles of endocytic origin found in different body fluids, can mediate intercellular communication. We hypothesized that microRNAs (miRNAs), a class of non-coding small RNAs of 18–25 nt which are known to be packaged in exosomes of human, bovine and porcine milk, may play important roles in the development of piglets. *Methods*: In this study, exosomes of approximately 100 nm in diameter were isolated from porcine milk through serial centrifugation and ultracentrifugation procedures. Total RNA was extracted from exosomes, and 5S ribosomal RNA was found to be the major RNA component. Solexa sequencing was used to explore miRNAs. *Results*: Solexa sequencing showed a total of 491 miRNAs, including176 known miRNAs and 315 novel mature miRNAs (representing 366 pre-miRNAs), which were distributed among 30 clusters and 35 families. Interestingly, we observed three miRNAs (ssc-let-7e, ssc-miR-27a and ssc-miR-30a) that could be generated from miRNA-offset RNAs (moRNAs). The top 10 miRNAs accounted for 74.5% (67,154 counts) of total counts, which were predicted to target 2,333 genes by RNAhybrid software. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses using DAVID bioinformatics resources indicated that the identified miRNAs targeted genes enriched in transcription, immunity and metabolism processes, and 14 of the top 20 miRNAs possibly participate in regulation of the IgA immune network. *Summary/conclusion*: Our findings suggest that porcine milk exosomes contain a large number of miRNAs, which potentially play an important role in information transfer from sow milk to piglets. The predicted miRNAs of porcine milk exosomes in this study provide a basis for future biochemical and biophysical function studies.

P8C-309=OP3-128

Release of microvesicles during haemapheresis correlates with surface roughness of adsorbent polymers

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P8C-310

Mesenchymal stem cell exosomes: from isolation to long time preservation

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Introduction: Mesenchymal stem cells (MSCs) are known to be an attractive tool in tissue engineering and cell therapy. Paracrine effects of these stem cells are more and more studied and could be used for disease treatments. Extracellular vesicles are secreted by cells into extracellular space under physiological or stress conditions. This study focuses on extracellular vesicles with nanometric scale (30–100 nm) named exosomes. The aim of this work is to evaluate by nanosizer and flow cytometry the appropriate conditions to store and preserve MSC exosomes. *Methods*: MSC culture is achieved in an exosome-free medium to eliminate exogenous nanovesicles. At passage 4, cells are serum starved during 48 h, and then conditioned medium (CM) is harvested. To isolate exosomes from CM, three steps are performed: ultracentrifugation, filtration (0.1 μm) and centrifugation on sucrose cushion. Exosome pellets are suspended in PBS and stored at room temperature (RT), 4°C, −20°C and −80°C from 24 h to 6 months. Nanoparticle size distribution is assessed by Nanosight, which allows high-resolution and real-time analysis. The presence of three exosome characteristic markers, CD9, CD63 and CD81, is evaluated by flow cytometry. *Results*: Samples analysed by Nanosight after isolation show a high concentration in nanoparticles with over 70% of particles into exosomes scale. Storage of suspended pellets at RT and 4°C over 5 days results in a loss of particles. Moreover flow cytometry dot plots show a positive staining for the three markers with CD9 expressed three times less than CD63 and CD81. At 4°C and RT the staining for the three markers is less important than fresh samples. Exosomes stored at −80°C seem to show consistent results with the results obtained for exosome pellets without storage. *Summary/conclusion*: Our preliminary results show that integrity of nanovesicles may be maintained. Temperature and time play an important role to preserve exosomes. Potential of MSC exosomes in therapeutics has been demonstrated; this is why long period storage would be helpful for further process, particularly in the field of regenerative medicine in order to use defined cellular products and not cells for therapy.

P8C-3110

Exosomal research: isolation and characterization of exosomes from human sera of normal and clinical subjects

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Introduction: The number of studies concerning bioactive vesicles has significantly increased in recent years. The newest family members of "bioactive vesicles" that function to promote intercellular communication are exosomes and microvesicles. These vesicles are probable nanodevices for biomarker discovery. With the potential of being an important biomarker, exosomal research

has opened the paths for analysis of its proteome and transcriptome. To test whether these bioactive vesicles could be used for targeted cancer therapy, we have conducted in vitro cytotoxicity-based evaluation of 12 endangered, pharmacologically important plant species on human cancer lines of diverse origin. Besides, we have evaluated the exosomes isolated from human sera to serve as biomarker for clinical purpose. *Methods*: Alcoholic and aqueous extracts from different plant parts of 12 pharmacologically important plants from Jammu region of India were prepared, and in vitro cytotoxicity assays were performed on human cancer cell lines from ovary, breast, colon, cervix, liver, lung, prostate and central nervous system. Exosomes were extracted from human sera from normal subjects as well as patients with different clinical states, that is, diabetes, hypertension, hypothyroidism, asthma, etc. Exosomes obtained by using Total Exosome Isolation Kit (Invitrogen) were spectrophotometrically analysed for protein and RNA confirming their presence. *Results*: According to their cytotoxicity against a number of human cancer cell lines, plant extracts from *Apium graveolens, Holarrhena antidysenterica, Mallotus philippensis* and *Nardostachys jatamansi* were fractionated and assayed. All showed anti-cancer activity but the ethanolic extract of *Mallotus philippensis* was found to be more pronounced. Analysis of proteins and RNA confirmed their presence in exosomes isolated from human serum samples obtained from normal subjects and patients. Protein samples from each such clinical and normal samples were subjected to SDS-PAGE. In all cases, two bands of M_r 22 K and 47 K appeared to be prominent in clinical samples, whereas polypeptide with Mr 60–66 K was conspicuous by its absence in clinical samples. *Summary/conclusion*: Molecular masses of exosomal proteins in clinical samples appeared to be different from those in normal samples suggesting clinical implication thereof as biomarker(s). Clinically significant results will be presented. Further, results obtained on in vitro cytotoxicity-based evaluation of 12 endangered, pharmacologically important plant species on human cancer lines of diverse origin suggest that bioactive vesicles could play an important role in targeted cancer therapy.

P8C-3111

Characterization of RNA from extracellular vesicles isolated by different methods

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Introduction: Extracellular vesicles (EVs), including exosomes, are a rich source of high-quality RNAs, released by all cells and present in all biofluids. Many current protocols to isolate EVs use ultracentrifugation; however, development of faster, more convenient methods with high specificity for vesicles and good RNA quality is an area of active research. Here, we present a characterization of EV RNA isolated by several different methods. *Methods*: Human plasma and serum EVs were isolated by ultracentrifugation or a novel spin column-based method, utilizing membrane affinity binding. The RNA was compared between isolation methods and to RNA extracted from direct lysis of plasma. The EVs were examined with electron microscopy and the extracted RNA was subjected to electrophoresis, qPCR and next-generation sequencing (NGS). *Results*: EVs contain both small and large RNAs – although the presence of large ribosomal RNAs (rRNA) can be masked in electrophoretic methods such as Bioanalyzer assays. The vast majority of rRNA and mRNA from plasma are found within vesicles. Both ultracentrifugation and affinity membrane isolation appear to extract nearly 100% of the large RNA from the samples. In contrast, some miRNAs are also found in the sample flow-through of the affinity membrane isolation, representing a population of miRNAs previously shown to occur in ''free'' circulating Ago2-complexes. The membrane of the EVs protects the RNA from degradation, allowing for stability of vesicle-derived RNA in plasma for several days at room temperature and multiple freeze/thaw cycles. The affinity membrane column was used to isolate plasma EV RNA from healthy and cancer patients for analysis by qPCR arrays and NGS, demonstrating the utility of exosomal RNA as a source of blood-based biomarkers. *Summary/conclusion*: Serum and plasma contain cell-free RNA, including miRNA, mRNA and rRNA, in quantity and quality sufficient to allow analysis by Bioanalyzer, qPCR and NGS. Both ultracentrifugation and affinity membrane isolation are efficient in isolating the large RNAs from human serum and plasma samples, and membrane affinity isolation appears to be selective for miRNAs contained in vesicles.

P8C-3112

Circulating miRNAs in human plasma

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Introduction: Circulating miRNAs can be isolated from a variety of biofluids and are the subject of growing interest for biomarker discovery in a wide range of diseases. It has previously been shown that miRNAs in human plasma exist either as ''free'' miRNAs bound to protein complexes (Ago2) or are contained in extracellular vesicles (EVs). These sources of plasma miRNA are generated by different biological processes and the ability to distinguish the 2 populations may aid in creating meaningful biomarker profiles. *Methods*: We characterized the ''free'' and EV-bound population of miRNAs with qPCR arrays, differentially isolated by membrane affinity binding. *Results*: While direct lysis of a whole plasma sample extracts both populations of miRNA, vesicle-bound miRNAs can be isolated by ultracentrifugation and binding to an affinity membrane. The Ago2-bound population of miRNAs is sensitive to RNase degradation after Proteinase K treatment of whole plasma while the vesicle-bound fraction remains protected by a lipid bilayer. The vesicle bound miRNAs on the other hand are susceptible to RNase digestion only when the vesicle membrane is disturbed with detergents. Here, we use qPCR arrays to establish the specific miRNomes present in both fractions. *Summary/conclusion*: Serum and plasma contains 2 populations of miRNAs, one inside vesicles, susceptible to RNase degradation after disturbing the membrane with detergents, and another in free protein complexes, susceptible to degradation following treatment with proteinase K. Membrane affinity isolation selectively withholds the membrane vesicles and leaves Ago2-bound miRNAs in the flow-through. The vesicle-derived miRNA profile is very different from the ''free'' miRNA profile, indicating the benefit of differentiating between the 2 populations when searching for defined miRNA biomarker profiles.

P8C-3113

RNA profiling of exosomes derived from the human body fluids

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Introduction: Exosomes are nanovesicles containing unique RNA and protein cargo, secreted by all cell types in culture and also found in abundance in body fluids, including blood, saliva, urine, CSF and breast milk. At the moment, the mechanism of exosome formation, the makeup of the cargo, biological pathways and resulting functions are incompletely understood. One of their most intriguing roles is intercellular communication – exosomes function as the messengers, delivering various effector or signalling macromolecules between specific cells. There is an exponentially growing need to dissect structure and the function of exosomes and utilize them for development of next-generation diagnostics and therapeutics.

Methods: Here we report novel exosome isolation strategies (from serum, urine, other body fluids) and characterization of exosomal RNA cargo using qRT-PCR (with TaqMan assays) and deep sequencing techniques (PGM and Proton platforms). *Results*: Analysis of the RNA content of exosomes derived from the body fluids and cell culture media revealed extremely diverse population of miRNA, mRNA, rRNA, piRNA, snoRNA, tRNA and other short ncRNA sequences (while very low levels of DNA were detected). Overall, exosomal cargo reflects the RNA content of the parental cell and thus is extremely valuable for diagnostics development. However, a number of RNA targets show significant differences in levels between the exosome and parental samples, thus opening a possibility of their utilization as positive and negative markers. The reason for these differences is not fully understood and will provide useful information regarding sorting of particular RNA sequences into exosomes. The data on exosome count and size distribution for various body fluids will be presented as well. *Summary/conclusion*: The work presented here is another step towards developing standardized techniques and protocols for isolation of exosomes and downstream analysis of their constituents. In a similar fashion, disease-specific RNA signatures residing within the exosomes can be discovered and used for development of superior, sensitive and minimally invasive diagnostic alternative to biopsies.

P8C-3114

Staining of microvesicles with lipid intercalating fluorescent dyes inhibited by excess plasma proteins

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Introduction: Previously we showed that HSA blocks binding of exosomes to the functionalized lipid moiety [1,2-dihexadeconyl-sn-glcero-3-phosphoethanolamine-N-(cap biotinyl)] on microbeads. The aims of these current studies were to determine whether a similar phenomenon occurs with exosome staining with fluorescent lipid intercalating dyes such as DiI (1,1′-dilinoleyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate), which has a similar structure to the lipid moiety used previously. *Methods*: In order to assess DiI binding to exosomes, small particle flow cytometry was performed using the Apogee A50 machine on exosomes isolated from prostate cancer cell culture and dilutions of patient plasma ranging from 1:60 to 1:600. In addition, we double stained patient plasma samples with anti-tetraspanin-FITC-conjugated antibodies and DiI and analysed by the Apogee. *Results*: Similar to previously noted plasma protein inhibition of lipid moiety binding to MVs, we show here that increasing plasma proteins decrease DiI staining. Specifically, staining was detected at plasma dilutions of 1:600 and 1:300 but was undetectable at higher concentrations of plasma. Double staining of plasma-derived MVs showed linear correlation to volume of plasma with R^2 values of 0.9987 and 0.8422 in a cancer patient plasma and normal plasma, respectively. Quantification of MVs in the cancer plasma was 241.5̂6/ml and only 18.6̂6/ml for the normal plasma. *Summary/conclusion*: These studies show that DiI dye is able to reliably stain exosomes but that hydrophobic plasma proteins can compete with/inhibit binding and reduce staining detected by the flow cytometer. Future studies should consider the amount of hydrophobic proteins present in samples when attempting to stain exosomes with fluorescent lipid intercalating dyes in complex biologic samples.

Arcadis room
Poster Session 9A - Isolation of EV
Chair: *Anita Böing and Dionne Tannetta* 13:00-14:00

P9A-312

Refining the gold standard of extracellular vesicle isolation: how to get more vesicles out of your sample

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Introduction: The most readily applied method to isolate extracellular vesicles is to perform differential ultracentrifugation, which generally requires an abundance of starting material and, as it often gives low yields, its efficiency can be questioned. The aim of this study was therefore to determine the efficiency of a 70-minute ultracentrifugation and to evaluate if the vesicular yield could be improved by performing longer centrifugations without losing purity. *Methods*: The final vesicle pelleting centrifugation was extended from the normal 70 minutes run to 155 minutes, 4 h 11 h and 37 h in an attempt to improve on vesicular yield. The RNA and protein quantity of isolates was established as a guideline for vesicle yield. Electron microscopy, western blot and flow cytometry were used to confirm the presence of exosomes by probing for the markers CD9, CD63, CD81 and TSG101. *Results*: An increase in centrifugation duration directly correlates to an increase in protein and RNA yields, with the ratio between the 2 suggesting a more pronounced increase of protein in comparison to RNA with extended centrifugation time. The presence of vesicles in the isolates after all centrifugations was confirmed by electron microscopy, western blot and flow cytometry collectively. Electron microscopy also verified the presence of significant amounts of vesicles in the supernatant remaining after a 70-minute centrifugation. *Summary/conclusion*: A 70-minute isolation, which is a common isolation duration, is insufficient to isolate a majority of vesicles present in a sample. With longer centrifugations, more proteins are however pelleted, although an approximately 2-fold extended run can be performed without any significant accumulation of proteins vs. RNA.

P9A-313

A comparison of vesicular isolation between traditional ultracentrifugation and a commercially available isolation kit

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Introduction: Recently, there has been an expansion of commercially available means for the isolation of extracellular vesicles. We have determined the yield of vesicular RNA isolation by a commercially available ''exosome-kit'' compared to traditional ultracentrifugation in terms of isolation efficiency. *Methods*: Isolations were performed both by differential ultracentrifugation and with Total Exosome Isolation Kit, a commercially available kit, offered by Invitrogen. Isolations with the kit were performed with 2 different procedures, one following the kit instructions and one modified to include ultracentrifugation steps. RNA and protein yields were measured and used as an indication of extracellular vesicular yield. The RNA profiles of isolates were established with a Bioanalyzer and used as a quality control for isolated vesicles. A second round of isolation using the kit was also applied to the supernatant of both kit and ultracentrifugation isolations, to see if more material could be attained with a second round of isolation. *Results*: The commercially available kit manages to isolate both RNA and protein at considerably higher quantities than if differential ultracentrifugation was applied. Furthermore, the general RNA profile (Bioanalyzer) is similar between the modified kit isolation and traditional ultracentrifugation, indicating that both methods isolate similar RNA-containing components. A second isolation round, using the kit, performed on the supernatant of a first round also revealed that the kit manages to almost entirely deplete the sample of material, while a conventional ultracentrifugation leaves much RNA containing material in the supernatant. *Summary/conclusion*: The kit isolates higher yields of RNA and protein than traditional ultracentrifugation, with similar RNA profiles between the two (if a slightly modified protocol is used with the kit). However, it remains to be established whether or not the same type of vesicles represent these samples.

P9A-314

Exosomes isolation: non-equilibrium zonal centrifugation vs. differential centrifugation. Advantages and perspectives

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Introduction: Differential centrifugation is going to be one of the most widely used methods for exosomes isolation, even though it is labour intensive and in most cases require large amount of source material. In the present work, we illustrate the theoretical consideration of microparticle sedimentation process with the results of the analysis of morphology, size distribution and amount of the objects sedimented at different centrifugation steps. *Methods*: HT29 cell culture supernatant was subjected to centrifugation at 500 g for 5 minutes, 2,000 g for 10 minutes, 10,000 g for 30 minutes and 100,000 g for 1 h. Measurements of particle size and concentration were done by NTA. TEM was used to determine the morphology of the particles. *Results*: According to our data, the essential part of the population of 70–100 nm spheric particles is sedimented at 10,000 g and such a loss is obviously to increase in case of using smaller tubes or rotors with smaller k-factors. On the contrary, the increase of rotor k-factor or of the particle path length (tube volume) leads to contamination of exosomes with population of bigger particles. To demonstrate the fact that exosomes themselves can be partly sedimented at 10,000 g, the population of exosomes non-contaminated with bigger particles was subjected to centrifugation at 10,000 g for 30 minutes. The analysis revealed the presence of identical particles in both the pellet and supernatant. To increase the yield and the purity of exosome population, we eliminated the 10,000 g centrifugation step and used non-equilibrium zonal centrifugation in sucrose. *Summary/conclusion*: Differential centrifugation fails to well separate the objects having similar sedimentation velocities as exosomes and microvesicles. We demonstrated that in accordance with theoretical predictions, the essential part of the population of 70–100 nm particles is sedimented at 10,000 g. We are discussing the possibility of increasing the yield and the purity of exosome population by using non-equilibrium zonal centrifugation.

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P9A-315

A novel isolation strategy for obtaining crude membrane vesicles from bovine skim milk

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Introduction: Bovine milks content of phospholipid membranes have largely been explored in the cream fraction, and known as the milk fat globule membrane that surrounds fat droplets. In skim milk, the population of phospholipid membranes is reported to constitute membrane vesicles with a soluble content known as exosomes and microvesicles. These vesicles contain various types of RNAs and proteins, suggested to transfer health-promoting messages from mother to offspring. However, the variety of the vesicles in milk is less understood and, additionally, complicated by the complexity of more pronounced milk components. Here we present a novel strategy for a short, gentle and non-denaturing isolation of skim milk-derived membrane vesicles. *Methods*: Untreated fresh bovine milk was defatted to remove milk fat globules. The resulting skim milk was subjected to ultracentrifugation. The resulting ochre-coloured soluble fraction above the casein pellet were further isolated from casein remnants by size-exclusion chromatography. Isolated membrane vesicles were investigated by electron microscopy, sucrose density centrifugation, western blotting and particle size analysis. *Results*: A crude phospholipid membrane fraction can be obtained from skim milk by ultracentrifugation. Casein micelle remnants as well as smaller protein components in the crude vesicle fraction can be successfully removed by size chromatography. Electron microscopy of the vesicle isolate reveals circular structures with membrane vesicle character. Sucrose density profiles of the isolate shows an enrichment of the membrane vesicle marker MFG-E8 at density 1.04 g/cm^3, but also fractions at higher (up to 1.2 g/cm^3) as well as lower density (down to 1.03 g/cm^3) exhibits significant MFG-E8 levels. Particle sizes in the range 50–300 nm is observed all over the gradient. The variety of the membrane vesicles is currently being investigated further by several means. *Summary/conclusion*: A new procedure for easy and gentle isolation of bovine milk membrane vesicles encompassing ultracentrifugation and size-exclusion chromatography has been established. The resulting vesicle isolate exhibits the general membrane vesicle characteristics and provides an appropriate start material from which the variety of milk vesicles can be investigated.

P9A-316

Characterization of extracellular vesicles isolated by size exclusion chromatography

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Introduction: In a search to decipher the role extracellular vesicles (EVs) play on pathological processes, novel methods for its detection and isolation are of paramount importance. Recently, at the ISEV meeting in Budapest, Rienk Nieuwland inferred that size exclusion chromatography (SEC) holds promise as a simple and efficient method for the isolation of plasma vesicles. Therefore, the aim of our study is to adopt a multifactorial platform to validate the potential of SEC to isolate pure plasma EVs in clinical samples. *Methods*: Platelet-free plasma (PFP) from 5 healthy donors were applied to a Sepharose CL-2B column and separated by size exclusion chromography. The eluated fractions (F) were then analysed by: (a) nanoparticle tracking analysis (NTA) for total vesicle concentration, (b) quantitative mass spectroscopy followed by functional annotation of the identified proteins, (c) extracellular vesicle (EV) array for capturing and phenotyping, (d) transmission electron microscopy (TEM) for morphology and (e) silver staining of SDS-PAGE gels, lipoprotein levels, protein and RNA to estimate purity. *Results*: We report that for fractions 7–10, a high particle count with a concomitant drop in protein and lipoprotein concentration is noted. Conversely, for fractions 17–25, we observe a 50% reduction in particle count by means of NTA with an increased total protein concentration. In addition, SEC-purified EVs retained RNA concentration and morphology. Furthermore, TEM demonstrated that SEC holds the potential of removing lipoprotein particles from plasma. EV array indicated that pooled fractions 17–25 exhibited higher EV content than fractions 7–10, although phenotypical details remained unchanged compared to control plasma. Mass spectroscopy revealed elevated apolipoprotein levels in the fractions 7–10 and augumented levels of EVs in fractions 17–20. *Summary/conclusion*: Our results accentuate that this method holds potential in the research field of EVs. Interestingly, our EV array and MS data suggests that a prerequisite for preserving the authenticity of NTA data is SEC purification of plasma samples, as it allows for removal of lipoproteins.

P9A-317

Biomechanical effects on microvesicle and nanovesicle production during isolation from blood

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Introduction: Micro and nanovesicles (MNVs), which are found in isolates from blood, are partly produced ex vivo, during and after blood sampling. Understanding the processes that underlay this production is important for interpretation of results derived from isolates as well as for understanding the processes that take place in vivo. In this work, we hypothesized that the concentration of MNVs in isolates reflects the shear stress energy dissipation during the sampling and centrifugation processes. This hypothesis was supported by a mathematical model and validated by experiments. *Methods*: In theoretical part, energy dissipation in the blood flow through the needle was calculated by a mathematical model based on the Poiseuille-Hagen law. It was considered that during centrifugation of blood at relatively low speed, the platelets move towards the bottom of the eprouvette and that those which reside in the top layer (subject to further processing) are the main source of MNVs in isolates. In experimental part, blood was collected from healthy human subjects in 2.7 ml tubes containing anticoagulant; MNVs were isolated by repetitive centrifugation and washing of samples and counted by flow cytometry. *Results*: It was found that the concentration of MNVs correlates with parameters that reflect shear stress (shear velocity at the needle wall, energy dissipation). Larger pressure difference that drives blood flow through the needle resulted in statistically significant larger concentration of MNVs in isolates. It was found that stronger centrifugation field correlates with lower concentration of MNVs in isolates. It was found that higher temperature during sample processing yielded lower concentrations of MNVs in the isolates. *Summary/conclusion*: Biophysical properties of blood plasma and blood cells are important in the determination of concentration and size of MNVs in isolates. The concentration is higher if blood is subjected to higher shear stresses during sampling and isolation.

P9A-318

Developing methods for isolating exosomes from human plasma for proteomics analysis

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Introduction: Isolating sufficient quantity and quality of exosomes from complex biological fluids, such as plasma, is a major challenge in exosome-based proteomics research. Removal of high-abundance non-exosomal proteins, such as human serum albumin (HSA), and co-isolating proteins is of paramount importance as these proteins may mask proteins of genuine exosomal association. Estimating sample purity has also been problematic. Our aim was to develop methods for generating highly pure plasma-derived exosomes and assessing the purity of the sample. *Methods*: Using a combination of qualitative and quantitative assessment, we have tested several approaches for isolation, including Optiprep™ gradients, gel filtration chromatography, dialysis, tangential flow filtration (TFF), pelleting and combinations of these. Human plasma [pre-cleared of platelets, 0.22 μm filtered, frozen (−80°C)] from healthy donors has been utilized as an exosome source material, and purity assessment methods have included ELISA-like assays for tetraspanins and HSA, nanoparticle tracking and total protein. *Results*: The isolation of exosomes from plasma using simple ultracentrifugation, TFF, dialysis, Optiprep™ gradients or combinations of these has been insufficient in removing the majority of plasma proteins and do not elevate purity, as measured by particle/protein ratio. The use of single-step Sepharose CL-2B size exclusion columns has however lead to the removal of >95% (n = 3) of protein and was effective in separating extracellular vesicle-associated tetraspanins from HSA. The particle/protein ratios of the samples also increased to 18–fold, suggesting increased vesicle purity. *Summary/conclusion*: The Sepharose CL-2B column approach is helpful as a first step for eliminating most of the contaminating plasma protein. Future work will involve combining column chromatography with other techniques such as Optiprep™ gradients. We are therefore continuing to refine our method to achieve a consistent protocol for isolating high purity exosomes from plasma for proteomics analysis.

P9A-319

Characterization of proteomic and RNA profiles of extracellular vesicles and non-vesicular secreted material

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Introduction: Characterization of extracellular vesicle (EV) contents is crucial for explaining EV-mediated biological effects. Thus, the used purification method must enable separation of vesicles from other non-vesicular secreted material as impurities could mislead attempts to define vesicle contents. Recently, we exploited an ultrafiltration and size exclusion liquid chromatography (UF-LC)-based strategy for 5–10 times more efficient isolation of pure vesicles compared to ultracentrifugation (UC) methods. Here we used UF-LC for fractionating conditioned medium (CM) to analyse the secretome on proteome and RNA level. *Methods*: For UC, CM was pelleted at 10^5 g. For UF-LC, CM was concentrated (10 or 100 kDa MWCO filters; Millipore) and fractionated (Sephacryl S-400 HR column; GE Healthcare). Proteome was analysed using LC-MS/MS. RNA was analysed using Bioanalyzer (Agilent), microRNA arrays and RT-qPCR. *Results*: Over 2,000 proteins were identified in EVs derived from N2a cells. Interestingly, translation-related proteins were highly abundant while miRNA processing/transport proteins were absent or of low abundance in EVs. Furthermore, the vesicle, protein and RNA distribution within LC fractions did not overlap. Bioanalyzer and qPCR data revealed considerable differences in vesicular and non-vesicular secreted material. While long RNAs were readily found in the vesicular fraction, the main miRNA peak did not coincide with the main vesicle peak, in line with proteomics findings indicating that the vast majority of miRNAs are non-vesicular. This was further supported by miRNA array analysis of MSC-derived EVs as an extra wash of the UC pellet dramatically reduced the number of detected miRNAs. *Summary/conclusion*: This study emphasizes the importance of a stringent EV purification protocol for the analysis of secreted protein and RNA material as highly abundant non-vesicular secreted proteins and RNA could easily distort the view on which molecules are associated with EVs and which not.

P9A-320

Large-scale isolation and characterization of bovine milk- and colostrum-derived exosomes

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Introduction: Exosomes present in bovine and human milk have been shown to harbour immune-modulating miRNAs. Exosomes have also been suggested as potential drug carriers. This spurred us to look into the untapped value of bovine milk and colostrum as source of exosome production. Here, we describe isolation and characterization of the milk- and colostrum-derived exosomes. *Methods*: Exosomes were isolated from raw bovine mature milk and colostrum by differential centrifugations at 4°C at 20,000 × g (30 minutes), 100,000 × g (100 minutes) and 120,000 × g (120 minutes). The particle size was measured by NanoSight and confirmed by SEM. Buoyant density of the milk- and colostrum-derived particles were determined by sucrose density gradient centrifugations. Exosome surface proteins were analysed by western blot, and RT-PCR was performed to identify immune related miRNA and mRNAs in exosomes. *Results*: The milk provided high yields of exosomes (>300 mg exosomal protein/100 ml) with 30–100 nm in size. The colostrum resulted in significantly higher yield (1.5- to 2-fold) than the milk. The milk-derived particles had lower buoyant density than the colostrum exosomes. The presence of select exosomal protein markers confirmed that the microvesicles isolated were indeed exosomes. Both the milk and colostrum exosomes carried vital surface proteins, with substantially higher levels of CD63 in colostrum exosomes. Immune-related miRNAs and proteins were significantly higher in the colostrum versus the milk exosomes. *Summary/conclusion*: Bovine mature milk and colostrum may serve as an economically viable source for producing large quantity of exosomes. The milk exosomes may serve as a scalable source in drug delivery due to its abundance and bio-distribution (Gupta et al. ISEV, 2014; Aqil et al. ISEV, 2014). The presence of higher levels of immune factors makes colostrum as a potential immune booster in infants and immune-compromised individuals.

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P9A-321

A novel synthetic peptide-based tool for microvesicle/exosome isolation: clinical applications

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Introduction: We have engineered and validated a synthetic peptide (Vn96*), which has specific affinity for canonical heat shock proteins

(HSPs), as a tool for the rapid isolation of exosomes or extracellular microvesicles (together referred to herein as eMVs) from cell culture media, human and animal body fluids. Most of the available methods of eMV isolation are based on physical characteristics (density and/or size separations) or multiple antibody affinity-based purification, which are not compatible with platform versatile high-throughput down-stream clinical applications. Given the presence of increased surface expression of HSPs on eMVs from pathological cells (e.g. cancers, acute ischemic stroke, allergy, diabetes, hypertension, infection, coronary artery disease), the discovery and validation of the Vn96 peptide as a tool to enrich ''pathological'' eMVs may have the potential for immediate and future diagnostic applications. Furthermore, the peptide chemistry for adaptation to various clinical assay platforms is well understood, robust and cost-effective. *Methods*: The eMV fractions isolated from conditioned media, plasma and urine using Vn96 peptide were compared to those obtained by ultracentrifugation or using a commercial purification reagent. These validation analyses were performed using nanoparticle tracking analysis, transmission electron microscopy, atomic force microscopy, immunoblotting, next-generation sequencing of miRNA cargo and proteome-based cellular component ontology analysis. *Results*: Our preliminary clinical data resulting from analyses of post-digital rectal examination urinary Vn96-captured eMVs from prostate cancer patients and healthy controls (with informed consent and medical ethics committee approval) demonstrated that we can detect and measure (via qPCR) a number of miRNAs previously identified by others in plasma and urine sediments where their expression was shown to be up-regulated in prostate cancer patients compared to healthy individuals. Similarly, mRNA analyses of Vn96-captured urinary eMVs demonstrated the presence of several known prostate cancer-associated markers (e.g. *PSA*, *PCA-3*, *FOLH1*, *PSMA*, *SPINK1*, *PSCA*, *CD24*, *GOLM1*, *ANXA3*, *TMPRSS2-ERG* fusion, *SLC45A3*). *PCA-3*/*PSA* mRNA ratios measured using Vn96 eMV RNA yielded improved specificity and accuracy for detecting prostate cancer compared to ratios measured using standard urine sedimentary procedures. *Summary/conclusion*: Together, our results suggest that Vn96-mediated capture of eMVs can improve disease diagnosis in the clinical setting.

*Patent pending.

Arcadis room
Poster Session 10C - Late Breaking
Chair: *Edit Buzás and Kenneth Witwer* 13:00-14:00

P10C-3210

Identifying unique proteins following immunoisolation of exosomes derived from the epididymis, prostate and seminal vesicle

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Introduction: Extracellular vesicles found in seminal fluid are produced by the various tissues that form the male reproductive tract. A few studies have shown that each region or tissue type releases exosomes containing a unique packet of information including proteins, mRNA and miRNA, that are acquired by the sperm as they pass through and are critical for maturation and egg fertilization. *Methods*: Based on published protein profiles of vesicles collected from each region (post-surgical), we identified key surface proteins specific to three regions of the tract: epididymis, prostate and seminal vesicle. Using antibody-labelled magnetic beads, we isolated vesicles from seminal fluid measured their relative concentration by flow cytometry and analyzed protein content by Orbitrap LC/MS. We are also developing a method to standardize exosome concentration before isolation using Nanosight Tracking Analysis. *Results*: Preliminary results indicate that the antibodies are isolating different amounts material from a single sample and that each population contains both shared and unique proteins. We have also confirmed the presence of vesicles in our isolations using transmission electron microscopy. *Summary/conclusion*: Magnetic bead immunoisolation is an effective tool for separating vesicle populations in any bodily fluid. By examining each population independently we can hopefully uncover novel proteins, especially in vesicle populations that are present in low concentration. This information may be critical in our understanding of male infertility and help us to establish new biomarkers to improve patient treatments and pregnancy outcome.

P10C-3211

Comparative analysis of proteomics data of plasma-derived exosomes

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Introduction: Exosomes are nanovesicles of 30–100 nm that can be produced by a plethora of cell types, are found in the circulation and are implicated in many processes of intercellular communication. Following up the characterization and description of the immunoprotective properties of reticulocyte-derived exosomes (*Rex*) in the murine malaria model of BALB/c mice infected with *Plasmodium yoelii* 17XNL, our present objective is the molecular characterization of *Rex* from human infections by *Plasmodium vivax*. However, (1) the obtention of reticulocyte-enriched preparations from patients is still limiting and (2) the state-of-the-art on proteomics analyses of reticulocyte-derived exosomes is still lacking more robust data, even though exosomes were first described in reticulocytes over 25 years ago. Therefore, we have started a comparative proteomic analysis of plasma-derived exosomes, isolated by different methodologies, to serve as a baseline for the future characterization of reticulocyte-derived exosomes from infections and the determination of the contribution of these vesicles to the overall population of plasma-derived exosomes. *Methods*: Platelet-free plasma was collected from healthy donors (n=3) and processed for exosome isolation by ExoSpin™ or sepharose size exclusion. The preparations were submitted to MS/MS in an orbitrap mass spectrometer, and protein identification was done with the Sequest algorithm. The resulting proteins from both preparations were compared among each other as well as to proteomic datasets from the literature and ExoCarta. *Results*: The meta-analysis of the combined proteomic data on plasma-derived exosomes shows a high variability among preparations in terms of (1) shared proteins, (2) presence of classical exosome markers and (3) possible contamination by apoptotic bodies. Also, the ubiquitous presence of the most abundant plasma proteins, irrespective of isolation methodology employed, may suggest that carry over of soluble proteins located outside the vesicles is a common feature of exosome isolation from plasma samples. *Summary/conclusion*: Altogether, these results present a comprehensive comparative proteomics analysis of plasma-derived exosomes and raise a few caution flags on the need to optimize processing and isolation of exosomes from plasma.

P10C-3212

Approaching the impact of an endurance race on extracellular vesicles purified from horse plasma

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Introduction: Extracellular vesicles (EVs) are heterogeneous-sized bilipid vesicles, first described in the early 80s. A large number of cells and tissues secrete EVs. The interest over EVs has increased since 2007, when their ability to carry functional genetic information among cells was reported. Here we describe our approach in investigating how endurance affects EVs in the plasma of horses and the population of miRNAs in EVs. *Methods*: Plasma samples were collected from 10 Arabian horses engaged in a 160 km race. Blood samples were collected via jugular venipuncture: the night before the race (T0), at the second veterinarian gate (66 km) (T1), at the end of the race (T2), 2 hours after the race (T3) and 15 hours after the race (T4). Extracellular vesicles were precipitated from 500 μL plasma using Exoquick (System Biosciences), and the pellet was resuspended in 200 μL of PBS 1X. Total protein quantification was performed, and vesicles were characterized using dynamic light scattering (DLS). Small RNAs were purified from 150 μL EVs using miRCury-Biofluids, following the instructions. Then, the purified small RNAs were characterized and quantified using Agilent Bioanalyzer – a small RNA chip. RNAseq libraries were constructed starting from 10 to 50 ng using TruSeq® small RNA sample preparation. *Results*: DLS showed two populations of vesicles after purification with Exoquick. T0 and T1 showed 97% of mass vesicles with 8.5 nm of radiusand 3% of mass vesicles with 60 nm of radius, respectively. T2, T3 and T4 showed 99.9% of mass vesicles with 53 nm radius in average. Total protein measurement of 5 μL of EVs ranges from 3.26 mg/mL to 4.84 mg/mL in all samples. The small RNA quantification of EVs ranges from 17 to 167 ng, with T3 showing a high concentration of small

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RNA. After the ligation of 5′ and 3′ adaptors, reverse transcription, PCR, gel purification and Bioanalyzer quantification, peaks of 147 nt and 157 nt were hardly seen. Library quantification using qPCR generates a final concentration of 0.5 nM and 0.8 nM. These libraries are, at the time of the submission, being sequenced on MySeq. *Summary/conclusion*: Based on size determination using DLS, Exoquick possibly purified HDL and exosomes from plasma as two main population of vesicles. RNAs smaller than 1,000 nt were purified from EVs in all animals and were characterized by Bioanalyzer. T3 shows statistically more amount of RNA compared with T0, T1, T2 and T4. Using low amount of small RNA as input to construct libraries for sequencing was a challenge. Further we will focus on identification of miRNAs and other regulatory RNAs that circulate in plasma extracellular microvesicles at different time points during extraneous endurance races.

P10C-3213

Erythrocyte-derived microvesicles: inhibition of Ca^{2+} channel activity to determine the implication of cytosolic Ca^{2+} levels in their release

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Introduction: Erythrocyte-derived microvesicles (eMVs) are naturally occurring entities resulting from biochemical and morphological changes to the plasma membrane in response to increased levels of intracellular calcium (Ca^{2+}). Circulatory Ca^{2+} levels are normally kept low to maintain normal cell function; however, stimulation and increased levels can be encountered during microcirculation affecting erythrocyte structure and stability, resulting in eMV release. This study investigates the effect on eMV production when pre-treating erythrocytes with different Ca^{2+} channel inhibitors. *Methods*: Erythrocytes were pre-treated with varying concentrations of Ca^{2+} channel inhibitors, and eMVs released were analysed by qNano and Guava easyCyte™ flow cytometry. *Results*: Higher concentrations of gadolinium chloride, a Ca^{2+} channel inhibitor, inhibit Ca^{2+} channel activity, completely halting eMVs release. p-Value of 0.03 suggests that inhibitor concentration significantly effects eMVs produced. *Summary/conclusion*: This preliminary work indicates that Ca^{2+} influx mediated by stretch-activated Ca^{2+} channels may be a major contributor to eMV release. Subsequent experimental studies with other Ca^{2+} channel inhibitors, nifedipine and mifebradil, are in progress to evaluate other channels. Investigating the role of Ca^{2+} channels in eMVs release may elucidate the fundamental mechanisms involved in their generation fully, to understand their functional role in the circulatory system of individuals in health and disease.

P10C-3214

Comparison of serum-derived exosome extraction methods and quantitative application in prostate cancer patients

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Introduction: Exosomes are emerging as a source of biomarkers with putative prognostic and diagnostic value. However, little is known about the efficiency, reproducibility and reliability of the protocols routinely used to quantify exosomes in human serum. *Methods*: We used increasing amounts of the same serum sample to isolate exosomes using two different methods: ultracentrifugation onto a sucrose cushion and ExoQuick™. Quantitative analysis of serum-derived exosomes was performed by determining protein concentration (BCA assay) and number of nanoparticles (Nanosight™ technology). Exosomes quality was assessed by Coomassie staining and western blotting for CD9, LAMP2 and Grp94 exosomal markers. Then quantity of serum-derived exosomes from prostate cancer patients (androgen sensitive, $n = 20$; castration-resistant, $n = 20$) was assessed using ExoQuick method. *Results*: Correlation between serum volume and number of isolated exosomes is significant for both methods when exosomes are quantified using protein concentration. However, when the number of nanoparticles is used to quantify exosomes, ExoQuick is the only reproducible and efficient method. CD9, LAMP2 and Grp94 exosomal markers are equivalently expressed in both methods. However, exosomes isolated using ultracentrifuge method are strongly contaminated with Albumin and IgG. Quantitative analyses on prostate cancer patients indicate that hormone-sensitive patients have higher amounts of serum-derived exosomes than castration-resistant patients ($p < 0.05$). *Summary/conclusion*: Exoquick™ is an efficient and reproducible method to isolate exosomes for quantitative studies, whereas ultracentrifugation is not. Moreover, high albumin contamination of ultracentrifugation-derived exosomes impairs the use of protein concentration as a mean to quantify serum-derived exosomes when ultracentrifuge is used to isolate exosomes.

P10C-3215=OP3-134

Proteomic profiling of outer membrane vesicles derived from uropathogenic *Escherichia coli* strain 536

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P10C-3216

Toward a multifaceted high throughput screen for exosome uptake pathways

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Introduction: Exosomes are small (30–100 nm) extracellular vesicles associated with processes including cellular communication, immunotolerance and stimulation, programmed cell death, coagulation, chemotaxis and anti-tumor homeostasis. These small vesicles have gained mounting attention as they not only stand to define a new paradigm of cellular and tissue regulation, but also entice researchers with a broad range of novel strategies for therapeutic intervention. Exploiting exosomes as drug delivery vehicles however, still demands for a greater understanding of their biology, in particular, of their interaction with recipient cells. *Methods*: Our work attempts to develop an unbiased multifaceted screen for exosome uptake modulators. By combining genetic, small molecule and secretome screening we aim to systematically dissect cofactors, receptors and uptake pathways that exosomes utilize for interacting with and entering into target cells. *Results*: Here we will describe the development of a high throughput exosome uptake assay, technical issues as well as initial results. *Summary/conclusion*: We anticipate that this work will contribute to a more systematic dissection of routes in exosome recipient cell interaction and thereby the general understanding of fundamental exosome biology as well as their application to novel therapies.

Scientific Program 2014 ISEV meeting
Saturday May 3rd, 2014
Oral Presentations

Registration 8:00-9:00

Willem Burger room
Symposium Session 10A - Late Breaking
Chair: *Douglas Taylor and Michiel Pegtel* 9:00-10:15

O10A-363

Regulation of exosomal export of miRNA by Human ELAV protein HuR in mammalian macrophage cells

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Introduction: Human ELAV protein HuR by binding the AU-rich elements (ARE) stabilizes mRNAs and inhibits miRNA action on target messages. HuR polymerizes on the target RNA and replaces miRNP bound to that mRNA. The fate of HuR-replaced miRNPs from target mRNA is not clear. In animal cells, miRNAs can be exported out of the cell via different mechanisms and exosomal export of miRNA is the key mechanism for intercellular transfer of miRNAs. *Methods*: We used mammalian macrophage cells and stimulated them with LPS to observe the changes in exosomal miRNA content. We have tested effects of HuR depletion or supplementation on exosomal export of miRNA by scoring miRNA content of secreted exosomes released from activated macrophage cells. *Results*: Here we have identified that the miRNPs replaced by HuR is destabilized in activated mammalian macrophage cells. We have identified that HuR promotes exosomal export of miRNAs by relocalizing miRNPs from P-bodies and promoting its accumulation in multivesicular bodies (MVBs), organelles that matures to exosomes in animal macrophages. *Summary/conclusion*: We have identified HuR as the primary factor required for exosomal export of miRNA in activated macrophage cells and observed that HuR-driven exosomal export of miRNA is essential for pro-inflammatory response.

O10A-364

Monocyte inflammatory responses to peripheral blood extracellular vesicles require scavenger receptors

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Introduction: Addition of extracellular vesicles (EVs) from RBC units to PBMCs induces a dominant pro-inflammatory cytokine response. We recently reported that monocytes are the main subpopulation of PBMCs that bind to EVs and are activated by the exosome (<220 nm) fraction. Here we present results examining the cell of origin of exosomes and microvesicles (MVs; >220 nm). The monocyte receptors required for exosome binding and monocyte activation were also explored. *Methods*: Differential centrifugation was used for purification of EVs from peripheral blood of five healthy donors. EVs were stained with PKH26 membrane dye, or for some experiments were separated by filtration into exosome and MV fractions. Exosomes and MVs were coated on 6 μm beads, blocked with bovine serum albumin, and stained with conjugated antibodies for RBC (CD235a), T cell (CD3), B cell (CD19), NK cell (CD16), monocyte (CD14), granulocyte (CD66b), endothelial cell (CD142), and platelet (CD41a) markers. To determine monocyte–EV receptor interactions, EVs were incubated with 0.01–1.0 μg annexin-V or anti-phosphatidylserine antibody for 30 min and were cultured with monocytes. Alternatively, monocytes were incubated with 0.01–1.0 μg anti-CD36, anti-CD163 or anti-CD206 antibodies for 30 min, and stained EVs were added to the cultures. Monocyte–EV binding and characterization of cell surface markers was measured by flow cytometry. Intracellular cytokine staining for TNF-α was performed on monocytes stimulated with exosomes or MVs. Multiplex assay was used to measure the level of pro-inflammatory cytokines in monocyte supernatants. *Results*: Exosome and MV fractions derived from blood were positive for the granulocyte marker (52.4 and 50.6% respectively, $p < 0.01$), and MV fractions were positive for the platelet marker (20.0%, $p < 0.01$). The percentage of other markers was less than 3% in both fractions of freshly isolated EVs. Monocyte–EV binding was reduced upon pre-incubation of EVs with recombinant annexin-V ($p < 0.05$) or anti-phosphatidylserine-neutralizing antibody ($p < 0.001$). Pre-incubation of monocytes with anti-CD36- or anti-CD163-neutralizing antibodies blocked monocyte–EV binding in a dose-dependent manner ($p < 0.001$). A neutralizing antibody against the scavenger receptor CD206 did not reduce monocyte–EV binding. The exosome fraction of EVs induced a stronger TNF-α response in purified monocytes compared to the MV fraction ($p < 0.05$). Exposure of monocytes to exosomes also led to the release of IL-1β, IL-6, IL-8, IL-12p70 ($p < 0.0001$), and GM-CSF ($p < 0.05$) into the culture supernatant. *Summary/conclusion*: Our results indicate that in peripheral blood, granulocytes give rise to both exosomes and MVs, while platelets significantly contribute to the MV but not exosome population. These EVs bind to monocytes through defined scavenger receptors and exert inflammatory effects on monocytes. Understanding how EVs interact with monocytes is the first step to harnessing these immunological mediators for therapeutic applications.

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O10A-365

Tumour-secreted microRNA-214 promotes immune evasion and tumour growth by inducing CD4 + CD25 + Foxp3+ regulatory T cells

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Introduction: An increased population of CD4 + CD25 + Foxp3+ regulatory T cells (Tregs) in the tumour-associated microenvironment plays an important role in cancer immune evasion. However, the underlying mechanism remains poorly understood. *Methods*: Cell culture and mouse model. *Results*: We observed increased expression and secretion of miR-214 in various types of human cancers and mouse tumour models. Tumour-secreted miR-214 was sufficiently delivered into recipient T cells by microvesicles (MVs) in vitro. In targeted mouse peripheral CD4+ T cells, tumour-derived miR-214 efficiently down-regulated phosphatase and tensin homolog (PTEN) and promoted Treg expansion. The miR-214-induced Tregs secreted higher levels of IL-10 and promoted tumour growth in nude mice. Furthermore, in vivo studies indicated that Treg expansion mediated by cancer cell-secreted miR-214 resulted in enhanced immune suppression and tumour implantation/growth. The MV delivery of anti-miR-214 antisense oligonucleotides (ASOs) into mice blocked Treg expansion and tumour growth. *Summary/conclusion*: Our study reveals a novel mechanism through which cancer promotes Treg expansion and immune suppression.

O10A-366

The extracellular RNA complement of *Escherichia coli* and its immunostimulatory properties

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Introduction: While growing in either a natural ecosystem or artificial conditions, bacteria secrete intracellular products into their extracellular milieu. Secretory products released from bacteria are not only involved in bacterial social behaviour, usually referred to as quorum sensing, but also in pathogenicity and inter-kingdom communication. Bacteria disseminate secreted products through their secretory systems via continuous or discontinuous passages across the bacterial membrane and by releasing outer membrane vesicles. Secreted biomolecules play important roles in microbe–microbe and host–microbe interactions. *Methods*: Using a combination of physical characterization and high-throughput sequencing, we have analysed the extracellular RNA complement of the enteric model bacterium *Escherichia coli* both within its growth medium and within its outer membrane vesicles, and have assessed the immunostimulatory properties of extracellular RNA in human primary macrophages. *Results*: Our results demonstrate that the majority of selectively exported RNAs are in the size range between 15 and 40 nucleotides derived from a pool of intracellular RNAs. A significant portion of the secreted RNAs is composed of specific cleavage products of functionally important structural non-coding RNAs, including tRNAs, 4.5S RNA, 6S RNA and tmRNA. In addition, the secreted RNA pool includes transcripts from cryptic prophages, intergenic and coding regions. We also demonstrate that the released bacterial RNA pool has immunostimulatory effects on human immune cells by up-regulating several cytokines, especially TNF-a, IL-6, IL-10 and CCL20. In particular, the release of TNF-α is significantly higher for the extracellular RNA complement than for intracellular RNA. *Summary/conclusion*: Our results highlight possible modulatory and/or regulatory roles for secreted bacterial RNAs in host–microbe interactions.

O10A-367

Extracellular vesicles from the choroid plexus mediate blood–brain communication during sepsis

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Introduction: Little is known about how the periphery communicates with the central nervous system (CNS) during systemic inflammation. The choroid plexus epithelium (CPE) is a unique single layer of epithelial cells situated at the interface of the blood and the cerebrospinal fluid (CSF), forming the blood–CSF barrier (BCSFB). In recent years, the BCSFB has gained increasing attention, especially its role in inflammatory and age-related diseases. The CPE is uniquely positioned to sense inflammation in the periphery and to signal to the brain via the CSF. *Methods*: We made use of the endotoxemia model (IP injection of lipopolysaccharide) to mimic systemic inflammation in BL6 mice and isolated CSF, CP and brain tissue at different time-points for further analysis (IHC, qPCR, western blot, etc). We performed miRNA and mRNA profiling, making use of the NanoString platform. Both TEM and 3D SEM were used to visualize the CP tissue. Additionally, we cultured primary CPE cells and studied them in vitro. Similarly, primary mixed cortical cultures were used. Intracerebroventricular injections were performed using a stereotaxic frame, and extracellular vesicles were stained with the membrane label PKH26. *Results*: Here, we identified CPE-derived extracellular vesicles (EVs), mainly exosomes, as a new mechanism of blood–CNS communication during systemic inflammation. Systemic inflammation induces a rapid decrease in CPE miRNA expression that is inversely correlated with increased levels of EVs and miRNAs in the CSF, secreted from multivesicular bodies (MVBs). The CPE-derived EVs cross the ependymal layer and are taken up by astrocytes and microglia, inducing miRNA target repression and inflammatory gene up-regulation. This way, CPE cells can sense and transmit signals about the peripheral inflammatory status to the CNS via the release of miRNA-containing EVs into the CSF, which transfer this pro-inflammatory message to recipient brain cells. *Summary/conclusion*: In conclusion, we identified an important role for CPE-derived EVs as a new mechanism of blood–CNS communication during systemic inflammation by transferring a pro-inflammatory message to the brain.

Jurriaanse room
Symposium Session 10B - Late Breaking
Chair: *TBA and TBA* 9:00-10:15

O10B-368

Dendritic cell-derived extracellular vesicles recruit mesenchymal stem/stromal cells

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Introduction: Immune cells, which migrate to injury locations, secrete mediators potentially involved in recruitment of other cell populations. Recent work from our team shows that dendritic cells (DCs) as well as NK cells and macrophages are able to recruit mesenchymal stem/stromal cells (MSCs) via paracrine action. Interestingly, immune cells, along with other cell populations, secrete abundant extracellular vesicles (EVs), including exosomes, which are reported to display specificity in their cellular targets and believed to act locally or systemically. Thus, this work aimed to explore the potential of DC-derived EVs for recruitment of MSCs. *Methods*: Primary human monocyte-derived DCs were obtained from healthy blood donors and MSCs from bone marrow samples. MSCs were characterized to follow the international society for stem cells criteria. Cells were maintained with 1% ultracentrifuged FBS for 3 days for EV production. EVs were collected by differential (ultra)-centrifugation (2,000 $\times$ g, 10,000 $\times$ g and 100,000 $\times$ g). Protein content was quantified and EVs were characterized by flow cytometry, upon coupling to latex beads, western blot (WB) and transmission electron microscopy (TEM). EVs were either labelled and added to MSC cultures, or placed in the bottom compartment of a transwell system, for migration assays, through gelatin-coated membranes (8 mm pores), which were analyzed after 8 h. Cells were fixed, non-invading cells removed, and nuclei stained with DAPI. Number of migrating cells was counted under a fluorescence microscope. *Results*: EVs displayed the exosome characteristic cup-like morphology by TEM and were positive for HLA-DR and CD63, while negative for calnexin, as analyzed by a combination of flow cytometry and WB. DC-derived EVs were readily internalized by MSC, to a greater extent than MSC-derived EVs. Internalization of DC-derived EVs was detectable 1 h after incubation and levels increased with incubation time. DC-derived EVs were also found to be able to recruit MSCs in a concentration-dependent manner, with significant cell recruitment being observed for 10 μg of EV protein. *Summary/conclusion*: In conclusion, using a primary human cell-derived system we show that EV produced by immune cells can be internalized by MSC and promote their recruitment, even in the absence of the EV-producing cell, which has important implications for the development of new tissue regeneration strategies.

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O10B-369

Exosomes in intercellular communication within the epidermal-melanin unit

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Introduction: In the thin outermost layer of the skin, the epidermal-melanin unit corresponds to the association of melanocytes with surrounding epidermal keratinocytes. Melanocytes are specialized in the production of the pigment melanin, which is synthesized within lysosome-related organelle called melanosomes, and then transferred to the keratinocytes to photoprotect the skin against the ionizing radiation. Keratinocytes can communicate with melanocytes via the release of soluble factors that participate in the regulation of the pigmentation, in particular after UVB exposure. We raised the hypothesis that keratinocytes could influence pigmentation through the release of extracellular vesicles (EVs). *Methods*: We have isolated and characterized EVs, of normal human keratinocytes (NHK) from different donors (Caucasian, Caucasian after UVB irradiation and Black) and incubated them with melanocytes in monoculture or in reconstructed epidermis. We have quantified the influence of EVs on pigmentation by measuring the melanin content and the tyrosinase activity of melanocytes. We have also investigated the expression of some pigmentation genes with qPCR. *Results*: We could observe that a co-culture of NHK and melanocytes induce a redistribution of MVBs of NHK close to the area of interaction of both cell types. Characterization of EVs revealed an endosomal origin and the labelling allows the observation of the interaction with melanocytes. Exosomes secreted by Black NHK or by Caucasian NHK stimulated by UVB and applied to melanocytes increased the pigmentation genes expression and the tyrosinase activity, resulting in enhanced melanin synthesis. *Summary/conclusion*: Our studies reveal an unexpected physiological role for exosomes in human pigmentation and open a new avenue to regulate pigmentation in normal and diseased states.

O10B-370

Release of pro-atherogenic microparticles from human macrophages stimulated by oxidized LDL uptake in vitro

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Introduction: Cardiovascular diseases, including atherosclerosis, are now the leading cause of mortality and morbidity in the west. Microparticles (MPs) are small ($<$1 micron) vesicles released from the plasma membrane of many activated or damaged cells by blebbing of the cell membrane. MPs display characteristic markers of the cells from which they are released, and may carry genetic material (mRNA, miRNA) or proteins to distant cells and tissues. Thus, they are thought to be involved in disease progression. Increased numbers of MPs have been measured in the blood of patients with atherosclerosis. The role of lipoprotein uptake on macrophage MP formation and their effects on the endothelium is not known. The aim of this study was (i) to measure MPs during differentiation of

THP-1 macrophages, (ii) determine whether uptake of lipoproteins increases MP release from THP-1 macrophages and (iii) determine how macrophage MPs influence endothelial oxidative stress and pro-inflammatory gene expression in vitro. *Methods*: THP-1 monocytes were differentiated using a standard protocol using PMA. Lipoproteins (VLDL, LDL, HDL) were isolated from human plasma and oxidized using a standard protocol. MPs were identified by annexin V staining and flow cytometry. MPs were purified by centrifugation at 17,000 × g and incubated with human umbilical vein endothelial cells (HUVEC). Reactive oxygen species (ROS) was measured using dihydrorhodamine 1,2,3 fluorescence. MCP-1 and IL-8 mRNA were measured using GeXP quantitative multiplex RT-PCR. MCP-1 secretion was measured by ELISA *Results*: Differentiation of THP-1 macrophages induced release of MPs into the cell culture supernatant, and this was further increased by incubation with native or oxidized LDL. MP from LDL- or ox-LDL treated macrophages induced ROS formation and expression of MCP-1 and IL-8 in HUVEC after 24 h incubation. *Summary/conclusion*: Uptake of oxidized LDL by human macrophages induces release of MPs that are capable of inducing pro-atherogenic signalling and gene expression in endothelial cells, suggesting that they could contribute to atheroma progression. Additional studies using MP from primary cultures of human monocyte-derived macrophages and purified from patients with hypercholesterolemia and atherosclerosis are necessary to further explore the function of MP in vascular inflammation and endothelial dysfunction.

O10B-371

Extracellular vesicles as protective vehicles for transmission of viral hepatitis

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Introduction: Hepatitis viruses have a tremendous global health impact, and spread of infection occurs at a high rate and via different routes of administration. For hepatitis C virus (HCV), blood–blood contact such as blood transfusion and needle sharing during intravenous drug use are well established routes of transmission. Infection occurring in situations of poor sanitation and overcrowding via a faeco–oral route of transmission is well established for hepatitis A virus (HAV) and hepatitis E virus but has not been established for HCV. The aim was to examine the possibility of HCV faecal transmission by release in bile and protection of virus in biliary exosomes. *Methods*: Human bile from gallbladders obtained from chronic HCV-positive and -negative patients were collected at the time of liver transplantation. Bile exosomes were isolated by serial ultracentrifugation steps. Huh7.5.1 cells harbouring JFH-1-derived infectious HCV virus were used as control. RNA from bile and exosomes were analyzed for HCV genomic RNA by real-time RT-PCR. *Results*: Electronmicroscopy showed the presence of exosomes, with a size ranging from 40–150 nm, derived from bile and Huh7.5.1 cells harbouring HCV infectious virus. Incubation of naive cells with these HCV positive exosomes resulted in a productive infection. Recent evidence for HAV indicates that this virus can be encapsulated in exosome-like vesicles, providing protection against the toxic environment of bile. For HCV, we found that virus in bile is enriched in the exosome-like vesicle fraction. Vesicles appear to provide protection of HCV to bile salt toxicity. *Summary/conclusion*: In conclusion, like for HAV our results indicate a potential for faecal transmission of HCV by release in bile in protective exosome-like vesicles.

O10B-372

TBA

van Weelde/Mees room
Symposium Session 10C - Late Breaking
Chair: *Dwijendra Gupta and Susanne Gabrielsson* 9:00-10:15

O10C-373

Mitochondria and EV secretion

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Introduction: Extracellular vesicles (EVs) regulate cell-to-cell communication due to their ability to modulate at a distance the properties of receptor cells. However, how their biogenesis is integrated into the cellular metabolic context is poorly understood. We are currently addressing the role of mitochondria during biogenesis and secretion of EVs. *Methods*: To address this, we have generated mitochondrial DNA (mtDNA)-depleted human T cells. We have analyzed the organization of the electron transport chain complexes in control and mtDNA-depleted cells by native blue gel, and by confocal microscopy we have studied the organization of mitochondria. The function of mitochondria has been assessed by simultaneously quantifying mitochondrial respiration and glycolysis in real time by using the Seahorse Extracellular Flux Analyser. We have isolated the EVs released by control and mtDNA-depleted cells by serial ultracentrifugation. Quantification of EVs has been performed by nanoparticle tracking analysis, western blot and flow cytometry. *Results*: mtDNA-depleted cells present decreased ROS production, decreased oxygen consumption and diminished mitochondrial ATP-derived synthesis. However, these cells retain the viability and the energetic content as compared to control cells, as assessed by overall ATP content and phosphorylation of AMPK, a metabolic sensor of the energy status of the cell. Surprisingly, we found that EV secretion is decreased in mtDNA-depleted cells. *Summary/conclusion*: These data suggest a novel link between mitochondria and EV secretion.

O10C-374

Characterization of the full RNA repertoire in exosomes by high-throughput sequencing

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Introduction: Exosomes contain a variety of different RNAs. Previously, RNAs in exosomes were profiled by microarrays and qRT-PCR, which are biased for certain sequences. Few high-throughput sequencing studies of the small RNA fraction of exosomes have been published, and most of these have focused only on the small RNA fraction. Here, we present strategies for full exosome transcriptome discovery using high-throughput sequencing. *Methods*: We used Illumina RNA-Seq to characterize the RNA in exosomes. We isolated exosomes by differential ultracentrifugation from the K562 leukaemia cell line. We then extracted total RNA and separated into fractions of different size. We prepared libraries from fractions of different sizes from exosomes and the corresponding K562 donor cells. *Results*: We report protein-coding transcripts, microRNAs and several other classes of non-coding RNAs present in the exosome fraction, including non-coding RNAs that were not previously reported in exosomes. Using these data, we present comparisons of RNA expression between the cellular and exosome RNA contents for different transcript types and discuss considerations in interpreting these data. *Summary/conclusion*: High-throughput sequencing allows for the characterization of the total transcriptome without bias for certain types of transcripts. We used RNA-Seq to characterize the full RNA repertoire in K562 cells and their exosomes.

O10C-375

Transcriptomic, proteomic and lipidomic analyses of two subpopulations of nanosized exosome-like vesicles

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Introduction: A variety of vesicles can be released by cells into its surrounding environment. These are usually classified into subgroups, such as exosomes, microvesicles and apoptotic bodies. The aim of this study was to further evaluate the possible presence of subpopulations within the subgroup of exosomes. *Methods*: Exosomes were analysed from the human mast cell line, HMC-1. Cell, debris and larger vesicles were eliminated by a 300 $\times$g and a 16,500 $\times$g centrifugation. The samples were divided in two before alternative ultracentrifugation, filtration and density gradient separations, performed in alternative orders. Different pellets were evident after these different separations, and the vesicles in the different pellets were (a) visualized by electron microscopy, (b) analysed for protein content (LC-MS/MS), (c) profiled for RNA cargo (Bioanalyzer and miRNA and mRNA content using Toray array) and (d) analyzed for lipid content (mass spectrometry). The two subpopulations were termed E1 and E2. *Results*: Two distinct nanosized, exosome-like subpopulations of vesicles could be identified. Both populations expressed common ''exosome-markers'' such as tetraspanins, annexins and rab proteins. However, the E1vesicles were $\sim$50 nm in size and had a higher density and contained primarily short RNAs, with no evidence of rRNA peaks. The E2 vesicles, by contrast, were $\sim$90 nm in size, had lower density and contained both small RNAs and prominent rRNA peaks. The protein content in the two subpopulations of vesicles was substantially different. The E1 vesicles contained more ceramide lipids, while the E2 vesicles contained more sphingomyelin. Furthermore, the miRNA and mRNA contents of the vesicle populations were dissimilar, and only the E2 mRNA correlated with the cellular mRNA whereas the mRNA of E1 vesicles did not. *Summary/conclusion*: The existence of distinct subpopulations of exosomes was demonstrated, highlighting the diversity of exosomes and their likely different functionality.

O10C-376

Therapeutic exosomes from human CD34+ stem cells target selective myocardial cells to deliver miR-126 promoting angiogenesis and repair of the ischemic heart

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Introduction: Locally transplanted human $CD34^+$ stem cells have been shown to improve exercise tolerance in patients with myocardial ischemia and promote angiogenesis in animal models. Earlier, in a first study of its kind, we have demonstrated a novel mechanism that therapeutically significant human $CD34^+$ stem cells secrete membrane-bound nanovesicles called exosomes (CD34Exo). CD34Exo are angiogenic and constitute a critical component of the pro-angiogenic paracrine activity of the cells. Here, we hypothesize that cell-free CD34Exo may mimic the beneficial effects of the cells and promote angiogenesis and ischemic tissue repair via transferring pro-angiogenic microRNAs, possibly to endothelial cells. *Methods*: We used a murine model of myocardial ischemia to determine the therapeutic efficacy of CD34Exo. Trafficking of exosomes was studied using confocal microscopy and flow cytometry, and RNA was analyzed by microRNA microarray and Taqman assays. *Results*: When injected into mouse ischemic myocardium, cell-free CD34Exo replicated the therapeutic activity of human $CD34^+$ cells by significantly improving the myocardial ischemia (ejection fraction, 42 ± 4 vs. $22 \pm 6\%$; capillary density, 113 ± 7 vs. 66 ± 6/HPF; fibrosis, 27 ± 2 v $48 \pm 7\%$; $p < 0.05$, $n = 7–12$) compared with a PBS control. Interestingly, confocal imaging and flow cytometry analyses of the exosomes-injected murine ischemic myocardial tissue revealed that CD34Exo were selectively internalized into endothelial cells and cardiomyocytes but not into fibroblasts. MicroRNA expression profiling and confirmatory tests indicated that CD34Exo are significantly enriched with pro-angiogenic miRNAs such as miR126. CD34Exo induced the expression of miR126 and several pro-angiogenic mRNAs in the exosomes-treated mouse ischemic myocardium, but did not affect the endogenous synthesis of miR126, suggesting a direct transfer. CD34Exo lacking in miR126 had decreased angiogenic activity in vitro and decreased pro-angiogenic gene expression in vivo, indicating that miR126 was important for CD34Exo function. Imaging using fluorescent miR126 confirms that CD34Exo directly transferred miR126 and possibly other yet to be identified moieties from its cargo selectively to endothelial cells and cardiomyocytes in the ischemic heart. *Summary/conclusion*: Our results reveal a novel molecular and trafficking mechanism of CD34Exo in vivo, that may be responsible for intercellular transfer of miRNAs from human $CD34^+$ stem cells selectively to endothelial cells and cardiomyocytes, inducing changes in gene expression, angiogenesis and myocardial recovery. Exosomes-shuttled miRNAs may signify amplification of stem cell function and may explain the therapeutic benefits associated with human $CD34^+$ cell therapy.

O10C-377

How can so little miRNA in exosomes, in fact possibly as little as one molecule of miRNA per exosome, affect the function of targeted cells?

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Introduction: We found that antigen (Ag)-specific B cell-derived exosomes could be "transfected" with miRNA-150 in vitro, and then washed at $100{,}000 \times g$ to remove free miRNA, to then dose-dependently suppress effector T cells mediating in vivo contact sensitivity (CS) in mice. *Methods*: The source of these exosomes likely is a rare subset of Ag-specific Ig variable-region AID (activation-induced deaminase)-mutated B1a cells activated early after immunization that we described previously. Suppression of the CS-effector T cells likely proceeds indirectly by the Ag-receptors on the B cell exosomes binding Ag-peptide in MHC complexes on the dendritic cell (DC) companions regulating the T cells as their TCR also binds the Ag/MHC complex on the DC. *Results*: Transfection of exosomes from this rare Ag-specific B1a B cell subset among the total of $5 \times 1{,}010$ exosomes used with as little as $50 \times 10–15$ M of miRNA-150 (femtomolar amounts), rendered them suppressive of the effector T cells mediating adoptive transfer of CS into naive recipients. Calculation of the number of miR-150 molecules transfected in the femtomolar amounts of pure purchased miR-150 indicated that as few as 1 out of 100,000 exosomes took up one molecule of the miRNA-150, even at 100% transfection efficiency. This suggests that the transfected exosomes from the rare B cell subset have unique features that enabled them to be transfected without electroporation or any assistance. Further, such exosomes with few to just one molecule of miR-150 apparently were suppressive. This raises the question of how so little miR-150 transferred by exosomes could act in the targeted cells, since it has been determined that at least 50 molecules of an miRNA are needed to function by binding the 3′ untranslated region of a targeted mRNA to inhibit translation, and note that a given miRNA can have multiple mRNA targets. *Summary/conclusion*: Thus, great fundamental questions for the field of miRNA functional transfer from exosomes to targeted cells arise from these puzzling findings: (a) How is it possible that such a small proportion of exosomes is transfected with miRNA-150?; (b) How can so few transfected exosomes effectively function to alter activity of the targeted cells?; (c) How can they influence mRNA translation at a dose of one molecule of miRNA-150 per exosome, whereas the minimally required dose of specific miRNAs is 50 copies per cell? Hypotheses will be put forward to potentially answer these questions by new experimentation, hopefully leading to testable theories to be presented and discussed.

Networking coffee — Arcadis room — 10:15-10:45

Willem Burger room

Plenary closing ceremony, prizes & announcement ISEV 2015 10:45-12:00

Wrap up ISEV 2014 - Clinical highlights

Fred Hochberg

Wrap up ISEV 2014 - Basic highlights

Esther Nolte-'t Hoen

ISEV 2015

Prizes Concluding remarks

Networking lunch — Arcadis room — 12:00-13:30

Departure — 13:30

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