NLSEV
Netherlands Society for Extracellular Vesicles

Abstract and Programme Book NLSEV2019

Third annual meeting of the

Netherlands Society for Extracellular Vesicles

(NLSEV)

Friday Nov 8th 2019

De Zalen van Zeven, Boothstraat 7, 3512 BT Utrecht

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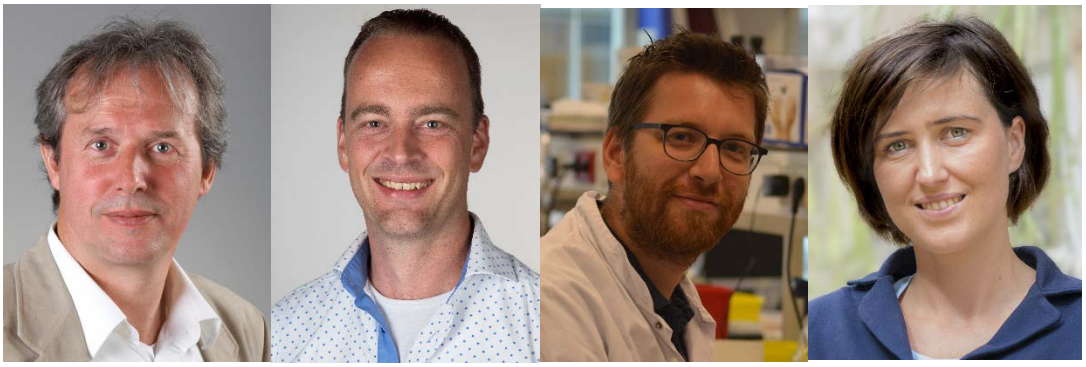
Abstract and Programme Book NLSEV2019



Dominique de Kleijn Joost Sluijter Pieter Vader Magdalena Lorenowicz

Local Organizing Committee

Programme

Program Third Meeting Netherlands Society for Extracellular Vesicles (NLSEV)

Friday November 8th 2019, De Zalen van Zeven, Boothstraat 7, 3512 BT Utrecht

9:00 – 9:30 **Registration**

9:30 – 9:45 **Welcome by Marca Wauben and Local Organizing Committee**

Chair Rienk Nieuwland

9:45 – 10:45 **Selected presentations**

Olivier de Jong (UMC Utrecht)

A novel CRISPR/Cas9-based reporter system enables detection of EV-mediated functional transfer of RNAs on a single-cell level

Estefanía Lozano-Andrés (Utrecht University)

Lipoprotein particles can influence extracellular vesicle labeling and fluorescence based detection by flow cytometry

Juan Pablo Rigalli (Radboudumc)

Modulation of tubular ATP levels by extracellular vesicles along the nephron: potential role in intrarenal communication and electrolyte reabsorption

Pepijn Beekman (Wageningen University)

Electrochemical quantification of EVs at physiological concentrations

10:45 – 10:55 **Sponsored talk (Izon)**

Camille Roesch

Single Particle Measurement for confidence in Biology; New information on samples purification.

10:55 – 11:30 **Coffee break & Posters meet and greet**

Chair Magdalena Lorenowicz

11:30 – 12:30 **Selected presentations**

Esther Drees (Amsterdam UMC, VUmc)

EV-bound plasma miR127-3p combined with serum TARC levels reflect metabolic disease activity in patients with classical Hodgkin lymphoma undergoing treatment

Martin van Royen (Erasmus MC)

EVQuant-micro; microfluidic assay for quantification of extracellular vesicles from clinical samples

Marije Kuipers (LUMC)

Glycosylated extracellular vesicles of the human parasite S. mansoni activate immune cells via interaction with DC-SIGN

Nazma Ilahibaks (UMC Utrecht)

TOP-EVs: Technology Of Protein delivery using Extracellular Vesicles

Programme

12:30 – 12:40 **Sponsored talk (Cell Guidance Systems)**
Inês Ferreira
Purification of high-quality exosomes using Exo-spin™ from Cell Guidance Systems: A novel application overview and new product announcement

12:40 – 13:50 **Lunch & Posters meet and greet**

Chair Michiel Pegtel

13:50 – 14:30 **Keynote lecture**
Neta Regev-Rudzki (Biomolecular Sciences, Weizmann Institute, Israel)
Malaria Parasite Networking

14:30 – 15:00 **Selected presentations**
Elena Martens-Uzunova (Erasmus MC)
A duplex quantitative real-time PCR assay for the detection of small non-coding RNA in urinary extracellular vesicles
Kyra Defourny (Utrecht University)
The Encephalomyocarditis virus Leader modulates autophagic pathways to promote the release of virions inside extracellular vesicles

15:00 – 15.10 **Sponsored talk (Beckman Coulter)**
Lutz Ehrhardt
Method transfer, scale up, standardisation & characterisation. A short update on EV solutions from the perspective of Beckman Coulter

15:10 – 15:40 **Coffee break & Posters meet and greet**

Chair Pieter Vader

15:40 – 16:20 **Keynote lecture**
Samir El Andaloussi (Laboratory Medicine, Karolinska Institute, Sweden)
Production, characterization and application of engineered EVs for biomedical applications

16:20 – 17:00 **EV-Delta networking**
Presentation and launch of EV-Delta
Presentation of consortia involving Dutch EV scientists:

1. TRAIN-EV Marca Wauben
2. IMMPROVE Irene Bijnsdorp
3. proEVLifeCycle Guido Jenster
4. Cancer-ID Edwin van der Pol
5. METVESII Rienk Nieuwland

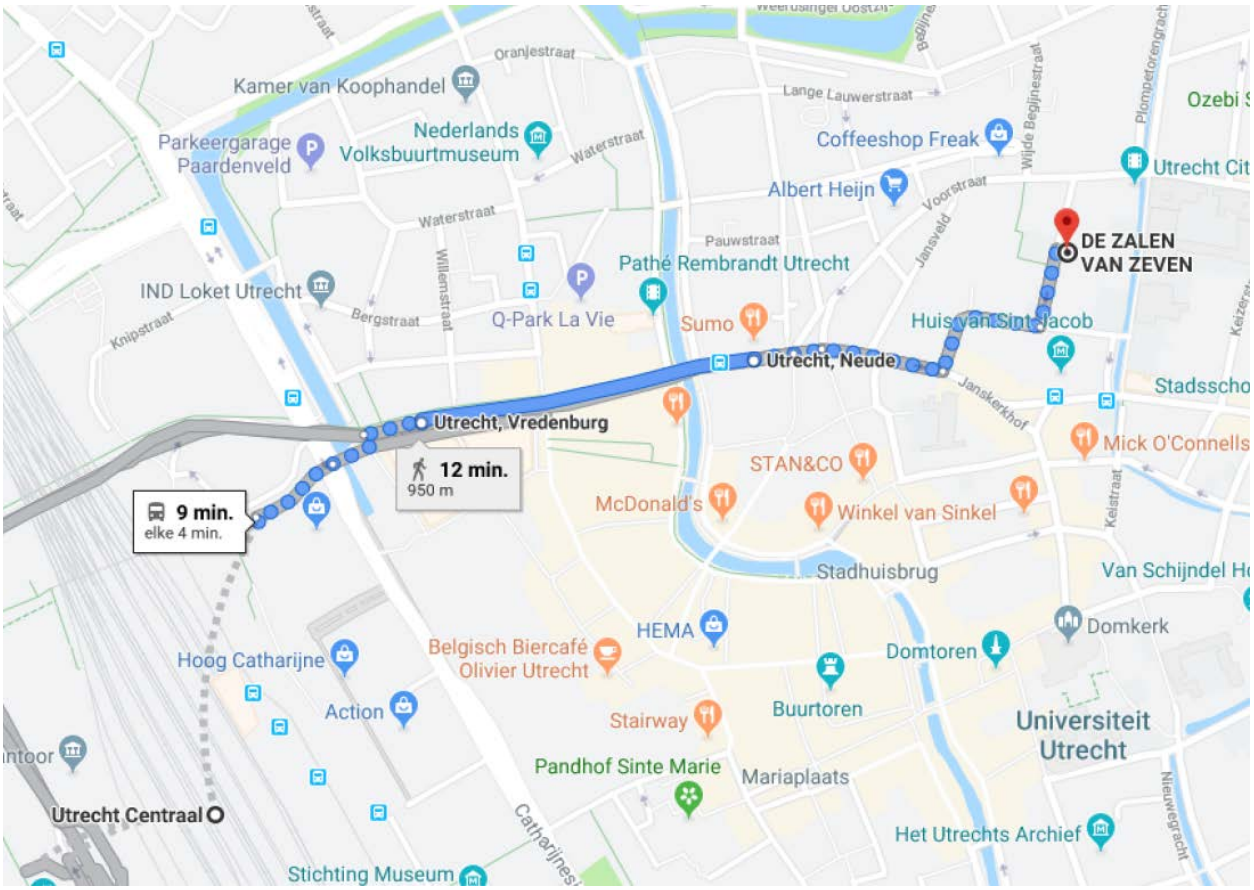
Programme

6. B-SMART Raymond Schiffelers
7. EXPERT Raymond Schiffelers
8. evFOUNDRY Martijn van Herwijnen

17:00 – 17:20 **NLSEV Announcements (Wauben) and Awards (LOC)**

17:20 – 19:00 **Drinks & Posters meet and greet**

Route



Oral Presentations

Keynote Speaker 1:

Neta Regev-Rudzki

Malaria Parasite Networking

Department of Biomolecular Sciences, Weizmann Institute of Science, Israel

Malaria, caused by a deadly parasite, is the most devastating parasitic disease, killing 1,000 children every day. The parasites transmission between humans via mosquitos involves a remarkable series of morphological transformations. We have obtained the first evidence that malaria parasites, while growing inside their host red blood cells, can communicate and transfer active cargo between them using Extracellular Vesicles (EVs). The secreted EVs are also capable of delivering cargo such as proteins, RNA and DNA to distal host cells, as monocytes, and by that the parasites use them to coordinate their own actions and to manipulate the host response. With malaria continuing to be a major global disease, advances toward understanding the basic biology of the parasite remain essential.

Keynote Speaker 2:

Samir El Andaloussi

Production, characterization and application of engineered EVs for biomedical applications

Laboratory Medicine, Karolinska Institute, Sweden

Extracellular vesicles (EVs) have emerged as important mediators of intercellular communication due to their ability to transfer bioactive lipids, proteins and different species of RNA into cells. Thus, EVs hold therapeutic potential in their own right and can additionally be harnessed for the delivery of macromolecular drugs. This presentation will cover the developments in producing, purifying and characterizing engineered EVs. Methods to determine PK and PD of EVs will be presented and how surface engineering can alter cell uptake and biodistribution in rodents. Finally, strategies to engineer producer cells in order to generate EVs harboring selected proteins for treatment of inflammatory diseases will be discussed.

Oral Presentations

NLSEV2019-O1 Olivier de Jong

A novel CRISPR/Cas9-based reporter system enables detection of EV-mediated functional transfer of RNAs on a single-cell level

Olivier G. de Jong [1, 2], Dan E. Murphy [1], Imre Mäger [2], Eduard Willms [2], Sander A.A. Kooijmans [1], Raymond M. Schiffelers [1], Samir El Andaloussi [3], Matthew J.A. Wood [2], Pieter Vader [1, 4]

1: Laboratory of Clinical Chemistry and Hematology, University Medical Center Utrecht. 2: Department of Physiology, Anatomy and Genetics, University of Oxford, United Kingdom. The Netherlands. 3: Department of Laboratory Medicine, Clinical Research Center, Karolinska Institutet, Sweden. 4: Department of Experimental Cardiology, University Medical Center Utrecht, The Netherlands.

Background: In recent years, multiple studies have shown that EVs play a role in intercellular communication through transfer of RNAs. Unfortunately, our understanding of the mechanisms regulating EV-mediated RNA delivery and processing is lacking, due to the absence of suitable readout systems for functional RNA transfer. Here, we describe a novel highly-sensitive CRISPR/Cas9-based reporter system that, for the first time, allows direct functional study of EV-mediated transfer of small non-coding RNA molecules on a single-cell level.

Methods: We generated a CRISPR/Cas9-based stoplight reporter system, in which eGFP expression is activated upon functional delivery of targeting single guide RNAs (T-sgRNAs). Donor cell lines were generated stably expressing either T-sgRNAs or non-targeting sgRNAs (NT-sgRNAs). Intercellular functional RNA transfer was assessed by measuring eGFP expression in reporter cells after direct co-culture, transwell co-culture, and upon addition of isolated EVs, using fluorescence microscopy and flow cytometry. The role of potential regulators of EV-mediated RNA transfer was assessed after RNAi-mediated target knockdown in reporter cells, prior to co-culture experiments.

Results: Expression of sgRNAs in donor cells and EVs was confirmed by RT-PCR. A significant activation of eGFP expression was observed in reporter cells after direct co-culture and transwell co-culture with donor cells expressing T-sgRNAs, but not NT-sgRNAs. Addition of EVs from cells expressing T-sgRNAs, and not NT-sgRNAs, also resulted in significant reporter activation. Reporter activation was substantially decreased after blocking EV production through addition of GW4869 or Rab27A knockdown in donor cells. Knockdown of multiple targets in endocytosis and/or intracellular membrane trafficking in reporter cells significantly decreased reporter activation, suggesting vital roles for these processes in EV-mediated RNA transfer.

Conclusion: Here we demonstrate a CRISPR/Cas9-based reporter system that for the first time allows the study of functional delivery of small non-coding RNAs with single-cell resolution. This novel approach allows the study of EV cargo processing in the context of functional RNA delivery, and may help to increase our understanding of the regulatory pathways that dictate the underlying processes.

Oral Presentations

NLSEV2019-O2 Estefanía Lozano-Andrés

Lipoprotein particles can influence extracellular vesicle labeling and fluorescence based detection by flow cytometry

Estefanía Lozano-Andrés 1, Sten F.W.M. Libregts1, Cláudio Pinheiro2,3, #, Guillaume Van Niel4, An Hendrix 2,3,#, Ger. J.A. Arkesteijn1, 5, Marca H.M. Wauben1

1 Department of Biochemistry and Cell Biology, Faculty of Veterinary Medicine, Utrecht University, Utrecht; 2 Laboratory of Experimental Cancer Research, Department of Human Structure and Repair Ghent University, Ghent; 3 Cancer Research Institute Ghent, Ghent; 4 Institute for Psychiatry and Neuroscience of Paris, Hopital Saint-Anne, Université Descartes, INSERM U1266, Paris; 5 Department of Immunology & Infectious Diseases, Faculty of Veterinary Medicine, Utrecht University, Utrecht.

Background: High-resolution flow cytometry (FC) allows for the detection of single extracellular vesicles (EV) and enables quantitative and qualitative characterization. EV in plasma have been associated with diseases, making them attractive for diagnosis and prognosis of patients. However, plasma contains a large number of different types of lipoprotein particles (LPP) and their presence may hamper robust flow cytometric analysis of EV since both entities partially overlap in biophysical properties such as size, density and molecular makeup. We here investigated whether these particles interfere with staining and detection of EV when generic fluorescent dyes are used for fluorescence-based FC.

Methods: Commercially available Chylomicrons, VLDL and LDL preparations were acquired and characterized by Dot Blot, ELISA and Electron Microscopy. EV were separated from conditioned cell culture media of the mouse 4T1 mammary carcinoma cell line by density-gradient ultracentrifugation and size exclusion chromatography. Isolated EV were characterized by Western blot, Nanoparticle Tracking Analysis and High-resolution flow cytometry. LPP were spiked-in presence of EV and labeled with PKH67 and/or a mouse CD9PE antibody. Next, samples were succumbed to density gradient floatation, after which FC-analysis was performed using a BD Influx optimized for detection of submicron-sized particles.

Results: We found that not only EV but also LPP can be labeled with the lipophilic dye PKH67. Importantly, the presence of LPP in the samples negatively affected labeling of EV with PKH67 and lead to an underestimation of the number of EV labeled with a specific antibody for a marker of interest. As the presence of LPP hampers both the generic fluorescent labeling and detection of EV during fluorescence-triggered flow cytometric analysis, these data illustrate that for fluorescence-based flow cytometric analysis of EV in plasma there is a need to separate EV from LPP before performing generic staining.

Conclusion: In order to perform reliable and reproducible fluorescence based FC-analysis of single EV from human plasma, samples should be carefully cleared from particles prone to incorporate the generic dye.
Funding: European Union’s Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No [722148] and STW-Perspectief Cancer-ID grant [14,191].

Oral Presentations

NLSEV2019-O3 Juan P. Rigalli

Modulation of tubular ATP levels by extracellular vesicles along the nephron: potential role in intrarenal communication and electrolyte reabsorption

Juan P. Rigalli (1), Eric Barros(1,2), Onno Arntz(3), Fons van de Loo(3), Cristian A. Carvajal(2), Rene Bindels(1), Joost Hoenderop(1)

1: Department of Physiology, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, Nijmegen, the Netherlands. 2: Department of Endocrinology, School of Medicine, Pontificia Universidad Católica de Chile, Santiago, Chile. 3: Department of Rheumatology, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, Nijmegen, the Netherlands.

Background: Purinergic signaling results from binding of extracellular ATP and its hydrolysis products to P1 and P2 receptors. While the acute regulation of epithelial sodium channel (ENaC) activity by ATP in the renal collecting duct is well-recognized, long-term regulation has been less investigated. Extracellular vesicles (EVs) released by tubular cells can be transported along the tubular lumen and interact with cells downstream. Although modulation of extracellular ATP levels by EVs has been described in extrarenal tissues, it is not known if a similar process takes place in the kidney.

Methods: We investigated the effect of EVs from proximal (HK2) and collecting duct (HCD) cells on extracellular ATP levels, αENaC expression and the underlying mechanisms. Extracellular ATP and gene expression were evaluated by chemiluminescence and RT-qPCR, respectively. Dependence on EV-uptake was evaluated using dynasore. The effect of flow on EV release was addressed using microfluidics chambers and nanotracking-analysis.

Results: ATP-hydrolyzing activity of EVs was determined in a non-cellular system. Only apically-released EVs from HCD cells decreased ATP. Following, HK2 cells were incubated with HK2 EVs, and HCD cells were incubated with EVs from HK2 and HCD cells. HK2 EVs added to HCD cells increased extracellular ATP (+25%*, uptake-dependent). Downregulation of the ectonucleotidase ENTPD1 (-22%*) may explain this observation. Conversely, HCD EVs decreased extracellular ATP in HCD cells (-40%*, uptake-independent), probably attributed to ATP hydrolysis by HCD vesicles. Differential proteomics of HK2 and HCD EVs pointed the protein 14-3-3 as potential mediator of ATP-hydrolysis by HCD EVs. While HK2 EVs decreased αENaC mRNA expression (-26%*), this was up-regulated by HCD EVs (+46%*). γ-S-ATP and apyrase mimicked the effects of HK2 and HCD vesicles on αENaC, respectively, supporting an inverse association between extracellular ATP levels and αENaC expression. Finally, flow-mediated release of EVs was observed only in HCD cells (+416%*).

Conclusions: EVs from proximal and collecting duct cells have a differential effect on αENaC expression via modulating purinergic-signalling, where the autocrine effect of HCD EVs might be sensitive to changes in pro-urinary flow. (*p<0.05, n=3-4)

Oral Presentations

NLSEV2019-O4 Pepijn Beekman

Electrochemical quantification of EVs at physiological concentrations

Pepijn Beekman [1,2], Dilu Mathew [3], Serge Lemay [4], Han Zuilhof [1], Wilfred van der Wiel [3], Séverine Le Gac [2]

1: Laboratory of Organic Chemistry, Wageningen University, 2: Applied Microfluidics for BioEngineering Research, University of Twente, 3: NanoElectronics, University of Twente, 4: BioElectronics, University of Twente

Introduction: Tumor-derived extracellular vesicles (tdEVs) are promising biomarkers for cancer patient management. An advantage of tdEVs over circulating tumor cells is their higher concentration in patient blood by 3-4 orders of magnitude (∽10^3-10^5 tdEVs /ml), giving more robust information while requiring smaller sample sizes. However, their small size and complex composition of blood samples require sensitive and selective detection methods. Here, we report electrochemical detection of tdEVs using a nano-interdigitated electrode array (nIDE) functionalized with cancer-specific antibodies and an antifouling coating. The EV detection mechanism is based on enzymatic conversion of p-aminophenyl phosphate (pAPP) by alkaline phosphatase (ALP) followed by redox cycling between nIDE fingers of the cleaved substrate, yielding a double signal amplification. The proposed sensing scheme gives a physiologically relevant limit of detection (LOD) of 10 EVs/µl.

Methods: Platinum nIDEs (120 nm width, 80 nm spacing, 75 nm height) were functionalized with an amino-undecanethiol monolayer, which was reacted with poly(ethylene glycol) diglycidyl ether. Anti-EpCAM antibodies were next immobilized to subsequently capture tdEVs. Anti-EpCAM-alkaline phosphatase conjugates were then introduced to yield ALP-tagged tdEVs. The non-electroactive pAPP was finally used to quantify the ALP concentration.

Results: With increasing tdEV concentration, an increase in redox current was measured, from average (n=3) 0.35 nA for 10 tdEV/µl to 12.5 nA for 105 tdEV/µl. This current is produced by the electroactive cleavage product of pAPP, which redox cycles between electrodes. As a negative control, the experiment was performed with incubation of platelet derived EVs (EpCAM negative), whereby also the signal did not significantly increase (background current ∽0.15 nA). The short migration distance in our nanoelectrode array yielded a factor ∽8 improvement compared to micro-electrodes (3µm width and spacing).

Conclusion: A sensitive sensor was developed for the detection of EVs at low concentrations. With an LOD of 10 tdEVs/µl and high selectivity towards tdEVs, our platform opens new avenues for screening patient blood samples.

Oral Presentations

NLSEV2019-O5 Esther Drees

EV-bound plasma miR127-3p combined with serum TARC levels reflect metabolic disease activity in patients with classical Hodgkin lymphoma undergoing treatment

E.E.E. Drees [1], J. Perez-Boza[1], N.J. Groenewegen[2], M.A.J. van Eijndhoven[1], L.I. Prins[1], S.A.W.M. Verkuijlen[1], X. Tran[1], A. Vallés-Martí[1], T. J. Molenaar[1], E. Aparicio-Puerta[3], M. Hackenberg[2-3], D. de Jong[1], J.M. Zijlstra[4]* and D.M. Pegtel[1-2]*

1: Amsterdam UMC, Vrije Universiteit Amsterdam, Department of Pathology, Cancer Center Amsterdam; 2: ExBiome B.V., Amsterdam; 3: Computational Epigenomics and Bioinformatics, Department of Genetics, University of Granada; 4:Amsterdam UMC, Vrije Universiteit Amsterdam, Department of Hematology, Cancer Center Amsterdam

Background: Circulating cell-free DNA, miRNA and proteins are considered as biomarkers for early cancer detection but the utility of liquid biopsies for response monitoring is less clear. Here we investigated whether extracellular vesicle (EV) associated miRNAs can act as therapy response indicators for patients with classical Hodgkin Lymphoma (cHL).

Methods: 193 samples derived from 31 patients with classical Hodgkin Lymphoma (cHL) were included in this study. EV-enriched fractions were isolated with size-exclusion chromatography (SEC). RNA was isolated using trizol. qRT-PCR was used for quantitative validation of the EV-bound miRNAs. EV-miRNAs and sTARC-levels as measured by ELISA were correlated with clinical parameters and treatment outcome as determined by FDG-PET. We performed small RNA sequencing on 44 plasma EV samples from cHL patients and healthy controls.

Results: Comparative analysis of small RNA sequencing profiles of plasma EVs from patients undergoing treatment and healthy controls identified miR21-3p, -155-5p, -24-3p, let7a and -127-3p. Of these miR127-3p remains elevated in patients with progressive disease. Antagonizing miR127-3p activity impairs growth and survival of HRS cells, consistent with their proposed role in repressing the cHL associated tumorsupressor A20. Quantitative RT-PCR validated elevated EV-miRNA levels in plasma from both newly diagnosed and relapsed/refractory cases. Long-term longitudinal plasma monitoring of 31 patients revealed that the EV-miRNA levels in complete (clinical and metabolic) responders drop as early as 1-2 treatment cycles and remain low in the following years unless a late relapse occurs. Notably, cHL-associated EV-miRNA levels are not related to liver and kidney function or age but weakly associated with leukocyte/thrombocyte counts and gender as does serum TARC, a validated cHL protein biomarker. Random effects modeling indicated that miR127-3p in combination with other miRNAs can predict ‘absence of active disease’ with a negative-predictive value (NPV) reaching 83%. Strikingly, combining EV-miR127-3p with sTARC increases accuracy and the NPV to 96%.

Conclusion: We propose that measuring EV-miRNA miR127-3p and sTARC early during therapy has potential to inform clinicians which cHL patients could be spared unnecessary treatment continuation.

Oral Presentations

NLSEV2019-O6 Martin van Royen

EVQuant-micro; microfluidic assay for quantification of extracellular vesicles from clinical samples

Serhii Mytnyk [1], Thomas A. Hartjes[3], Guido W. Jenster[2], Martin E. van Royen[3] and Volkert van Steijn[1]

1: Delft University of Technology, Delft, the Netherlands; 2: Department of Urology, Erasmus Medical Center, Rotterdam, the Netherlands; 3: Erasmus Optical Imaging Centre, Department of Pathology, Erasmus Medical Center, Rotterdam, The Netherlands.

Background: To optimally use the potential of extracellular vesicles (EVs) as biomarkers for various diseases there is a need for fast, inexpensive and accurate methods for EV characterization in clinical samples. We recently developed EVQuant, a (semi-) high-throughput microscopy based assay for quantification and characterization of EVs in research and clinical samples. Our aim is to further develop it into a simple assay that requires limited resources/expertise, enabling its wide dissemination across the community. Here we present an epifluorescence microscopy based microfluidic assay for the simultaneous quantification of the concentration and the size distribution of urinary EVs in minimally processed clinical (prostate cancer) samples.

Methods: EVs were labelled using general fluorescent lipid membrane stains and/or immunofluorescent antibodies. Liquid samples were injected into shallow PDMS microchannels with a known depth. Because the microscope the measurement volume is precisely defined it allows us to determine the EV concentration. In addition, this approach enabled us to determine their size distribution by tracking 2D-diffusion of individual EVs, without the need of advanced equipment such as a confocal microscope.

Results: We have successfully validated our approach by applying it to suspensions of fluorescent nanoparticles of defined sizes and known concentrations. When applied to EVs, the developed method allowed accurate concentration measurements over a wide range, as confirmed by comparison with data obtained from EVQuant analysis using immobilized EVs. Moreover, single particle tracking analysis provides a quick and accurate diameter estimate of individual urinary EVs with a size down to 50nm.

Conclusions: We developed EVQuant-micro, an assay for EVs concentration and size measurement without the need for complex equipment, significantly decreasing analysis times and making this method a promising tool in preliminary EV diagnostics in clinical environment.

This work was supported by the IMMPROVE Alpe d'HuZes grant of the Dutch Cancer Society (EMCR2015-8022)

Oral Presentations

NLSEV2019-O7 Marije Kuipers

Glycosylated extracellular vesicles of the human parasite S. mansoni activate immune cells via interaction with DC-SIGN

M.E. Kuipers [1,2], E.N.M. Nolte-'t Hoen [2], A.J. van der Ham [1], A. Ozir-Fazalalikhan [1], D.L. Nguyen [1], C.M. de Korne [1], R.I. Koning [3], J.J. Tomes [4], K.F. Hoffmann [4], H.H. Smits# [1], C.H. Hokke# [1]

1: Department of Parasitology, LUMC, Leiden, 2: Department of Biochemistry & Cell Biology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, 3: Department of Cell & Chemical Biology, LUMC, Leiden, 4: IBERS, Aberystwyth University, Aberystwyth, United Kingdom, #these authors contributed equally

Background: Parasitic helminths such as Schistosoma mansoni release excretory/secretory (E/S) products to establish and maintain a successful infection within their host. Only recently it has become clear that extracellular vesicles (EVs) are among these E/S products. The E/S includes a variety of glycoproteins that induce immunomodulatory effects on dendritic cells (DCs), macrophages, and other host cells. However, interaction of S. mansoni EVs with host immune cells remains to be explored. Since glycoproteins often modulate immune responses in a glycan-dependent manner and involve C-type lectin receptors (CLRs) such as the mannose receptor (MR) and DC-SIGN, we additionally investigated the role of those CLRs in the interaction of S. mansoni EV with host cells.

Methods: EV released from cultured S. mansoni larvae were fluorescently labelled and incubated with human monocyte-derived DCs (moDCs) for 2h, with or without pre-incubation of EGTA or antibodies blocking DC-SIGN or MR. EV internalization was measured by flow cytometry and confocal microscopy. N-glycans on the surface of EVs were analysed by mass spectrometry. MoDC were incubated with EVs in the presence of IL-1β&TNFα and/or anti-DC-SIGN/isotype control followed by the analysis of DC activation and cytokine production by ELISA, flowcytometry, and qPCR.

Results: S. mansoni EVs were internalized by moDC in a dose- and calcium dependent manner. Interestingly, blocking DC-SIGN almost completely inhibited EV internalization, but blocking MR had no effect. Glycans on the surface of intact EVs included complex type N-glycans with terminal Galβ1-4(Fucα1-3)GlcNAc (LewisX) motifs, well-known ligands for DC-SIGN. Incubation of moDCs with EVs increased the expression of costimulatory molecules CD80 and CD86, and the regulatory molecule PD-L1. Furthermore, IL-12 and IL-10 mRNA expression and secretion were increased, which was partly DC-SIGN dependent.

Conclusions: These results provide evidence for glycan-dependent interaction of S. mansoni-released EVs with host immune cells and reveal a role for DC-SIGN and EV-associated glycoconjugates in immune modulation by parasites.

Oral Presentations

NLSEV2019-O8 Nazma Ilahibaks

TOP-EVs: Technology Of Protein delivery using Extracellular Vesicles

Zhiyong Lei [1, 2], Arif Ibrahim Ardisasmita[1], Nazma Ilahibaks[1], Pieter Vader[1, 2], Raymond Schiffelers[2], Joost Sluijter[1,3]

1.Experimental cardiology Laboratory, UMC Utrecht; 2.Clinical Chemistry and Haematology, UMC Utrecht, 3.Regenerative Medicine Center, UMC Utrecht

Background: Emerging CRISPR/Cas9 genome editing technologies have created new possibilities for many hereditary diseases without curative treatment at the moment. The most challenging issue for the clinical application of the genome-editing approach is how to deliver the CRISPR/Cas9 system effectively and especially safely to the diseased organ. With safety issues by using different viral vectors, cell-derived extracellular vesicles (EVs) appear to follow nature's choice for intracellular transportation of protein, if we can overcome the limitations in active protein-loading.

Method: By exploiting ligand-induced membrane association technology, we engineered cells with the protein of interest targeted to the inner membrane of the cell and subsequently packed into EVs. After taken up by the recipient cell, in the absence of the ligand, membrane-associated proteins are released from the membrane to fulfil their function into the target cell. This Technology Of Protein delivery using Extracellular Vesicles is named TOP-EVs.

Results: Using GFP, Cre recombinase and Cas9 as proof of concept, we show that we can actively load protein-of-interest into TOP-EVs with unprecedented efficiency and effectively deliver intracellularly these proteins to a broad range of cell types. Using Cre TOP-Evs on Cre reporter cells, we also investigate the mechanism of TOP-EVs uptake as well as the influence of different storage methods on the function of these EVs.

Conclusion: TOP-EVs enable highly effective intracellular functional protein delivery, which open up many potential applications that require intracellular/membrane protein delivery such as genome editing.

Oral Presentations

NLSEV2019-O9 Elena Martens

A duplex quantitative real-time PCR assay for the detection of small non-coding RNA in urinary extracellular vesicles

Elena S. Martens-Uzunova [1], Natasja Dits [1], Guido Jenster [1]; Laboratory of Experimental Urology, Department of Urology, Erasmus Medical Center, Erasmus University, Rotterdam, The Netherlands

Background: Extracellular vesicles (EVs) have emerged as a novel promising source of cancer biomarkers. EVs are produced and shed into different body fluids including blood and urine by virtually any normal or malignant cell. It is evident that the molecular content of EVs is highly resembling their cell of origin. Therefore, tumor cell originating EVs can be explored as a rich source of minimally invasive, liquid biopsy RNA biomarkers for prostate cancer and other malignancies.

In this study, we build upon our previous work to develop a quantitative real-time PCR (qPCR) assay for the simultaneous detection of small noncoding RNA present in urinary EVs in order to evaluate their use as minimally invasive PCa biomarkers.

Methods and Results: We developed custom TaqMan Advanced assays (TermoFisher) against candidate biomarker microRNAs, tRNA fragments (tRFs), snoRNAs and snoRNA-derived RNAs (sdRNA) and tested their performance by absolute quantification qPCR. Urinary EVs were captured by precipitation and Total RNA was isolated directly from urinary EVs captured by precipitation kit (Norgen Biotek). cDNA was synthesized from the obtained total RNA or from equimolar mix of synthetic small RNA targets using TaqMan advanced universal cDNA synthesis kit (ThermoFishrer). To enable the simultaneous detection of two different RNA targets in the same PCR reaction, we designed TaqMan probes with either FAM or VIC labeling and tested their performance against a mix of synthetic RNA templates and in total RNA material isolated from urinary EVs. To establish efficiency and accuracy all assays were tested as uniplex reaction with only FAM or only VIC probe in the reaction mix. Subsequently, duplex reactions including different combinations of FAM and VIC assays were combined and efficiency and accuracy were compared to the uniplex setting. After optimization best performing duplex combinations were tested against total RNA derived from urinary EVs.

Conclusions: Our results demonstrate that duplex qPCR is an efficient method for the simultaneous detection of small RNA in the same reaction mix. This makes possible the evaluation of candidate small RNA biomarker combinations in urine and opens the door to the development of a small RNA based, minimally invasive liquid biopsy test for PCa.

Oral Presentations

NLSEV2019-O10 Kyra Defourny

The Encephalomyocarditis virus Leader modulates autophagic pathways to promote the release of virions inside extracellular vesicles

Susanne G. van der Grein (1), Kyra A.Y. Defourny(1), Huib H. Rabouw(2), Frank J.M. van Kuppeveld(2), Esther N.M. Nolte-'t Hoen(1).

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Background: Recent data indicate that naked viruses belonging to the picornaviridae family can be released from host cells via enclosure in extracellular vesicles (EV). EV cloak secreted virus particles in a host-derived 'envelope', thereby altering virus-host interactions. As a result, EV can affect antiviral immune responses and disease severity. This presses the need for a better understanding of the formation and function of EV-enclosed viruses. Previously, we demonstrated the presence of the autophagosome marker LC3 in EV isolates from Encephalomyocarditis virus (EMCV) infected cells, suggesting the involvement of a secretory autophagy pathway in EV-mediated virus release. Little is known about the viral and host factors that regulate this process. Here, we have assessed the role of the EMCV Leader, a viral protein that is dispensable for replication but is required for symptomatic disease.

Methods: Cells were infected with wildtype virus or a mutant carrying an inactive Leader. EV produced during the infection were isolated using differential ultracentrifugation and density gradient purification. EV were characterized by high resolution flow cytometry and their infectivity determined using end-point dilution assay. In addition, the fate of autophagosomes in infected cells was monitored using a reporter assay for autophagosome-lysosome fusion and analysis of the secretion of autophagosomal proteins.

Results: Inactivation of the EMCV Leader strongly reduced the extracellular release of EV-enclosed virus. Whereas autophagosomes are typically degraded, we show that this process is blocked by the Leader. Instead, autophagosomes fuse with the plasma membrane, as indicated by the secretion of autophagy marker LC3 during infection with the wildtype but not the mutant virus. Pharmacological reactivation of degradative autophagy in infected cells resulted in a strong reduction in the release of EV and EV-enclosed virus. Similarly, the reduced release of EV-enclosed virus in the absence of the Leader could be partially reversed by drugs that promote the secretion of autophagosomes.

Conclusions: Our data supports a role for secretory autophagy in the release of EV-enclosed virus particles, a pathway that is regulated by the EMCV Leader. These findings highlight an unconventional route for EV formation that intersects with autophagosomal compartments and that may contribute to viral pathogenesis.

Poster Presentations

NLSEV2019-P2 Suzy Varderidou-Minasian

Mesenchymal stromal/stem cell-derived extracellular vesicles promote human cartilage regeneration by control of autophagy

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Background: Osteoarthritis (OA) is a rheumatic disease leading to chronic pain and disability with no effective treatment available. Recently, allogeneic human mesenchymal stromal/stem cells (MSC) entered clinical trials as a novel therapy for OA. Increasing evidence suggests that therapeutic efficacy of MSC depends on paracrine signalling. Here we investigated the role of bone marrow MSC-derived extracellular vesicles (BMMSC-EVs) in cartilage repair and show that BMMSC-EVs promote cartilage regeneration in vitro.

Methods: OA chondrocytes were treated with inflammatory mediators such as tumor necrosis factor alpha (TNF-alpha) to induce inflammation. Hereafter, bone marrow MSC-derived extracellular vesicles (BMMSC-EVs) are added to investigated the role of BMMSC-EVs in cartilage repair

Results: Treatment of OA chondrocytes with BMMSC-EVs induces production of proteoglycans and type II collagen and promotes proliferation of these cells. MSC-EVs also inhibit the adverse effects of inflammatory mediators on cartilage homeostasis. Our data show that BMMSC-EVs downregulate tumor necrosis factor alpha (TNF-alpha) induced expression of pro-inflammatory cyclooxygenase-2, pro-inflammatory interleukins and collagenase activity in OA chondrocytes. The anti-inflammatory effect of BMMSC-EVs involves the inhibition of NF□B signaling, activation of which is an important component of OA pathology. Our findings indicate that BMMSC-EVs have ability to promote human OA cartilage repair by reducing the inflammatory response and stimulation of OA chondrocytes to produce extracellular matrix, the essential processes for restoring and maintaining cartilage homeostasis.

Conclusion: Autophagy, a cellular homeostatic mechanism for the removal of dysfunctional cellular organelles and macromolecules, is essential to maintaining chondrocytes survival and differentiation. Accumulating evidence indicates that the expression of autophagy regulators is reduced in osteoarthritic joints, which is also accompanied by increased chondrocyte apoptosis. Our preliminary data indicate that BMMSC-EVs carry mRNA of natural autophagy inducers and promote autophagy in OA chondrocytes. Therefore, we hypothesize that MSC-EVs exert their beneficial effects on cartilage regeneration by restoring the expression of autophagy regulators.

Taken together, our data demonstrate that MSC-EVs can be important mediators of cartilage repair and hold great promise as a novel therapeutic for cartilage regeneration and osteoarthritis.

Poster Presentations

NLSEV2019-P3 Yong Hu

Extracellular vesicles in human milk expose tissue factor and promote coagulation

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Tissue factor (TF), the transmembrane receptor of coagulation factor VII, initiates coagulation. TF is associated with extracellular vesicles (EVs) in saliva, seminal- and amniotic fluid under normal, physiological conditions. It is unknown whether human milk also contains such TF-exposing EVs.

Human milk was collected from six healthy nursing adult women. Milk was fractionated by ultracentrifugation and size exclusion chromatography (SEC), and the ability of milk or fractions thereof to trigger clotting of plasma was studied with and without inhibitory antibodies against TF or coagulation factor VII(a). The association of TF and EVs was investigated by flow cytometry, surface plasmon resonance imaging (SPRi), cryo-electron microscopy, and Western blot.

Addition of milk to plasma shortened the clotting time. When milk was first subjected to ultracentrifugation, the TF- and factor VII(a)-dependent procoagulant activity was recovered in the milk pellet. When milk was fractionated by SEC, fractions 8 and 9 contained the highest TF-dependent procoagulant activity. Flow cytometry, SPRi, and Western blot confirmed that these fractions contain EVs because these fractions contained the highest levels of the common EV markers tetraspanin CD9 and CD63. The presence of TF in these fractions was detectable by Western blot.

In conclusion, we demonstrate that EVs in human milk expose TF and promote clotting. Although the function of such EVs needs to be studied further, we hypothesize that milk-derived EVs protect the “milieu interieur” by promoting a hemostatic envelope that accelerates haemostasis and thus might contribute to wound healing.

Poster Presentations

NLSEV2019-P4 Marije Kleinjan

Characterization of extracellular vesicles isolated from differently processed bovine milk

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Introduction: Recently we found that cell-derived extracellular vesicles (EV) in raw milk have a strong immune modulatory capacity. We here investigated how processing of bovine milk affected the EV quantity and composition.

Methods: EV from homogenized, pasteurized, Ultra Heat Treated (UHT) milk and raw bovine milk were isolated by differential centrifugation followed by sucrose density gradient centrifugation. Density gradient fractions 4-6, 7-9 and 10-12 were pooled and analyzed by flow cytometry, cryo EM and western blot and small RNA concentrations were determined by Bioanalyzer.

Results: Quantitative single EV-based flow cytometry showed no differences between EV numbers in pasteurized and raw milk. However, pasteurization combined with homogenization resulted in a strong reduction of EV in fraction 7-9, while in UHT milk the number of EV was drastically reduced. These results were confirmed by cryo EM. Western blotting showed that the general EV markers CD9 and CD63 were most prominent in fraction 7-9 of all kinds of milk, except for UHT treated milk where no protein signals could be detected. For MHCII a shift in signal was observed from fractions 7-9 in raw milk to fractions 4-6 after pasteurization. This could be indicative for lost or damaged MHCII positive EV populations induced during milk processing. Furthermore, pasteurization also resulted in loss of small RNAs in fractions 7-9, but not in fractions 4-6. Moreover, homogenization of milk affected the distribution of MFG-E8 throughout the gradient.

Conclusion: Depending on the type of processing, differential effects on the total EV population or on EV subsets were observed. Homogenization most likely affected mainly the MFG membranes in milk, while UHT treatment had the most detrimental effect on EVs. Hence processing of raw milk can impact both the amount and integrity of milk EV. Currently we are investigating the consequences of bovine milk processing on the immune modulatory capacity of milk EV.

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Poster Presentations

NLSEV2019-P5 Manon Mulders

TGFβ cooperates with exosomal RNA to drive pro-metastatic cancer inflammation

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Introduction: Extracellular vesicle-mediated communication between tumor and stromal cells has a critical role in tumor progression in osteosarcoma. We previously demonstrated that tumor-secreted exosomes drive tumor growth and metastasis formation by reprogramming mesenchymal stem cells (MSCs) in the bone marrow environment. This effect was determined by a membrane-bound form of TGFβ on the surface of the vesicles, strongly inducing pro-metastatic IL-6 production in MSCs. Because EVs are complex entities carrying a variety of signals in the form of proteins, lipids and nucleic acids to recipient cells, we hypothesized that besides TGFβ other EV components contribute to generate a pro-metastatic environment. Defining these components and their modality of action could provide new insights into novel therapeutic strategies to counteract the metastatic process.

Methods: The global alterations of the MSC gene expression profile induced by osteosarcoma EVs in the presence or absence of a TGFβ inhibitor were analyzed by RNA-seq. Small RNA-sequencing was used to define the RNA classes enriched in osteosarcoma EVs. To study exosomal RNA-induced alterations we transfected exosomal RNA in MSCs. The effect of TGFβ inhibition on metastasis formation was evaluated in vivo using an orthotopic mouse model.

Results: RNA-seq analysis shows that osteosarcoma EVs modulate pathways involved in cell plasticity, differentiation and inflammation. Based on their enrichment in the presence or absence of TGFβ inhibition, pathways were classified as TGFβ-dependent or -independent. Interestingly, the majority of pathways related to inflammation were TGFβ-independent, and all modulated genes within these pathways were TLR3-induced or TLR3-signaling related. Small RNA-sequencing of osteosarcoma EVs highlighted the presence of inflammatory RNA species potentially able to activate RNA sensors, including TLR3, in MSCs. Finally, intracellular delivery of purified exosomal RNA induced TGFβ-independent chemokines, suggesting that exosomal RNA can activate innate immune response in a TLR3-dependent manner. Preliminary in vivo data shows that TGFβ inhibition already alone reduces metastasis formation.

Conclusion: Our study suggests that TGFβ and exosomal RNA cooperate to shape a pro-metastatic environment. Future studies will be aimed at interfering with the TGFβ- and TLR3-mediated alterations with combination therapy to counteract metastasis formation in osteosarcoma.

Poster Presentations

NLSEV2019-P6 Marieke Roefs

Towards identification of protein cargo mediating cardiac repair by cardiac progenitor cell-derived extracellular vesicles

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Background: Stem and progenitor cell transplantation therapy holds great promise for regenerating damaged heart tissue. However, there is limited understanding of what actually drives the improvement of cardiac function after cellular administration. Several lines of evidence suggest that its efficacy is mainly caused by secreted paracrine factors including extracellular vesicles (EVs). Indeed, cardiac progenitor cell (CPC)-derived EVs have been shown to protect the myocardium against ischemia/reperfusion injury in several preclinical models. However, the underlying mechanisms for CPC EV-mediated cardioprotection remain elusive.

Methods/Results: In an attempt to increase EV release, CPCs were stimulated with calcium ionophore or vehicle for 24 hours. EVs were isolated from serum-free conditioned medium using size exclusion chromatography. The concentration and size of EVs was assessed using nanoparticle tracking analysis and EVs were functionally characterized based on endothelial cell activation assays. Surprisingly, we found enhanced phosphorylation of ERK and AKT by western blotting analyses and increased wound closure in a scratch assay after stimulation with CPC EVs, but not after stimulation with calcium ionophore CPC EVs. This indicates that the ability of EVs to stimulate pro-survival pathways and induce endothelial cell migration was lost after CPC exposure to calcium ionophore. We utilize this observation as a springboard to unravel protein-mediated effects of CPC EVs on the endothelium. Proteomic composition of both EV populations was compared using liquid chromatography tandem mass spectrometry analysis. A total of 2030 unique proteins were identified in at least one of the EV samples. Among them, almost 40 proteins were revealed as candidates based on relative enrichment in CPC EVs compared with calcium ionophore CPC EVs. Gene Ontology analysis demonstrated that these CPC EV-associated proteins were mainly involved in extracellular matrix organization and degradation, ECM proteoglycans and integrin cell surface interactions.

Conclusion: A specific set of EV proteins is identified that may be functionally responsible for activation of endothelial cells upon exposure to CPC EVs. Further studies will reveal their specific involvement in angiogenesis and the cellular and molecular mechanisms of cardioprotection. This may lead to a better mechanistic understanding of the use of CPC EVs as cell-free therapeutics for cardiac repair.

Poster Presentations

NLSEV2019-P7 Nader Kameli

Characterization of faeces-derived bacterial membrane vesicles and the impact of their bacterial origin on inflammatory responses

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Background: The human gastrointestinal tract harbours a diverse and complex microbiome, which interacts in a variety of ways with the host. There's compelling evidence that gut microbial dysbiosis, defined as an alteration of diversity and abundance in intestinal microbes, is an etiological factor in IBD. Membrane vesicles (MVs), which are nano-sized particles released by bacteria, have been found to interact with the host and modulate the development and function of the immune system. As a result MVs have been suggested to play a critical role in both health and disease. In this study we developed a method to isolate, characterize and assess the immunoreactivity of heterogeneous populations of MVs from fecal samples of healthy volunteers.

Methods: MVs were isolated by using a combinations of ultrafiltration and size exclusion chromatography from 10 fecal samples. (Quantification and verification were done with tunable resistive pulse sensing (TRPS) and bead-based flowcytometry, while transmission electron microscopy was used to confirm the presence of MVs. Real time 16s PCR was used on bacterial or MVs DNA to check for the presence and ratio of the most common phyla (Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria). THP-1 cells were used to study the inflammatory character of the MVs; 1*105cells/ml were differentiated and exposed for 24 h to 108 particles/ml of isolated MVs. TNF-α was measured by ELISA.

Results: We could successfully isolated 2*109-2*1010 particles/ml from 0.5 gram of feces. Bead-based flowcytometry in combination with TRPS provide a valuable method for (semi-)quantitative measurements of MVs derived from both Gram+ and Gram- bacteria. PCR results showed differences in the relative abundance between bacteria and the MVs. Moreover, MVs evoke the release of TNF-α by THP-1 cells in a dose dependent matter. Also, a significant positive correlation was found between Actinobacteria/γ-Proteobacteria vesicles and the release of TNF-α.

Conclusion: It has become increasingly clear that MVs could provide an additional layer to the definition of homeostasis or dysbiosis of the microbiota. The current study supports their potential involvement in the intestinal homeostasis or inflammatory disorders and provides incentives for future research.

Poster Presentations

NLSEV2019-P8 Leyla Ayse Erozenci

Protein profiles in urinary exosomes to detect prostate cancer

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To improve prostate cancer (PCa) diagnosis, an accurate minimally-invasive test is preferred. Prostate cancer secreted factors can be found in urine, including extracellular vesicles (EVs). Since EVs are derived of the cell of origin, profiling EVs may provide more sensitive and accurate PCa biomarkers.

We selected 40 urine samples from control (age-matched n=12), early stage (n=17) and advanced (n=11) PCa patients. Urinary EVs were isolated using the VN96-peptide. Proteomics profiling was performed using gel-LC-MS/MS. Data analysis was performed with STRING, Cytoscape, GeneSetEnrichement analysis tool, and DAVID gene ontology software. Independent validation of identified proteins was performed using ELISA. To normalize the expression of the candidate proteins, we measured urine PSA and creatinine for prostate secreted correction, and CD63 and CD9 for EV number correction. A high depth was obtained using LC-MS/MS proteomics, with >3000 proteins identified in the urinary-EVs.

The profiles were enriched for exosome-markers, indicating that we indeed isolated exosomes/ small EVs. Urinary EVs from PCa patients showed an increase in multiple endosomal pathways and an upregulation of a notable number of RAB G-coupled proteins. Candidate proteins were selected based on significance ($p<0.05$), fold change (>2), involvement of (prostate) cancer development/growth. Five candidate proteins underwent validation in a group of 32 patients (to be extended to 68 patients), in small volume of urine: ~4 ml. From the 5 selected proteins, 4 proteins showed a significant increase between control, early and advanced stage disease.

In conclusion, new potential urinary biomarkers for prostate cancer were identified that require large scale validation. Validation of urine biomarkers require normalization (e.g. PSA and EV-number) in order to accurately measure the proteins for clinical application.

Poster Presentations

NLSEV2019-P9 Alba Martín-Barreiro

Thermo-ELISA fluorescence immunoassay for the detection of extracellular vesicles

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Background: The specific and sensitive detection of (subpopulations of) extracellular vesicles (EVs) in biofluids is a challenge. ELISA-like assays are standard practice in the clinic for high throughput biomarker analysis. Adapting such a platform into a sensitive EV detection assay for small numbers of disease-associated EVs in a point-of-care device, would be an important step forward.

Methods and Results: We developed an ELISA-like immunoassay based on a new sensing technology that uses detection antibodies coupled to gold nanoprisms (AuNPrs). These anisotropic nanoparticles exhibit localized surface plasmon resonance in the near infrared region (NIR). The analytes (EVs) are captured in a well using specific immobilized antibodies, after which the AuNPrs-conjugated detection antibodies are allowed to bind to the captured EVs. Irradiation of the wells by a NIR laser source, generates the release of heat proportional to the analyte concentration. These temperature changes can be detected with an infrared camera and quantified using image analysis (ThermoELISA immunoassay). In this way, the ThermoELISA greatly increases the sensitivity (70-fold) with respect to the classic colorimetric ELISA and decreases the limits of detection (LOD) and quantification (LOQ).

This methodology requires thermal sensors to quantify the signal, which is not yet a standard clinical readout technology. For this reason, we made use of the temperature-dependency of fluorescent dyes and have the local heat produced by AuNPrs, reduce fluorescence of dyes (conjugated to detection antibodies) as the assay readout. The combination of both transductions aims to simplify the detection, increase the sensitivity of the method and further reduce the LOD and LOQ. The heat-fluorescence transduction of different fluorophores (AF488, fluorescein, gold nanoclusters) with AuNPrs has been evaluated, showing a significant and easily measurable reduction of fluorescence upon heat production.

Conclusions: The ThermoELISA is a highly sensitive detection method and can be converted to a fluorescence readout. We are adapting the assay to detect of EVs in urine for the diagnosis of prostate cancer. Using specific antibodies and cancer-specific nanobody-phages, labeled with the selected fluorophores and AuNPrs, we expect to be able to identify the few cancer-derived EVs in the large pool of normal urinary EVs.

Poster Presentations

NLSEV2019-P10 Tom Keulers

GABARAPL1 is required for the secretion of pro-angiogenic extracellular vesicles during hypoxia

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Hypoxia is a hallmark of solid tumours and is associated with tumour progression and therapy resistance. In response to hypoxia, tumour cells secrete pro-angiogenic factors to induce blood vessel formation and restore oxygen supply to the tumour. Extracellular vesicles (EVs) are emerging as mediators of intercellular communication in the tumour microenvironment. EVs have the ability to reprogram recipient cells by shuttling biological information such as nucleic acids and proteins, which contributes to tumour progression, angiogenesis and metastasis formation.

In this study we demonstrate that during hypoxia, cells secrete a unique subset of EVs with pro-angiogenic properties, characterized by GABARAPL1 expression on the EV surface. GABARAPL1 is a member of the LC3/GABARAP proteins family, which expression is induced during hypoxia, suggesting a role during these conditions. Silencing GABARAPL1 with inducible knockdown models perturbs GABARAPL1+EV secretion and results in decreased tumour growth due to decreased vascularisation and enhanced necrosis. Additionally, targeting GABARAPL1 directly after tumour irradiation resulted in enhanced tumour regrowth delay

Exosomes are small EVs (70-100nm) which are released into the extracellular environment by fusion of late endosomes/ multivesicular bodies (MVB) with the plasma-membrane. IHC and EM experiments demonstrated that silencing GABARAPL1 resulted in deformed MVB and decreased RAB7 activity, suggesting that GABARAPL1 is involved in the endocytic pathway/ endosome maturation. The exact role of RAB proteins in EV secretion remains to be elucidated.

GABARAPL1 is expressed on the EV surface, and is therefore accessible for targeting with antibodies. GABARAPL1+EVs elicit pro-angiogenic responses in vitro by inducing tube-formation in HUVEC cells. This effect can be fully blocked by GABARAPL1 blocking antibodies. Since we can also detect increased levels of GABARAPL1+EVs in the blood of cancer patients, this could open therapeutic opportunities for concurrent therapies to control tumour growth is therefore interesting to pursue as therapeutic target.

Poster Presentations

NLSEV2019-P11 Sander Kooijmans

A novel capture-and-release platform uncovers functional heterogeneity between extracellular vesicle subpopulations with distinct surface signatures

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Background: Extracellular vesicles (EVs) exist as subpopulations with heterogeneous content and surface composition. This heterogeneity may also be reflected in functional differences between EV subpopulations. For example, given that the EV surface dictates interactions with their environment, EV uptake and processing by recipient cells may differ between subpopulations with different surface profiles. Unfortunately, tools to isolate and functionally compare EV subpopulations based on their surface signature are currently not available. In this study, we showcase novel capture-and-release technology to isolate intact EV subpopulations based on their surface profile and compare their properties.

Methods: EVs were isolated from MDA-MB-231 or SKOV-3 cells using tangential flow filtration(TFF) and size exclusion chromatography (SEC). EV subpopulations expressing specific surface markers were isolated using a novel magnetic bead-based capture-and-release platform. Released EVs were characterized by Nanoparticle Tracking Analysis (NTA), transmission electron microscopy (TEM) and western blotting. Uptake of fluorescently labeled EV subpopulations by various cell types was examined using flow cytometry.

Results: MDA-MB-231 and SKOV-3 EVs isolated by TFF and SEC showed classical EV features, including presence of canonical EV marker proteins, heterogeneous size distribution by NTA and typical “cup-shaped” morphology as examined by TEM. After separating these EVs based on their surface makers using novel capture-and-release technology, EV subpopulations with distinct characteristics were obtained. EV subpopulations appeared intact as demonstrated by TEM, but differed in their mean size and size distribution. Furthermore, western blot analysis revealed that EV subpopulations differed in their canonical EV protein content. Moreover, EV uptake by target cells differed between EV subpopulations and between target cell types.

Conclusions: In this work we present novel capture-and-release technology to isolate intact EV subpopulations based on their specific surface marker expression. We used this platform to show differences between EV subpopulations in terms of protein composition, size distribution and cellular uptake. In theory, the platform is applicable to any surface marker of interest, enabling its use to clarify relationships between the surface signature of EVs and their functionality. Additionally, the platform may be adapted to facilitate enrichment of EVs with desirable characteristics for therapeutic purposes.

Poster Presentations

NLSEV2019-P12 Caitrin Crudden

Inhibiting extracellular vesicle release from breast cancer cells to combat drug resistance

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Background: Despite advances in detection and therapy, breast cancer remains the second leading cause of cancer-related death in women. A large proportion of patients that initially respond to therapy eventually relapse due to resistance mechanisms, that ultimately underpin disease mortality. Production of extracellular vesicles (EVs) is upregulated upon transformation, and has been shown to drive metastatic outgrowth and been linked to therapy resistance. It appears that cancer EV production may be a consequence of an altered metabolism, representing a new vulnerability.

Methods: To address whether selective inhibition of the pathways that drive EV production could sensitive breast cancer cells to therapy, we used paired sensitive/resistant cell sub-lines generated by incremental exposure to pharmaceuticals in vitro. We used HER2 overexpressing cell (BT-474) sub-lines resistant to HER2 monoclonal antibody trastuzumab, and HER2/EGFR tyrosine kinase inhibitor lapatinib, alongside a HER2 low/absent cell line (MCF-7) resistant to the chemotherapeutic paclitaxel. Employing bioluminescence assays to quantify EV release, glucose consumption and lactate secretion, we interrogated the links between EVs, metabolism and drug resistance.

Results: Our panel of breast cancer cell lines upregulate EV release, glucose consumption and lactate production, when compared to a non-tumorigenic breast epithelial control. Both EV release and aerobic glycolysis were upregulated in BT-474 sub-lines displaying resistance to both trastuzumab and lapatinib. Mechanistic links between the two systems are suggested by experiments showing that selective inhibition of EV release returns glucose consumption/lactate production to the levels of sensitive lines. Although the aerobic glycolysis rates appeared upregulated in the MCF-7 paclitaxel resistant sub-line, this mechanism of resistance did not translate into an upregulation of EV release.

Conclusion: Our results suggest that EV production appears linked to the mechanism of resistance underlying HER2 targeting, both by blocking antibody and tyrosine kinase inhibitors. In these instances, inhibition of EVs may represent a feasible approach to combat acquired resistance.

Poster Presentations

NLSEV2019-P13 Britta Bettin

Stable, ready-to-use, pre-labeled EVs for testing new standardization procedures of EV concentration measurements

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Introduction: Extracellular vesicles (EVs) in body fluids are being explored as biomarkers of diseases. Many laboratories use flow cytometers (FCMs) to characterize single EVs, but the measured concentrations of EVs are incomparable between instruments. To improve comparability, development of reference materials and methods are needed. Additionally, validation of new reference materials and methods requires stable biological test samples. Our aim is to develop stable, ready-to-use, and well-characterized biological test samples containing EVs.

Methods: Urine and plasma were collected from healthy donors, and EVs were labeled with lactadherin-FITC. Labeled EVs were isolated by size-exclusion chromatography, after which the isolated EVs were mixed with the cryopreservation agents dimethyl sulfoxide (DMSO), ExoCap, or trehalose, frozen in liquid nitrogen, and stored at -80°C for 1 day up to 5 months. After thawing, EV concentrations were measured by a calibrated FCM (Apogee A60-Micro).

Results: The measured EV concentrations stored for 1 day decreased 27% (p=0.04; mean of the 3 cryopreservation agents) in plasma and 35% (p=0.05) in urine, relative to the fresh starting materials. After 5 months of cryopreservation, the concentration of plasma-derived EVs decreased 2.0% (DMSO and Exocap) and 8.5% (trehalose) compared to 1 day of storage. The concentration of urine-derived EVs decreased of 6-18% after 5 months cryopreservation compared to 1 day of storage.

Conclusions: These preliminary results show that pre-labeled plasma-derived EVs can be stored for up to (at least) 5 months when using cryopreservation agents. Although urine-derived EVs seem less stable, other cryopreservation agents will be tested. These optimized biological test samples will be key to validate newly developed reference materials and methods.

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Poster Presentations

NLSEV2019-P14 Jennifer Perez-Boza

Circulating EV-bound microRNAs to discriminate between cancer patients and healthy volunteers

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Background: Liquid biopsies are a non-invasive alternative to surgical biopsies enabling clinicians to gain information about patient status through a simple blood or urine sample. The advantage of EV-bound microRNAs versus other types of circulating biomarkers (such as ctDNA) is that they provide a more complex view of the systemic response to disease. Our aim is to find a circulating microRNA EV-bound signature to discriminate between healthy volunteers and patients with solid cancers.

Methods: In the training cohort, 9 healthy donors (HDs), 7 esophageal cancer (ESO) and 13 colorectal cancer patients (CRC) were included. EVs were purified from volumes ranging between 1 and 3mL of plasma using size exclusion chromatography and total RNA was isolated with TRIzol following the manufacturer's instructions. A set of microRNAs were detected as a pre-sequencing quality control. Samples that passed the quality control were sequenced (SE, 50bp) using TruSEQ Small RNA Sequencing (Illumina). Trimming and mapping of the libraries against the human miRNome was performed using sRNAbench. We conducted differential expression analysis, hierarchical clustering, ratio research and LASSO regression to find candidate microRNA biomarkers to discriminate between samples from healthy and cancer patients. The findings were validated in a second cohort with a new set of 7 healthy volunteers and 14 additional samples from patients with head and neck squamous cell carcinoma (HNSCC).

Results: We identified a 8-miR signature from circulating microRNAs in plasma that can discriminate between patients with esophageal cancer, colorectal cancer and healthy controls. We validated these results in a second cohort with a new cancer type confirming the potential of a complex signature to discriminate between cancer and non-cancer samples. At the same time, our analysis revealed a series of microRNAs unique to each cancer type as well as a set of microRNAs associated with the presence/absence of cancer.

Conclusions: We have proved the potential of circulating EV-bound microRNA profiling to find complex microRNA signatures to discriminate between patients with cancer and healthy volunteers.

Poster Presentations

NLSEV2019-P15 Laura Varela

Development of a biobanking protocol for robust quantitative and qualitative analysis of synovial fluid-derived extracellular vesicles from healthy and diseased subjects

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Background: Extracellular vesicles (EV) are known to play a role in intercellular communication between cells in both health and disease states. Increasing evidence accumulates exhibiting how EVs could play a role in joint diseases. We are interested to unveil the (patho)physiological roles of EV in synovial fluid. Previously, we defined a storage and isolation protocol for EV from healthy equine-derived synovial fluid (SF). Nonetheless, in the processing of both equine and human-derived SF samples from inflamed joints, we encountered increased aggregation and clotting problems despite the lack of blood contamination by visual inspection. Since these aggregates interfered in quantitative and qualitative EV analysis, due to trapping of EV, we now explore a strategy to optimize the SF storage and EV isolation protocol to enable reliable EV analysis in samples derived from healthy and diseased subjects. We hypothesized that the aggregate structures might be caused by a combination of fibrin clots and DNA, which could be formed e.g. after neutrophil activation.

Methods: SF was obtained from human psoriatic arthritis patients and equine LPS-induced acute synovitis. Thawed cell-free SF samples or fresh SF samples were incubated with hyaluronidase, DNAse I and or EDTA/PBS. EV isolation consisted of differential (ultra)centrifugation followed by sucrose density ultracentrifugation. Collected fractions were analyzed for the formation of aggregates and for EV markers by western blotting.

Results: SF samples from inflamed synovia are prone to develop clot-like aggregates. Although we could demonstrate EV at the proper buoyant density despite the presence of aggregates, we also detected EV markers in high buoyant density fractions. Currently, we are investigating whether addition of DNAse I an EDTA could prevent aggregate formation.

Conclusion: For robust and reliable analysis of SF-derived EV from healthy and inflamed synovia a modified protocol for biobanking of SF and isolation of EV needs to be developed to prevent aggregation.

Poster Presentations

NLSEV2019-P16 Ana Merino

Membrane particles from Mesenchymal Stromal Cells enhance the endothelial regeneration under inflammatory conditions

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Background: Cardiovascular (CV) risk factors such as inflammation have been strongly linked to the dysfunction of endothelial cells (EC), and to an increase in CV events. Mesenchymal stromal cells (MSC) are widely studied as regenerative therapy. However, MSC are large and get trapped in the capillary networks of the lungs after intravenous infusion, where they have a short survival time. Recent work demonstrated that inactivated MSC which lost their capacity to secrete factors maintain their regulatory capacity, suggesting that cell membrane dependent interactions with immune cells are responsible for the regulatory effects. We propose a new cell-free therapy based on the generation of small plasma Membrane Particles (MP) from adipose tissue MSC. We previously showed that MP were effective in reducing the inflammatory phenotype of monocytes. In the present study we investigated the effect of MP on the EC regeneration.

Aim: Study the therapeutic potential of MP as regenerative therapy for EC under inflammatory condition.

Methods: MP from MSC were generated by hypotonic shock and extrusion. Two ratios of EC:MP (1:50000, 1:100000) were tested in an endothelial cell model of inflammation (HUVEC + 25ng/ml of TNFα). The taking up of MP by HUVEC was analyzed by flow cytometry and confocal microscopy. Toxicity was examined at 24, and 48h by apoptosis assay. Monocyte adhesion, transmigration to HUVEC monolayer, and angiogenesis were analyzed by microscopy. The endothelial integrity was evaluated by colorimetric assay.

Results: Both confocal microscopy and flow cytometry showed that within 24 hours >90% of HUVEC have taken up MP. Moreover, the MP appear to end up in the lysosomes of the HUVEC. The toxicity experiment showed that even the highest concentration of MP does not induce apoptosis in HUVEC. Monocytes do not adhere more firmly to the HUVEC after treating them with MP and reduce the number of monocytes that cross the endothelial barrier. MP enhanced the angiogenesis on HUVEC by increasing the number of angiogenic structures compared to the non-treated HUVEC. In the endothelial integrity assay, MP restore the endothelial cell barrier integrity from TNFα-induced leakiness.

Conclusion: Under inflammatory conditions MP enhance the regenerative capacity of endothelial cells.

Poster Presentations

NLSEV2019-P17 Wouter Woud

Imaging flow cytometry: a platform for quantification and phenotyping of human plasma derived extracelular vesicles

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Background: Extracellular Vesicles (EVs) are released by all types of cells and are shown to reflect the physiological status of their source cell. They can be found in bio-fluids such as plasma and urine and are subject to intensive research as potential biomarkers for diseases. Commonly used techniques such as Nanoparticle Tracking Analysis (NTA) or Electron Microscopy are limited in their ability to quantify or phenotype EVs and as such new and improved methods for (single) EV analysis are highly desired by the field. We present a novel platform that allows for the quantification, visualization and phenotyping of single, human plasma derived EVs by utilizing Imaging Flow Cytometery. This technique combines the speed, sensitivity and phenotyping abilities of cytometry with the detailed imagery of microscopy.

Methods: We developed a staining protocol for plasma based on sample dilution. Staining was performed with CFSE, a die that becomes fluorescent after esterase cleavage, to identify a '100% total EV pool'. Secondary stainings were performed using two identical clones of anti-CD31 (same concentration) conjugated with different fluorophores to assess the quantitative and discriminative capabilities of the ImageStreamX (ISX). Detergent treatment was used to discriminate between nanoparticles and artefacts. Acquisition was performed by running each sample for 5 minutes on an ISX Mark II Imaging Flow Cytometer with settings: 40x magnification, low fluidics speed, and appropriate lasers turned on and set on maximum power. Data analysis was performed using improved masking settings within the IDEAS® software.

Results: ISX analysis of human plasma of a healthy individual demonstrated three populations of EVs, namely CFSE+, CD31+ and CFSE+/CD31+. It was observed that a great number of CFSE+ events in fact were not EVs but rather artefacts. CFSE+/CD31+ EVs were detected and CD31-positivity was confirmed with a second antibody within the found population. Post-acquisition analysis with improved masking of the acquired images greatly increased the softwares' sensitivity to detect dimly fluorescent particles, allowing quantification and phenotyping of single EVs.

Conclusion: This work shows the potential of imaging flow cytometry to be a platform suitable for the quantification, visualization and phenotyping of EVs in complex samples such as plasma.

Poster Presentations

NLSEV2019-P18 Onno Arntz

An Optimized Size Exclusion Chromatography Isolation Protocol to Minimize Impurities in Plasma-derived Extracellular Vesicles Samples of Patient's own Immunoglobulins and Antibody-based Therapeutics

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Background: Extracellular vesicles (EVs) present in body fluids can be isolated with different techniques and the optimal isolation method depends on the research questions to be addressed. All conventional methods used for EV isolation from plasma (p) like ultracentrifugation (UC), polyethylene glycol precipitation (PEG), size exclusion chromatography (SEC) are known to co-isolate immunoglobulins (Ig). The presence of Ig's may interfere in functional assessment of pEVs and for this further optimization of the isolation method is needed.

Results: pEVs were isolated by 4 methods of 4 different Rheumatoid Arthritis (RA) patients who received Tumor-necrosis-factor (TNF)-α antibody (etanercept) therapy. Particle-, protein-, IgG-concentration and inhibition of TNF-α was determined by resp. NTA, BCA, ELISA and a TNF-α sensitive reporter cell line. UC and PEG isolated pEVs showed the highest protein and IgG levels. pEV samples isolated by SEC or commercial SEC qEV columns showed the lowest protein and the highest particle concentration although the highest TNF-α inhibition was observed in these samples. To minimize the presence of etanercept we optimized the SEC method by elongate column length from 56mm to 222mm (SEC-2.0). pEVs isolated by SEC-2.0 or by conventional SEC showed no difference in particle concentration although IgG concentrations were significant diminished (>90%). Interestingly, SEC-2.0 pEV samples showed no inhibition of TNF-α anymore. To also confirm the presence of patient's own Ig, in pEV samples obtained from 32 blood donors by SEC-2.0 IgG levels were assessed by ELISA. By NTA using a fluorescence antibody against IgG IgG+ pEVs were determined. A significant positive correlation was found in the presence of IgG+ particles and IgG concentration suggesting no free IgG presence in these samples.

Conclusion: This study shows for the first time the presence of biologicals in pEVs isolated from antibody treated RA patients by conventional methods and the necessity to improve the pEV isolation method. SEC-2.0 isolation resulted in pEV samples without unbound antibodies, yet we confirmed the presence of IgG on pEVs. To study the functional role of pEVs, we advise to check the isolated pEV fraction on the presence of biologicals that patients received.

Poster Presentations

NLSEV2019-P19 Nazma Ilahibaks

Biofabrication of Cell-Derived Nanovesicles: a Potential Alternative to Extracellular Vesicles for Regenerative Medicine

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Extracellular vesicles (EVs) are mediators of intracellular communication by transferring functional biomolecules from their originating cell to recipient cells. This intrinsic ability gained EVs scientific interest in the field of drug delivery as therapeutic delivery vehicle. Furthermore, EVs derived from stem- or progenitor cells can act as paracrine mediators to promote repair and regeneration. Despite substantial research efforts into EVs for therapeutic applications, their use remains limited by the lack of highly efficient and scalable production methods.

Here we present the biofabrication of cell-derived nanovesicles (NVs) as scalable, efficient and economical production alternative to EVs production. We show that NVs have a similar in size and morphology as EVs. Yet, NVs do not share similar EVs surface markers such as CD63 and Alix. Nevertheless, in vitro uptake experiments show that NVs are internalized by cardiac fibroblasts, endothelial- and cardiomyocyte progenitor cells. Regarding NV functionality, this is the first report, to the best of our knowledge, which successfully demonstrates functional protein delivery by NVs delivering Cre recombinase to Cre-loxP reporter cells. Finally, we assessed the functionality of cardiomyocyte progenitor cell-derived (CPC) NVs by investigating its capacity to phosphorylate mitogen-activated protein kinase 1/2 (MAPK1/2)-extracellular signal-regulated kinase 1/2 (ERK1/2) in primary human mammary epithelial cells. We demonstrate that CPC – NVs are capable to phosphorylate ERK1/2 to a similar extent as CPC-EVs. Thus, CPC- NVs as –EVs are capable to activate an important pathway involved in angiogenesis in the wound healing process, cell survival and migration.

Taken together, these observation indicate that NVs have similar delivery and functionality capability as EVs. Hence, NVs have the potential to be applied for various biomedical applications such as therapeutic delivery and regenerative medicine.

Poster Presentations

NLSEV2019-P20 Alberta Giovanazzi

Human milk-derived extracellular vesicles modulate Toll like receptor 3 activation at various molecular levels

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During early life, milk supports the post-natal innate immune responses of the child by providing maternal components. Besides activating maternal components, we previously found that human milk-derived extracellular vesicles (EV) can suppress innate immune responses initiated by endosomal Toll-like receptors (TLR). One such intracellular receptor is TLR3, which recognizes virus-derived double stranded RNA. This study aimed to unravel the molecular mechanism of direct effects of milk EV on TLR3 expression and function.

Oral cavity-derived epithelial cells, endogenously expressing TLR3, were co-cultured with physiological concentrations of purified milk EV or EV-depleted milk control in presence of a TLR3 agonist, after which TLR3 mRNA and protein were determined. A comprehensive genomic and proteomic analysis performed on milk EV linked miRNAs and proteins to the TLR3 signaling pathway.

Upon co-culture with milk EV, the expression of TLR3 gene transcript and other TLR-related genes was downregulated. Genomic analysis identified several miRNAs with known associations with these genes. Furthermore, proteomic analysis revealed the presence of several inhibitory proteins capable of modulating TLR3 signaling, including TLR3 proteolytic cleavage. Since interference with TLR cleavage affects optimal signal transduction we analyzed the presence of TLR3 isoforms and found that both full length and cleaved TLR3 isoform levels were altered.

Milk EV-mediated modulation of TLR3 responses in epithelial cells can occur both during and after TLR3 biosynthesis. This indicates that milk EV can have direct effects on TLR3 expression and function by interfering via multiple molecular regulatory mechanisms. To better understand the physiological role of milk-EV in the development of the innate immune system, we are currently investigating the molecular mechanisms of EV-modulatory effects on different cell types and after different TLR triggers.

Poster Presentations

NLSEV2019-P21 Nathalie Lak

Tumor-specific biomarkers in extracellular vesicles in pediatric neuroblastoma

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Background: Neuroblastoma is the most common extracranial solid tumor in pediatric patients. Current diagnostic strategies involve imaging and histopathology of tumor tissue and bone marrow. These modalities are also performed for detection of minimal residual disease (MRD). In 2008, Stutterheim et al demonstrated that PHOX2b is a specific mRNA marker for neuroblastoma that can be used for detection of MRD in peripheral blood and bone marrow. In this study, we aimed to determine if detection of neuroblastoma-specific RNA and DNA markers is feasible in plasma and if there is a diagnostic potential for extracellular vesicles (EV) in this disease.

Method: Blood was collected in EDTA tubes and was centrifuged to separate plasma from cell pellet. RNA was isolated from whole blood, plasma and cell pellet. Using Size Exclusion Chromatography (SEC), extracellular vesicles were isolated from 500ul plasma. We confirmed the presence of EV with Western Blot, NanoSight and electron microscopy (EM). We isolated RNA and DNA from the different SEC fractions. To detect neuroblastoma-specific RNA, PHOX2b was measured by RT-qPCR. To detect neuroblastoma-specific DNA, ALK F1174L mutation and methylated RASSF1a were measured by respectively digital droplet PCR and RT-qPCR.

Results: From 48 samples taken from patients with neuroblastoma, PHOX2b was detected in 44% of the whole blood, in 31% of the cell pellet and in 8% of the plasma. From one neuroblastoma sample, we isolated EV. By NanoSight and EM we confirmed the presence of EV in the vesicle-rich fractions. We demonstrated the presence of CD9, CD63 and TSG101 in the vesicle-rich fractions of neuroblastoma patients by Western Blot. CD81 was only present in the higher fractions, containing no vesicles but only proteins. The vesicle-rich fractions contained both tumor-derived DNA and mRNA. However, tumor-derived DNA levels were higher in the vesicle-poor, protein-rich fractions.

Conclusion: Plasma from patients with neuroblastoma contains tumor-specific RNA and DNA. Especially the tumor-specific RNA is enriched in EV. This finding suggests that cell-free mRNA from plasma is conserved in EV and implies a novel role for cell-free RNA from liquid biopsies for diagnostic approaches.

Poster Presentations

NLSEV2019-P22 Sabine Bartel

Human airway epithelial extracellular vesicle miRNA signature is altered upon asthma development

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Background: miRNAs are master regulators of signalling pathways critically involved in asthma and are transferred between cells in extracellular vesicles (EV). We aimed to investigate if the miRNA content of EV secreted by primary normal human bronchial epithelial cells (NHBE) is altered upon asthma development.

Methods: NHBE cells were cultured at air-liquid interface and treated with Interleukin (IL)-13 to induce an asthma-like phenotype. EV isolations by precipitation from basal culture medium or apical surface wash were characterized by Nanoparticle Tracking Analysis, Transmission Electron Microscopy and Western Blot, and EV-associated miRNAs were identified by a RT-qPCR-based profiling. Significant candidates were confirmed in EVs isolated by size exclusion chromatography from nasal lavages of children with mild-to-moderate (n=8) or severe asthma (n=9), and healthy controls (n=9).

Results: NHBE cells secrete EVs to the apical and basal side. 47 miRNAs were expressed in EVs and 16 thereof were significantly altered in basal EV upon IL-13 treatment. Expression of miRNAs could be confirmed in EVs from human nasal lavages. Of note, levels of miR-92b, miR-210 and miR-34a significantly correlated with lung function parameters in children (FEV1FVC%pred and FEF25-75%pred), thus lower EV-miRNA levels in nasal lavages associated with airway obstruction. Subsequent Ingenuity Pathway Analysis predicted the miRNAs to regulate Th2 polarization and dendritic cell maturation.

Conclusion: Our data indicate that secretion of miRNAs in EVs from the airway epithelium, in particular miR-34a, miR-92b and miR-210, might be involved in the early development of a Th2 response in the airways and asthma.

Poster Presentations

NLSEV2019-P23 Melissa Piontek

Mechanical properties of extracellular vesicles derived from immortalized adipose stromal cells incubated under normoxic and hypoxic oxygen conditions

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Background: Because of their immunosuppressive, anti-inflammatory, and regenerative properties adipose stromal/stem cells (ASCs) are of great interest in the field of regenerative medicine, e. g. in cell-based therapeutics and treatments of diseases. The pro-regenerative and pro-angiogenic abilities of extracellular vesicles (EVs) produced by ASCs have been previously reported. However, little is known about the mechanics of these EVs and the effect on properties of the EVs produced by stressed cells. Here, we address the influence of the culture conditions (normoxic and hypoxic), and thus the stress applied on immortalized ASCs, on the mechanical properties of EVs produced by those cells.

Methods: The culture medium of differently incubated immortalized ASCs (normoxic and hypoxic oxygen concentration) was collected during two consecutive days, each. The EVs were isolated following an ultracentrifugation protocol. Fresh and frozen samples were investigated by AFM nanoindentation, an approach to probe the mechanical properties of ASC-derived EVs.

Results: Preliminary data was acquired on ASC-derived EVs cultured under normoxic and hypoxic conditions. We discuss factors as size, deformation upon adhesion, stiffness, bending modulus as well as sample quality for AFM measurements.

Conclusions: AFM measurements on EVs require good sample quality in terms of isolation. First measurements were conducted to investigate the mechanical properties of EVs produced by cells under normoxic oxygen conditions and under stress (hypoxic oxygen conditions). Further experiments will be needed for systematic conclusions on fresh and frozen EVs.

Poster Presentations

NLSEV2019-P24 Joëlle Klazen

The effect of vitamin D on the extracelular vesicle communication in bone metastasis

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Background: Bone is a preferential site for cancer metastases, especially from prostate and breast cancer. Here, metastasized cells are resistant to current therapies and create a vicious cycle of negative influences on bone metabolism to stimulate cancer cell growth. Compounds that restore bone integrity, like vitamin D, may be promising therapies in bone metastases. In co-cultures of human osteoblasts (SV-HFO) and bone metastatic prostate cancer cells (PC-3), we found restored bone formation and decrease in cancer cell number after vitamin D (1,25 (OH)2D3) treatment. To study the mechanisms of this possitive effect, we focused on the effects of vitamin D on PC-3 cell derived extracellular vesicles (EVs). Tumor cell-derived EVs are important messengers in the creation of the metastatic niche in bone. We investigated the effect of vitamin D on PC-3 cell EV production, EV mRNA content and interaction with SV-HFOs.

Methods: EVs from vitamin D treated and untreated GFP-expressing PC-3 cells were isolated by differential ultracentrifugation (20,000xg and 100,000xg fractions), and counted by high-resolution flowcytometry (BD influx) and microscopy-image-guided counting (EVQuant). mRNA content was analyzed by high-throughput sequencing. EV-uptake by human osteoblasts (SV-HFO) was measured by flowcytometry (BD Accuri C6).

Results: Vitamin D treatment of the PC-3 cells resulted in a decreased number of cells with a significantly higher number of EVs produced per cell in the 100,000xg fraction only. This 100,000xg EV fraction of the vitamin D treated PC-3 cells had different preferentially packaged mRNA compared to the 100.000xg EVs of the untreated PC-3 cells. Furthermore, EVs from vitamin D treated PC-3s were taken up less by osteoblasts than EVs from untreated PC-3 cells.

Conclusion: Vitamin D changes EV production by bone metastatic prostate cancer cells. It affects their extracellular vesicle count, content and contact with bone. Our data identify mechanisms by which vitamin D may alter the EV communication from metastatic cancer cells to bone cells, and thereby possibly reduce bone metastasis.

Poster Presentations

NLSEV2019-P25 Tom Driedonks

Y-RNA subtype ratios in plasma extracellular vesicles are cell type specific and candidate biomarkers for inflammatory diseases

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Background: Major efforts are made to identify changes in the microRNA (miRNA) and messenger RNA content of patient plasma to discover novel disease-associated biomarkers. MiRNA in plasma can be associated to various macromolecular structures, including extracellular vesicles (EV), lipoprotein particles (LPP) and ribonucleoprotein particles (RNP). Besides miRNA, plasma contains various other non-coding RNA species, of which some are contained in EV. Members of the Y-RNA family have been detected in EV from various cell types and are among the most abundant non-coding RNA types in plasma. We previously showed that shuttling of full-length Y-RNA into EV is modulated by TLR-activation of EV-producing immune cells. This suggested that Y-RNAs may have potential as biomarker for immune-related diseases.

Methods: We separated RNA-containing structures in plasma based on differences in size, density, and resistance to protease/RNase treatment. Using RT-qPCR, we quantified full-length Y-RNA subtypes (Y1, Y3, Y4) in EV from various blood-related cell types cultured with or without LPS-stimulation. In plasma samples from a human endotoxemia study, inflammation-induced changes in Y-RNA were assessed in vivo.

Results: Plasma Y-RNA was mainly found in EV (early SEC-fractions, density 1.11-1.18 g/ml). In contrast, miRNAs were either found in LPP (e.g. miR-122), both in EV and LPP (e.g. miR-16 and miR-21), or in EV (e.g. miR-150). EV-enclosed Y-RNA was resistant to enzymatic degradation, while LPP-bound miRNAs were degradation sensitive. We discovered that EV released by different blood cell types varied in Y-RNA subtype ratios. These ratios remained stable upon LPS-stimulation of the EV-producing cells. In endotoxemia plasma samples, the neutrophil-specific Y4/Y3 ratios and PBMC-specific Y3/Y1 ratios changed significantly during systemic inflammation. Importantly, the plasma Y-RNA ratios strongly correlated with the number and type of immune cells during the inflammation process.

Conclusions: Cell type specific 'Y-RNA signatures' in plasma EV can be determined without prior EV-enrichment, and may be further explored as rapid test to diagnose inflammatory responses or other immune-related diseases.

Poster Presentations

NLSEV2019-P26 Mirthe Dekker

Extracellular vesicle protein levels to detect stress induced ischemia in women presenting with stable chest pain

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Purpose: diagnosis of stable ischemic heart disease (IHD) is complicated, especially in females. Currently, no blood test is available. We assessed if plasma Extracellular Vesicle (EV) protein biomarkers could identify stress induced ischemia as surrogate marker of stable ischemic heart disease in patients presenting with chest pain in the outpatient clinic.

Methods: We analyzed 281 patients with stable chest pain referred for 82Rb PET/CT. Myocardial perfusion was evaluated semi quantitatively according to the 17 segment model of the AHA. Blood samples were collected before PET/CT and Plasma EVs were isolated in 3 plasma sub-fractions (LDL, HDL, TEX) and proteins, identified by proteomics, were quantified in each of these sub-fractions using immuno-bead assays. We used a model (determined with backward selection) to determine the best combination of EV proteins on top of clinical parameters to detect stable IHD.

Results: SerpinG1 and Cystatin C in the HDL fraction showed a significant association with stress-induced ischemia. These biomarkers significantly increased the AUC of the clinical model consisted of history of coronary artery disease from 0.73 to 0.78, P-value <0.001.

Stratified analysis on gender showed that the added value of the biomarkers is completely adjudicated to the effect in females with an AUC increase from 0.73 to 0.87 (P value < 0.001, p value males 0.128).

Conclusion: We identified an EV based biomarker signature that on top of clinical history of CAD is able to diagnose stable ischemic heart disease in females presenting with chest pain.

Poster Presentations

NLSEV2019-P28 Ger Arkesteijn

Improved scatter based flow cytometric detection of particles in the 100 nm range by reduction of optical background signals

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Flow cytometry has become an indispensable tool in biological research. Besides multi-parameter analysis of cells in biological samples, flow cytometry is widely used to sort and purify sub-sets of cells from a heterogeneous population at high speed based on single cell characteristics. For decades, the design of flow cytometers fulfilled the demands to process cells or cell-sized particles. With the research field of extracellular vesicles (EVs) rapidly expanding, there is an increased demand to address heterogeneity of EV populations in biological samples. Although flow cytometry would be the ideal technique to do so, the available instruments are in general not equipped to optimally detect the dim light scatter signals generated by submicron-sized particles like EVs. Whereas sideward scattered light (SSC) and fluorescence are currently used as a trigger signal to identify EVs within samples, the forward scatter light (FSC) parameter is often neglected due to the lack of resolution to distinguish EV-related signals from noise. However, after optimization of FSC detection by adjusting the size of the obscuration bar, we recently showed that certain EV-subsets could only be identified based on FSC. This observation made us to further study the possibilities to reduce noise detection and enhance FSC-detection of submicron-sized particles.

By testing differently sized obscuration bars and pinholes in front of the FSC detection lens, we generated a matrix that allowed us to determine which combination resulted in the best and most robust signal-to-noise ratio regarding FSC detection of submicron-sized particles.

We found that a combination of an 8 mm obscuration bar and a 200 µm pinhole reduced background noise in a reproducible manner to such extent that it allowed a robust separation of 100 nm polystyrene beads from noise within the FSC channel, and even allowed triggering on FSC without the presence of massive background noise signals when both beads and EVs were measured.

These technical adaptations thus significantly improved FSC detection of submicron-sized particles and provide an important lead for the further development and design of flow cytometers that aid in detection of submicron-sized particles.

Poster Presentations

NLSEV2019-P29 Corina-Adriana Ghebes

Expansion of umbilical cord blood-derived hematopoietic stem cells by novel components, identified in bone-marrow niche cell-derived extracellular vesicles

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Introduction: Ex vivo expansion of umbilical cord blood (UCB) derived hematopoietic stem and progenitor cells (HSPCs) is a promising approach to accelerate hematopoietic recovery in patients eligible for UCB stem cell transplantation. Extracellular vesicles (EVs) represent uncovered means of intercellular communication via the transfer of bioactive lipids, proteins and RNAs that can regulate cell fate of EV-target cells. Previous work, performed within our group, showed that bone marrow (BM) derived mesenchymal stromal cells (MSC) can be used as a feeder layer for ex vivo expansion of HSC. In this study we explored whether MSC exert their function by the release of EVs and whether we can develop an innovative EV-component based approach to ex vivo expand clinically relevant numbers of UCB-derived HSC for therapeutic purposes.

Methods: UCB derived CD34+ HSPCs were ex vivo cultured in serum-free medium supplemented with cytokines (SCF and FLt-3) with or without EV-derived from human MSC of different origin (fetal and adult bone marrow). EVs were isolated from cells conditioned medium by sequential centrifugation steps at low (0.3k-2k) and high speed (20k-100k). We assessed the capacity of the HSPC to proliferateand to generate colony forming units. To elucidate the key regulatory components present in EVs we performed protein sequencing library and compared stimulatory versus non-stimulatory EVs.

Results: We identified that BM stromal cells of different origin show distinct HSPC support potential. Adult MSC-derived EVs (stimulatory) show a significant increase in HSPC proliferation when compared to fetal MSC-derived EVs (non-stimulatory) and cytokine control. Protein sequencing analysis comparing stimulatory and non-stimulatory EVs identifies a total of 2664 proteins, with 42 differentially expressed proteins found in stimulatory EVs, respectively 94 in non-stimulatory EVs. Functional protein analysis using STRING, suggests the involvement of fatty acid oxidation proteins in stimulation of HSPC proliferation. Contrarily, involvement of TGF-beta signalling proteins suggests a diminished effect of fetal MSC-derived EVs on HSPC proliferation.

Conclusion: Our study gives insights into the complex biological role of EVs in HSPC-stromal cell crosstalk and promotes the utility of EVs and their cargo as therapeutic agents for expansion studies.

Poster Presentations

NLSEV2019-P30 T. Qin

Variability in isolation and characterization of extracellular vesicles using different methodologies

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Extracellular vesicle (EV) based biomarkers holds great potential in disease diagnosis. However, methods for isolation and detection of EVs vary highly between research groups. Using different EV isolation methods might have great impact on the EV profile discovered in downstream analysis. In this study we therefore characterized three different EV isolation methods, based on their yield, variability and reproducibility.

EVs were isolated from pooled plasma of six healthy volunteers and concentrated SK-N-SH (neuronal) cell derived culture media. Nanoparticle tracking analysis (NTA) was used to measure the particle concentration and size distribution of the different EV isolates. Total protein concentrations of each yield were measured using the BCA assay. Western blotting was used to characterize EV enriched protein markers (CD9, CD63, and Flotillin-1) in the isolates as well as contaminating proteins (ApoE and Albumin). EVQuant (Erasmus MC) was used to measure the quantity of total and CD9 and CD63 labeled EVs.

NTA measurements showed particles matching the size of EVs, with all three isolation methods. However, CD9, CD63 and Flotillin-1 measurements of these samples showed great variability between the different methods, as well as between plasma and SK-N-SH cell culture samples. This might indicate that different isolation methods tend to enrich different subpopulations of EVs. Furthermore, none of the tested methods provided isolated EVs that included all the tested EV protein markers. Interestingly, the particle to protein ratios were higher in plasma derived qEV fractions compared to the Exoquick and Exo-spin methods.

Overall, we found great variability between the three used methods with respect to EV isolation. These findings implicate that the choice of isolation method can have great impact on the downstream EV analysis.

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NLSEV2019 Izon

Single Particle Measurement for confidence in Biology; New information on samples purification

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Introduction: Extracellular Vesicles (EVs) derived from biological fluids possess extensive heterogeneity with regards to size, number, membrane composition and cargo. Tremendous research interest exists towards development and use of EV fraction of biofluids as rich sources of diagnostic and prognostic biomarkers. High precision fractionation of the nano-biological content of biofluids can dramatically reduce background, increase purity, and inform on the biology of the biomarkers and therapeutic biomolecules.

Method: Size exclusion chromatography (SEC) is the most standardizable technique, already widely used for the purification of EVs from biofluids. Significant improvement to the use of SEC is possible through automation and precision. Here, we developed a range of SEC columns of various sizes, with 2 resin types, separating down to 35nm or 70nm. We also developed a low-cost prototype automatic fraction collector (AFC) that adds high precision, improves repeatability, speeds up workflow. RFID tags are proposed to ensure high quality of data capture and transfer. Moreover, Tunable Resistive Pulse Sensing technology was used for accurate, high-resolution particle analysis (size, size range, concentration, and electrophoretic mobility) and normalization.

Results: SEC columns provide a convenient, reproducible, and highly effective means of eliminating >99% of non-vesicular protein from biological fluid samples, and separating exosomal and non-exosomal volumes for further downstream analysis. 35 nm pore sized SEC gel leads to increased resolution, higher yield and one fraction earlier elution of EVs from plasma compared to the 70 nm pore size. Use of AFC allowed precise massbased measurements and tunability within 30ul of volume exiting the column. Most importantly, due to the additional functionality provided by AFC, the EV field needs to revisit the way that fraction numbers, post-SEC are used. That will be replaced with a more logical framework, wherein the void volume is measured and disposed of, and precise volumes are used instead of the somewhat arbitrary fraction numbers.

Conclusion: Thus, the qEV-AFC platform allows for QA, high-precision EV volume collection and minimizes samples processing related reproducibility issues for clinical studies.

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NLSEV2019 Cell Guidance Systems

Purification of high-quality exosomes using Exo-spin™ from Cell Guidance Systems: A novel application overview and new product announcement

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Exosome isolation from cerebrospinal fluid (CSF) is commonly investigated to discover potential protein candidates for diagnostics in central nervous system diseases. However, the isolation step is still challenging and an efficient method for isolating exosomes from small volumes of CSF needs to be identified. Our Exo-spin™ kit has been successfully used in a proteomics study with exosomes isolated from CSF from multiple sclerosis patients. In addition to exosome isolation, Exo-spin™ columns can be used to remove excess detection dye. Moreover, Exo-spin™ mini-HD, a new size exclusion chromatography column for high-definition exosome isolation, will be announced.

NLSEV2019 Beckman Coulter

Method transfer, scale up, standardisation & characterisation. A short update on EV solutions from the perspective of Beckman Coulter

Lutz Ehrhardt, Senior Marketing Manager Centrifugation Europe