NLSEV
Netherlands Society for Extracellular Vesicles

Abstract and Programme Book NLSEV-2021

Fourth annual meeting of the

Netherlands Society for Extracellular Vesicles (NLSEV)

Friday November 12th 2021

KIT Royal Tropical Institute, Mauritskade 63, 1092 AD

Amsterdam

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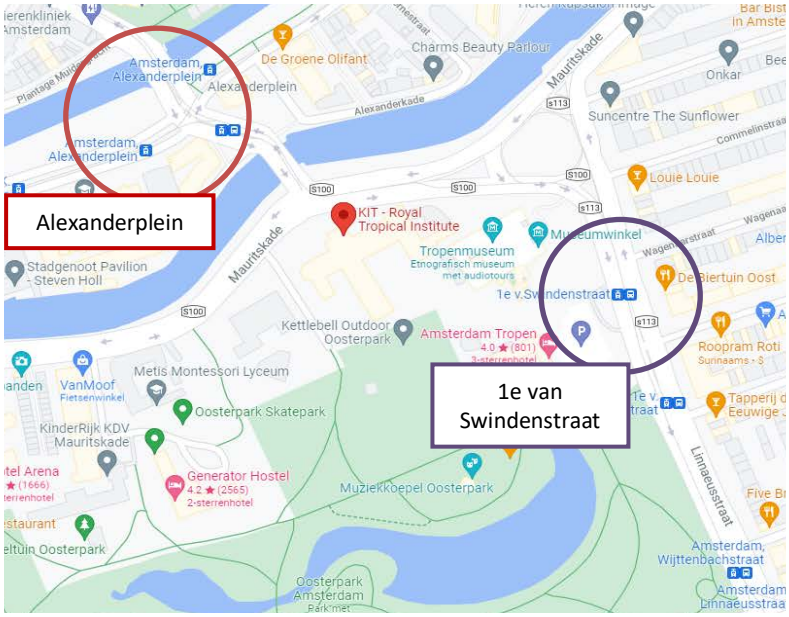
Practical information

Route to KIT by public transport (recommended):



Travel to tram stops Alexanderplein or Linnaeusstraat 1e van Swindenstraat, which are both right in front of the KIT:

From Amsterdam Central Station:
Tram 14 → tram stop Alexanderplein

From Amsterdam Sloterdijk:
Tram 19 → tram stop Alexanderplein

From Diemen:
Tram 19 → tram stop Linnaeusstraat 1e van Swindenstraat

Route to KIT by car:
Take the Watergraafsmeer/Diemen (s113) exit from the A10 ring road. At the end of the road choose the exit heading to Centrum/Watergraafsmeer. Continue through via the Middenweg that turns into the Linnaeusstraat. Koninklijk Instituut voor de Tropen is located at the end of this street on the corner of Mauritskade. A limited number of paid KIT parking spaces is available in the Linnaeusstraat, which need to be reserved upfront. Plenty of paid street parking is also available in the surrounding area. Alternatively, a parking garage is two tram stops away from the KIT (tram 19):
Q-Park Oostpoort, Polderweg 92, 1093 KP Amsterdam.

COVID-19 regulations
In line with Dutch government policy, it is mandatory for all attendants of NLSEV-2021 to present a valid proof of COVID-19 vaccination, negative test or recovery from COVID-19 using a digital or printed COVID certificate. After approval of the certificate, no additional safety measures apply in the KIT building.

Programme

Programme

9:00 – 9:30 **Registration**

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

9:45 – 10:35 **Keynote Lecture** *Chairs: Esther Nolte 't Hoen and Raymond Schiffelers*

Clotilde Théry *Institut Curie Centre de Recherche, Paris, France*

Exosomes, other extracellular vesicles and co-isolated factors in cross-talk between tumors and the immune system

10:35 – 11:05 **Selected presentations: EV biogenesis & release**

Frederik Verweij *Utrecht University, Utrecht, The Netherlands*

ORP1L ER Membrane Contact Sites support endosomal RAB conversion for exosome secretion

Laura Varela *Utrecht University, Utrecht, The Netherlands*

Acute joint inflammation induces a sharp increase in EV release and modifies the phospholipid profile of synovial fluid-derived EVs

11:05 – 11:40 **Coffee break & Posters meet and greet**

11:40 – 12:25 **Selected presentations: EV technology** *Chairs: Rienk Nieuwland and Marca Wauben*

Nazma Ilahibaks *UMC Utrecht, Utrecht, The Netherlands*

TOP-EVs: Technology of CRISPR/Cas9 Protein delivery through Extracellular Vesicles

Olivier de Jong *Utrecht University, Utrecht, The Netherlands*

Extracellular-vesicle mediated delivery of CRISPR/Cas9 by targeted modular aptamer-based loading and UV-activated cargo release

Martijn van Herwijnen *Utrecht University, Utrecht, The Netherlands*

Pre-processing of bovine milk prior to EV isolation is essential for purity, but various protocols affect colloidal and functional properties of milk EVs

12:25 – 12:35 **Sponsored presentation: IZON Science**

Extracellular Vesicles: Automating your Research for Isolation and Accurate Single Particle Characterisation

12:35 – 13:50 **Lunch & Posters meet and greet**

13:50 – 14:50 **Selected presentations: EV Biomarkers** *Chairs: Fons van de Loo and Kasper Rouschop*

Aleksandra Gąsecka *Amsterdam UMC, Amsterdam, The Netherlands*

Leukocyte extracellular vesicles as the first biomarkers to predict outcomes in patients undergoing percutaneous aortic valve replacement

Programme

Cristina Gómez-Martín *Amsterdam UMC, Amsterdam, The Netherlands*
EV-miRNA biomarkers signatures for treatment response monitoring in refractory Multiple Myeloma patients
Wouter Woud *Erasmus MC Rotterdam, Rotterdam, The Netherlands*
Extracellular vesicle subsets released during normothermic machine perfusion are associated with human kidney characteristics
Domenico Maisano *University "Magna Graecia" of Catanzaro, Catanzaro, Italy*
A novel phage display based platform for exosome diversity characterization

14:50 – 15:00 **Sponsored presentation: NanoView**
ExoView - Next Generation Product Launch - Customizable assays for detecting up to 5 biomarkers on individual EVs and viruses

15:00 – 15:10 **Sponsored presentation: Dispertech**
Nanocet: Measure size of single external vesicles with high resolution

15:10 – 15:20 **NLSEV General Assembly**

15:20 – 16.05 **Coffee break & Posters meet and greet**

16:05 – 16:55 **Keynote Lecture** *Chairs: Michiel Pegtel and Pieter Vader*
David Lyden *Weill Cornell Medicine, New York, USA*
Extracellular vesicles and particles promote metastatic organotropism and systemic disease

16:55 – 17:40 **Selected abstracts 4: EVs in cancer**
Caitrin Crudden *Amsterdam UMC, Amsterdam, The Netherlands*
Inhibiting EV release from breast cancer cells combats drug resistance
Tom Keulers *Maastricht University, Maastricht, the Netherlands*
Secretion of pro-angiogenic extracellular vesicles during hypoxia is dependent on the autophagy-related protein GABARAPL1
Venkatesh Rao *Technische Universität Dresden, Dresden, Germany*
TIMP1-dependent remodelling of the extracellular matrix is mediated by extracellular vesicles in colorectal liver metastases

17:40 – 17:50 **NLSEV Awards & Closing (Local Organizing Committee)**

17:50 – 19:00 **Drinks & Posters meet and greet**

Oral Presentations

Oral presentations

Keynote Speaker 1:

Clotilde Théry

INSERM U932, Institut Curie Centre de Recherche, PSL Research University, Paris, France

Exosomes, other extracellular vesicles and co-isolated factors in cross-talk between tumors and the immune system

All cells, including tumors, release diverse types of extracellular vesicles (EVs), which can transfer complex signals to surrounding cells. Tumor-derived EVs have been often described as promoting tumor growth and/or metastasis, in particular by manipulating the anti-tumor immune responses. However, different types of EVs, originating from different subcellular compartments (e.g. endosome-derived exosomes, plasma membrane-derived ectosomes or microvesicles), are likely to bear different functions. Specific markers to distinguish these different classes of EVs are still difficult to define. Furthermore, in many EV isolation methods, EV preparations also contain co-isolated components, that can contribute to the EV functions studied. Consequently, the specific composition and functions of subtypes of tumor-derived EVs and the other extracellular nanoparticles still need to be defined. We will present our recently developed approaches to identify putative markers of subtypes of EVs among heterogeneous EV populations released by tumor cells, ways to distinguish functions of EVs and co-isolated non-EV components within EV preparations, and our recent observations that, in triple-negative breast cancer, tumor-derived EVs may carry anti-tumoral, and thus beneficial functions for the patient.

Oral Presentations

Keynote Speaker 2:

David Lyden

Meyer Cancer Center, Weill Cornell Medicine, New York, USA

Extracellular vesicles and particles promote metastatic organotropism and systemic disease

In 1889, Stephen Paget first proposed that organ distribution of metastases is a non-random event, yet metastatic organotropism remains one of the greatest mysteries in cancer biology. A growing number of studies demonstrate that tumor-derived microvesicles, referred to as exosomes, may alter the tumor microenvironment at future sites of metastasis promoting pre-metastatic niche formation, and thus creating a favorable “soil” for incoming metastatic “seeds.” However, by what mechanism, and whether their role is significant in tumor progression remains unknown. We have recently demonstrated that exosomes released by lung-, liver- and brain-tropic tumor cells preferentially fuse with resident cells at their future metastatic sites, such as fibroblasts and epithelial cells in the lung, Kupffer cells in the liver, and endothelial cells and microglia in the brain. We found that tumor-derived exosome homing to organ-specific cell types prepares the pre-metastatic niche. Proteomic profiling of exosomes revealed distinct integrin expression patterns associated with each organ-specific metastatic site. Whereas exosomal α6β4 integrin was associated with lung metastasis, exosomal integrin αvβ5 was linked to liver metastasis and αvβ3 was associated with brain metastasis. Tumor cells secrete a heterogenous population of exosomes. To further characterize and determine the functional contribution of the heterogenous population of exosomes, we employed the state-of-art technique of asymmetric field flow fractionation system. From exosomes derived from different tumor types, our work revealed two major exosomal subpopulations whose hydrodynamic diameters are 60-80nm (exosome small; Exo-S) and 90-120nm (exosome large; Exo-L), respectively. Importantly, we report a novel nanoparticle population, defined as exomeres, that are distinct from conventional exosomes based on their remarkably different morphology and smaller size (<50nm). For isolation of exosome and exomere profiling, we developed a modified protocol based on differential ultracentrifugation to isolate exosomes/exomeres from viable human sources, such as surgically resected tumors and adjacent tissues as well as from bodily fluids, such as plasma. The diversity of these samples necessitates robust and highly reproducible protocols and refined isolation technology, as as asymmetric flow field-flow fractionation. Specific isolation protocols allows for preparation of exosomes and exosomes for various downstream applications, including proteomic profiling. Thus, proteomic profiling of exosome subpopulations can serve as potential biomarkers for early cancer detection, prognosis and determining treatment response.

Oral Presentations

NLSEV2021-O1: Frederik Verweij

ORP1L ER Membrane Contact Sites support endosomal RAB conversion for exosome secretion

Frederik J. Verweij* [1,2,6], Maarten P. Bebelman [1,3,4], Mickael Couty [1], Anaïs Bécot [1], Roberta Palmulli [1,2], Xavier Heiligenstein [2], Julia Sires-Campos [2], Graça Raposo [2], D. Michiel Pegtel [3], Guillaume van Niel [1,5]

**: Presenting author; 1: Institute for Psychiatry and Neurosciences of Paris, Hopital Saint-Anne, Université de Paris, INSERM U1266, Paris, France; 2: Institut Curie, PSL Research University, CNRS, UMR144, Paris, France; 3: Department of Pathology, Cancer Center Amsterdam, Amsterdam University Medical Center, Amsterdam, The Netherlands; 4: Division of Medicinal Chemistry, Amsterdam Institute for Molecules Medicines and Systems, VU University, Amsterdam, The Netherlands; 5: GHU Paris Psychiatrie et Neurosciences, Hôpital Sainte Anne, Paris, France; 6: Division of Cell Biology, Neurobiology and Biophysics, Utrecht University, Utrecht, The Netherlands*

Exosomes are endosome-derived Extracellular Vesicles (EV) involved in intercellular communication. They are generated as intraluminal vesicles within endosomal compartments that fuse with the plasma membrane (PM). The molecular events that generate secretory endosomes that lead to the release of exosomes are not well understood. We identified the compartment of origin of CD63 positive exosomes as a subclass of non-proteolytic endosomes at prelysosomal stage. These compartments undergo a Rab7a/Arl8b/Rab27a GTPase cascade to fuse with the plasma membrane (PM). Dynamic ER-LE Membrane Contact Sites MCS via ORP1L have the distinct capacity to modulate this process by affecting LE motility, maturation state and small GTPase association. Thus, exosome secretion is a multi-step process regulated by GTPase switching and MCS, highlighting the ER as a new player in exosome-mediated intercellular communication.

Oral Presentations

NLSEV2021-O2: Laura Varela

Acute joint inflammation induces a sharp increase in EV release and modifies the phospholipid profile of synovial fluid-derived EVs

Laura Varela *[1], Chris van de Lest [1,2], René van Weeren [1], Marca Wauben [2]
**: Presenting author; 1: Division of Equine Sciences, Department of Clinical Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands; 2: Division of Cell Biology, Metabolism & Cancer, Department of Biomolecular Health Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands*

Background: Extracellular vesicles (EVs) are essential for intercellular communication and are secreted by all types of cells. They are modified after an acute inflammatory insult. Most joint disorders have inflammation as their common denominator. This study aimed to investigate the lipidome profile of EVs from synovial fluid collected from healthy horses and animals with a Lipopolysaccharide (LPS)-induced synovitis at 5h post-induction (acute inflammation phase), 24h, and 48h (resolution phases). We analyzed the typical biomembrane-derived non-neutral lipids, i.e., glycerophospholipids and sphingolipids.
Methods: Equine synovial fluid (SF) was collected pre-LPS-injection from the middle carpal joints of Warmblood horses at 0 hours, followed by synovitis induction with an intra-articular injection of 3.5 ng LPS from E. coli. Subsequently, SF was harvested at 5 hours, 24 hours, and 48 hours. EVs were isolated by differential ultracentrifugation at 10,000g/200,000g and purified according to buoyant density by gradient-ultracentrifugation. Lipids were extracted by the Bligh & Dyer method and analyzed using the Orbitrap Fusion mass spectrometer. Subsequent data processing, visualization, and statistical analyses were performed using R packages and/or GraphPad software.
Results: We identified more than 150 lipid species within nine lipid classes. During synovitis, there is an increase of EVs present in the synovial cavity that at 24 hours declines until reaching baseline levels. Moreover, healthy EVs showed a different lipid profile than the 5h and 24h EVs from inflamed joints, whereas at 48h, the lipid profile was more similar to 0h EVs. Moreover, the amount of hexosylceramide (HexCer) in 5h, 24h, and 48h EVs rose compared to 0h EVs. In addition, phosphatidylethanolamine (PE) relatively increased. At the same time, phosphatidylserine (PS), phosphatidylcholine (PC), and sphingomyelin (SM) decreased in the composition of EVs from both 5h and 24h EVs compared to 0h and 48h EVs. We further identified that specific lipid species played a key role in changing the healthy lipid profile into the inflammatory profile and later into the resolution lipid profile in SF.
Conclusion: An inflammatory stimulus augments the number of EVs present in the synovial cavity during acute synovitis. Moreover, inflammation profoundly impacts the phospholipid bilayer composition of SF-EVs.

Oral Presentations

NLSEV2021-O3: Nazma Ilahibaks

TOP-EVs: Technology of CRISPR/Cas9 Protein delivery through Extracellular Vesicles

Nazma F. Ilahibaks*,# [1], Arif Ibrahim Ardisasmita [1]#, Zhiyong Lei [2]#,Pieter Vader [1,2] and Joost Sluijter[1,3]
**: Presenting author; #: Authors contributed equally; 1: Laboratory of Experimental Cardiology, Department of Cardiology, University Medical Center Utrecht, 3584 CX Utrecht, The Netherlands; 2. Department of Clinical Chemistry and Hematology, University Medical Center Utrecht, 3584 CX Utrecht, The Netherlands; 3: Circulatory Health Laboratory, Regenerative Medicine Center, University Medical Center Utrecht, University Utrecht, 3584 CX Utrecht, The Netherlands*

Background: The discovery of the gene-editing system clustered regularly interspaced short palindromic repeats (CRISPR) combined with the *S. pyogenes* CRISPR-associated endonuclease protein 9 (Cas9) has opened new doors in gene therapy. The CRISPR/Cas9 system is a robust, highly efficient and simplictic method to mediate site-specific genome modification. It application in cardiovascular disease may cure or prevent genetic diseases in the future. Despite its advantages, CRISPR has its own inherent safety and efficacy limitations which need to be resolved for its clinical application. Effective CRISPR/Cas9 delivery without persistent acitivity in target cells causing undersired off-target effects remains challenging. To address these challenges, we investigated extracellular vesicles (EVs) mediated CRISPR/Cas9 ribonucleoprotein (RNP) delivery. EVs are endogenous nanocarriers and are considered to have high biocompatibility, low immungenocity and intrinsic targeting abilities. We harness EVs delivery capabilities by loading CRISPR/Cas9 RNP inside via chemical-inducible dimerization system together with the vesicular stomatitis virus glycoprotein (VSV-G), creating TOP-EVs. Via TOP-EVs mediated CRISPR/Cas9 RNP delivery we aim to develop a safe and effective gene therapy whereby the endogenous mediated delivery enabling transient genome-editing reduces risk of off-target effects.
Method: The protein loading inside EVs is facilicated via chemical inducible heterodimerization system FRB-FKBP together with VSV-G. TOP-EVs are produced by transfection of the chemical inducible heterodimerization system FRB-FKBP-Cas9, VSV-G and/or sgRNA plasmid in HEK293FT producer cells. Post-transfection, rapamycin is added to the medium of the producer cells. After 72h, the TOP-EVs are isolated from the conditional medium via differential ultracentrifugation and characterized via western blot, nanoparticle tracking analysis and reporter assays.
Results: We confimed Cas9 containing TOP-EVs produced by HEK293FT cells via Western blot and Nanoparticle Tracking Analysis. We found the TOP-EVs system promotes CRISPR/Cas9 loading inside EVs up to a 8-fold. Consequently, TOP-EV mediated CRISPR/Cas9 delivery induce genome editing with ±30% efficiency in Cas9-stoplight reporter cells. Furthermore, the TOP-EVs can be employed for protein delivery in multiple cell types including myoblast, induced pluripotent stem cell-derived fibroblast human umbilical vein endothelial cells and HepG2 cells. Evidence shows TOP-EV uptake is facilitated by clathrin-dependent endocytosis and micropinocytosis.
Conclusion: TOP-EVs are a promising delivery system which can be harnassesed for therapeutic protein delivery and CRISPR/Cas9-mediated genome editing.

Oral Presentations

NLSEV2021-O4: Olivier de Jong

Extracellular-vesicle mediated delivery of CRISPR/Cas9 by targeted modular aptamer-based loading and UV-activated cargo release

Omnia M. Elsharkasy [1], Olaf L. Cotugno [1], Ingmar Y. de Groot [1], Willemijn S. de Voogt [1], Jerney J. Gitz-François [1], Raymond M. Schiffelers [1], Pieter Vader [1, 2], Olivier G. de Jong* [1, 3].
**: Presenting author; 1: CDL Research, University Medical Center Utrecht, The Netherlands; 2: Department of Experimental Cardiology, University Medical Center Utrecht, The Netherlands; 3: Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, The Netherlands.*

Background: CRISPR/Cas9 is a prokaryotic endonuclease capable of targeting and editing genomic sequences with high specificity and efficiency. As such, CRISPR/Cas9 holds tremendous therapeutic potential for the treatment of genetic pathologies. One of the major hurdles for the development of CRISPR/Cas9-based therapeutics is the intracellular delivery of the Cas9-sgRNA ribonucleoprotein (RNP) complex because of its large size, negative charge, and immunogenicity. Extracellular vesicles (EVs) hold the potential to overcome this hurdle due to their biocompatibility and intrinsic capability of highly efficient intercellular transfer of RNA molecules and proteins.
Methods: To facilitate targeted loading of the RNP complex, sgRNAs with high-affinity MS2 coat protein-interacting aptamers were generated and expressed alongside Cas9 and EV-enriched proteins fused to the MS2 coat protein. The MS2 coat protein, lacking the Fg loop to prevent capsid formation, was cloned in tandem on the N-terminus of CD9, CD63, CD81 and ARRDC1 or the C-terminus of Δ687-PTGFRN or a myristoylation sequence, linked by a UV-sensitive photocleavable protein (PhoCl). Cas9 loading and UV-mediated PhoCl cleavage were measured by Western Blot analysis. To study Cas9 delivery, we used a previously published fluorescent stoplight reporter system which is activated by Cas9 activity (De Jong et al, Nat Commun. 2020).
Results: EV loading of Cas9, as well as UV-mediated cleavage of the PhoCl fusion proteins, was confirmed by Western Blot analysis. Using EVs with MS2-PhoCl-CD63 fusion proteins we observed efficient Cas9 delivery (14.5%), but only after UV-treatment of EVs and co-expression of the VSV-G glycoprotein. Comparing RNP delivery efficiency using various EV-targeted fusion proteins revealed that CD9 and the myristoylation sequence showed notably high delivery of Cas9, followed by CD63, Δ687-PTGFRN, CD81, and lastly ARRDC1. Western Blot analysis revealed that these results strongly correlated to Cas9 loading in EVs.
Conclusions: Here, we describe a novel modular platform for EV-mediated loading and delivery of Cas9 RNPs. Our results demonstrate that EVs are indeed capable of functional Cas9-RNP delivery and that Cas9 loading and delivery was strongly dependent on the targeted loading protein that was employed. Moreover, these data indicate that additional modifications for regulated cargo release and endosomal escape strongly increase Cas9-RNP delivery.

Oral Presentations

NLSEV2021-O5: Martijn van Herwijnen

Pre-processing of bovine milk prior to EV isolation is essential for purity, but various protocols affect colloidal and functional properties of milk EVs

Martijn J. C. van Herwijnen [1]*, Marije Kleinjan [1], Francesca Loria [2], Andres Lõhmes [2], Natasa Zarovni [2], Anna Carnerup [3], Tommy Nylander [3], Andrea Ridolfi [4], Marco Brucale [4], Francesco Valle [4], Lucrezia Caselli [5], Lucia Paolini [6], Paolo Bergese [6], Marca H. M. Wauben [1]

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Background: Although bovine milk is rich in extracellular vesicles (EVs), other abundant components like casein micelles hamper the isolation of pure EVs. Pre-clearing milk by precipitation or disruption of caseins overcomes this issue. However, the impact of pre-clearing on EV integrity and function has not been investigated. We compared three different protocols used to reduce casein micelles to untouched milk and analyzed the effects of pre-processing on subsequently isolated EVs.
Methods: Raw bovine milk was subjected to differential centrifugation after which milk remained untouched, or caseins were cleared by precipitation due to acidification with hydrochloric acid (HCl), or caseins were disruption by addition of EDTA or sodium citrate. EVs were further purified using density gradient floatation and Size Exclusion Chromatography (SEC). EV purity was assessed by Augmented COlorimetric NANoplasmonic (CONAN), total protein, phospholipid and cholesterol; presence of EV markers by Western blotting (WB); morphology and size by Cryo-Electron Microscopy, Atomic Force Microscopy (AFM), Nano Tracking Analysis (NTA) and Dynamic Light Scattering (DLS). Additionally, colloidal properties including Zeta potential, membrane stiffness and adhesion to lipid surfaces were determined. Functionality of EVs was analyzed in a Xanthine Oxidase activity assay and an in vitro T cell modulation assay.
Results: Clearing casein micelles from bovine milk prior to density gradient separation and SEC greatly enhanced the purity of the milk EV samples in all tested methods, with acidification giving the purest samples. However, precipitation of caseins by acidification influenced size, lipid surface interactions and gave a loss in vitro functionality. EDTA treatment also affected EVs in their size, but also membrane stiffness and T cell modulation. Only sodium citrate treatment did not influence most of the evaluated EV properties.
Conclusions: Using a comprehensive analysis, we were able to show that pre-processing of bovine milk is needed and greatly enhances purity of milk EV samples, but can also influence the functionality and colloidal properties of milk EVs. Therefore, for isolation of bovine milk EVs, we advise to carefully consider which pre-processing to apply. Based on our results we recommend the use of sodium citrate for the clearance of caseins in bovine milk.

Oral Presentations

NLSEV2021-O6: Aleksandra Gąsecka

Leukocyte extracellular vesicles as the first biomarkers to predict outcomes in patients undergoing percutaneous aortic valve replacement

Aleksandra Gąsecka* [1,2,3], Edwin van der Pol [2,3,4], Krzysztof J. Filipiak [1], Janusz Kochman [1], Zenon Huczek [1], Radosław Wilimski [1], Rienk Nieuwland [2,3]
** Presenting author; 1: 1st Chair and Department of Cardiology, Medical University of Warsaw, Poland; 2: Laboratory of Experimental Clinical Chemistry; 3: Vesicle Observation Center; 4: Biomedical Engineering and Physics, Amsterdam University Medical Center, University of Amsterdam, Amsterdam, The Netherlands*

Background: Transcatheter aortic valve implantation (TAVI) is a novel treatment for aortic stenosis (AS), associated with better outcomes than surgical aortic valve replacement in high-risk patients. However, up to 29% of patients annually experience major adverse cardiac and cerebrovascular events (MACCE) after TAVI. MACCE are mostly caused by atherothrombosis, i.e. formation of platelet aggregates (thrombi) on ruptured atherosclerotic plaques. Because platelets release extracellular vesicles (EVs) during thrombus formation, we hypothesized that EVs are a biomarker to predict MACCE after TAVI.
Methods: This was a multicentre, prospective clinical study. Venous blood was collected 1 day before TAVI and at hospital discharge (n=57, mean age 79.9+6.4 years, 49% male). Flow cytometry (Apogee A60-Micro) was used to determine concentrations of plasma EVs labelled with markers for endothelial cells (CD146), leukocytes (CD45), platelets and megakaryocytes (CD61) and activated platelets (CD62p). Analysis of flow cytometry data files was fully automated. Rosetta Calibration (Exometry) and Flow-SR were used for diameter and refractive index determination. Wilcoxon signed rank test was used to compare EV concentrations before and after TAVI. The predictive value of EVs for MACCE and the cut-offs were calculated using a receiver operating characteristic curve. Logistic regression model incorporating EV concentrations and clinical characteristics was used to determine the best model for MACCE prediction.
Results: Concentrations of EVs from activated platelets increased, whereas from leukocytes decreased after TAVI, compared to the measurement before (p=0.06, p=0.04, respectively). Among 55 patients discharged from the hospital, 14 patients experienced MACCE (25%) during the median 15 months of observation. Increased baseline concentration of leukocyte EVs and male gender were the only independent predictors of MACCE in multivariable analysis (OR 4.01, 95% CI 0.77 - 23.77, p=0.04 for leukocyte EVs; OR 6.84, 95% CI 1.41 - 33.17, p=0.03 for male gender).
Conclusions: We identified increased concentrations of leukocyte EVs as new candidate biomarkers to predict MACCE after TAVI. Leukocyte EVs could be used to augment risk stratification in this patient cohort. The next step is to validate the clinical applicability of EVs to predict post-TAVI MACCE in a large-scale clinical trial.

Oral Presentations

NLSEV2021-O7: Cristina Gómez-Martín

EV-miRNA biomarkers signatures for treatment response monitoring in refractory Multiple Myeloma patients

Cristina Gómez-Martín*[1,2],Esther E.E. Dress[1], Nils Groenewegen[1,3], Catharina G. M. Groothuis-Oudshoorn [4], Inger Nijhof [5], Johan de Rooij[2,3], Niels van der Donk [5], D. Michiel Pegtel[1]
**: Presenting author; 1: Department of Pathology, Cancer Center Amsterdam, Amsterdam UMC, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands; 2: You2Yourself B.V., Enschede, The Netherlands 3: ExBiome B.V., Amsterdam, The Netherlands; 4: Department of Health Technology and Services Research, Technical Medical Centre, University of Twente, Enschede, The Netherlands; 5: Department of Hematology, Cancer Center Amsterdam, Amsterdam UMC, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands*

Background: Multiple Myeloma is a very aggressive cancer type that despite several therapeutic advances remains largely incurable. Several new treatments are being developed but it remains crucial to determine as early as possible if patients respond to treatment so if it is not the case a new one can be applied. The most commonly used marker for this is the M-protein level in both blood and urine. However, this IgG protein produced in great quantities by myeloma cells, has very low specificity and sensitivity. MiRNAs are well known for their role in negative regulation of gene expression. Moreover, its expression is often deregulated in disease and of high interest for therapeutic purposes[1] as they are shed by cancer cells into the circulation, both free and bound to EVs. We propose a different approach to the response to therapy measurement problem using EV-miRNAs signatures.
Methods: We isolated EV-MiRNAs from blood samples from patients responding and not-responding to treatment with Isoseek[2] protocol, perfomed miRNA profiling with sRNAbench and finally Lasso regression models were built.
Results: We have found that when comparing the EV-miRNAs profiles of 16 Multiple Myeloma patients at Progressive Disease (PD) stages to 16 patients that were into remission (RM) and after Lasso Modelling we can get a model that with only 9 miRNAs can perfectly separate both groups. We tested this signal in an independent dataset (18 PD vs 21 Remission) and with an AUC of 0.889 we can clearly validate it and separate patients that are not responding to treatment from those who actually do it.
Conclusions: We present here a tentative model based on only 9 miRNAs that can be used to predict the response to treatment in Multiple Myeloma patients with high sensitivity and specificity. Therefore EV-miRNAs seem a promising new biomarker approach of response to treatment in patients with refractory multiple myeloma.

[1]Rupaimoole, et al. MicroRNA therapeutics: Towards a new era for the management of cancer and other diseases. Nature Reviews Drug Discovery 16, 203–221 (2017).
[2]Eijndhoven, M. A. J. van et al. Unbiased and UMI-informed sequencing of cell-free miRNAs at single-nucleotide resolution. bioRxiv 2021.05.04.442244 (2021).

Oral Presentations

NLSEV2021-O8: Wouter Woud

Extracellular vesicle subsets released during normothermic machine perfusion are associated with human kidney characteristics

Wouter W. Woud* [1], Asel S. Arykbaeva [2,3], Ian P.J. Alwayn [2,3], Ana Merino [1], Carla C. Baan [1], Robert C. Minnee [4], Martin J. Hoogduijn [1] , Karin Boer [1]

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Background: Extracellular Vesicles (EVs) represent stable, tissue specific nano-sized particles that reflect the conditional state of their tissue of origin. Normothermic Machine Perfusion (NMP), aimed at restoration of cellular metabolism and function to organs, offers the possibility to assess graft status prior to transplantation through analysis of biomarkers in the perfusion fluids. Here, the dynamic release and phenotype of kidney EVs released during NMP were analyzed to examine whether EVs could function as a potential biomarker for assessing kidney quality before transplantation.

Methods: Eight discarded kidneys (~13 ± 5 hours of cold ischemia, age 68 ± 7 (mean ± standard deviation), all male) were perfused in a closed system at 37 0C for 6 hours. Perfusates were taken before and at 1, 3 and 6 hours and examined with Nanoparticle Tracking Analysis (NTA) and Imaging Flow Cytometry (IFCM). For IFCM, perfusates were stained with the tetraspanin EV markers CD9, CD63 or CD81, or a mix of the three markers in combination with CFSE to identify, quantify and characterize EVs.

Results: Analysis of perfusates with NTA revealed that the majority of nanoparticles present in the perfusates are <240 nm. Using IFCM, we selectively studied these small (≤ 240 nm) nanoparticles. For CFSE and the mix of tetraspanin double-positive EVs, we observed a ~700 / 740 / 560 fold increase compared to EV levels before perfusion at 1, 3 and 6 hours of NMP, respectively. Especially after 1 hour of NMP, double-positive EV levels were found to be positively correlated with donor age whilst negative correlations were found for cold ischemia time. Furthermore, tetraspanin CD81 was found to represent the majority (~70%) of the excreted double-positive EV (CD9: ~15% / CD63 <10%).

Conclusion: EVs are excreted during NMP with highest excretion levels during the first hour of perfusion. Tetraspanin CD81 is predominantly present on these EVs. The characterization of the excreted EVs as well as their correlation with clinical parameters provide a starting point to study their role as potential biomarkers of kidney quality.

Oral Presentations

NLSEV2021-O9: Domenico Maisano

A novel phage display based platform for exosome diversity characterization

Domenico Maisano*[1], Selena Mimmi [1], Eleonora Vecchio [1], Annamaria Aloisio [1], Nancy Nisticò [1], Giuseppe Fiume [1], Ileana Quinto [1], Enrico Iaccino [1]

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Background: Exosomes are a subtype of extracellular vesicles, 30-150 nm in diameter, with an essential role as mediators of both physiological and pathological processes. By now, a wide array of efficient protocols for exosomes purification have been published, unfortunately without fulfilling the need for rigor and standardization to shed light on exosomes diversity. During the last years, we successfully validated the screening of random peptide libraries as a method to identify phage-derived peptide binders for the idiotypic determinants of the immunoglobulin B Cell Receptor (IgBCR) which was shared also on the surface of relative tumor derived exosomes (TDE). To verify the suitability of this technology even in absence of an unequivocal exosome marker, in this work we adopted the Eμ-myc transgenic mice lacking in immunoglobulin expression.
Methods: A random phage-display library (CX7C) was intravenously injected into Eμ-myc tumor-bearing mice and 20 independent phages were randomly chosen after three biopanning cycles. Phages binding capability was validated by FACS and confocal analysis on tumor cells in vitro and by in-vivo imaging on tumor bearing mice in vivo. Then, we used phage (ϕ) 8 to specifically sort tumor derived exosomes, and, by qRT-PCR, we analyzed their miRNA cargo composition.
Results: FACS and confocal microscopy analyses indicated ϕ8 as the best tumor cells binder within selected phages. Moreover, ϕ8 showed the capability to target and recognize, in vivo, the tumor mass in mice as showed by in-vivo imaging. Then, electron and atomic force microscopy results indicated ϕ8 specificity for TDE-trapping and sorting. Lastly, we analyzed the expression of a predefined set of miRNAs in total, CD63-positive, and ϕ8-positive exosomes, highlighting that ϕ8-positive exosomes showed an independent miRNA expression profile with respect to total and CD63-positive exosomes.
Conclusions: Here we presented an alternative way to select and sort a specific exosome subpopulation, with differences in miRNAs enrichment with respect to other exosomes subpopulations. Adapting our validated approach to the enormous possibilities offered by patient-derived xenograft models, the reported in vivo phage-display-based platform could represent the cornerstone for a comprehensive molecular characterization of disease-related exosomes."

Oral Presentations

NLSEV2021-O10: Caitrin Crudden

Inhibiting EV release from breast cancer cells combats drug resistance

Caitrin Crudden* [1], Maarten Bebelman [1,2], Katinka Langedijk [1], Margriet Jonk [1], Connie Jimenez [3], Bart Westerman [4], Michiel Pegtel [1].
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Background: Despite the advent of targeted therapies for breast cancer treatment, a large proportion of patients will eventually relapse due to resistance mechanisms. There are currently no drugs available that selectively target the most malignant subpopulations of drug-resistant breast cancer cells and re-sensitizing such cells would hugely benefit patients. Extracellular vesicle (EV) release has been linked to therapy resistance, and crucially, has been a central modality across a wide range of mechanisms of resistance, drugs and cancer types, whereby EV release appears to give a survival advantage. It is therefore conceivable that selective inhibition of the pathways that drive EV production or release from cancer cells has therapeutic potential. We hypothesized that aggressive and resistant cancer cells activate EV production/release pathways which are distinct from non-malignant cells. In this project we aimed to identify clinically feasible inhibitors of EV release to combat drug resistance.
Methods/Results: We compiled a panel of human breast cancer cell lines spanning disease sub-type and drug resistance, finding that EV secretion is indeed increased in aggressive, resistant and metabolically-rewired (preference for glycolysis) cell lines, compared to non-malignant or sensitive controls. Since the commonly used exosome marker CD63, is itself involved in biogenesis, we sought a reporter system that limited exogenous interference. We developed an endogenous bioluminescent reporter for EV secretion that could sensitively and robustly report EV secretion, amenable for high-throughput screening. Using CRISPR/Cas9-technology we generated HA-NanoLuc-CD63 knock-in MCF7. We performed a broad-spectrum kinase inhibitor screen of 400 compounds, and validated potential hits for toxicity, pharmacokinetics and cellular morphology changes. In support of our original hypothesis, we found that Bemcentinib, identified as a putative inhibitor of EV secretion in MCF7, increases the sensitivity of a paclitaxel-resistant MCF7 subline to paclitaxel-toxicity. In a long term clonogenic assay, co-treatment of Bemcentinib and Paclitaxel synergize to prevent paclitaxel-resistant MCF7 from producing progeny, when compared to either agent alone.
Conclusion: We find evidence that blocking underlying EV secretion pathways is feasible by kinase control, and able to resensitize resistant cells to first-line agents, representing an alternative treatment strategy.

Oral Presentations

NLSEV2021-O11: Tom Keulers

Secretion of pro-angiogenic extracellular vesicles during hypoxia is dependent on the autophagy-related protein GABARAPL1

Tom G. Keulers* [1], Sten F. Libregts [2], Kim G. Savelkouls [1], Johan Bussink [3], Hans Duimel [4], Marijke I. Zonneveld [1], Karel Bezstarosti [5], Jeroen A. Demmers [5], Carmen Lopez Iglesias [4], Marc Vooijs [1], Marca Wauben [2], Kasper M.A. Rouschop [1]

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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. Exosomes and microvesicles, collectively termed 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. 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. In this study we demonstrate that the autophagy-related protein GABARAPL1, is required for endosomal maturation and cargo loading of EVs during hypoxia.
Furthermore, we demonstrate that GABARAPL1 is expressed on the surface of EVs released during hypoxic conditions and that these GABARAPL1+EVs have pro-angiogenic properties. Silencing GABARAPL1 in inducible knockdown models perturbs GABARAPL1+EV secretion and results in decreased growth of xenografted tumours due to decreased vascularisation and enhanced necrosis. Additionally, targeting GABARAPL1 directly after radiotherapy resulted in enhanced tumour regrowth delay, demonstrating the therapeutic potential of these observations.
Since GABARAPL1 is expressed on the EV surface, it is 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. Tthis could open therapeutic opportunities for concurrent therapies to control tumour growth is therefore interesting to pursue as therapeutic target.

Oral Presentations

NLSEV2021-O12: Venkatesh Rao

TIMP1-dependent remodelling of the extracellular matrix is mediated by extracellular vesicles in colorectal liver metastases

Venkatesh Rao* [1], Qianyu Gu [1], Christoph Kahlert [1].
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Background: The molecular reprogramming of stromal microarchitecture by tumour-derived extracellular vesicles (EVs) is proposed to favour pre-metastatic niche formation. TIMP1 overexpression has been implicated in invasion and metastasis, although its role in the remodelling of the liver metastatic milieu remains undefined. Our study aimed to elucidate the role of extravesicular tissue inhibitor of matrix metalloproteinase-1 (TIMP1EV) in pro-invasive extracellular matrix (ECM) remodelling of the liver microenvironment to aid tumour progression in colorectal cancer.
Methods: TIMP1 expression was examined using immunohistochemistry (IHC) on a panel of 81 primary tumours (colorectal cancer; CRC) and 80 colorectal liver metastases resections (CRC liver MET). Using primary fibroblast cultures, we investigated whether CRC-derived extracellular vesicles (CRC-EVs) induced TIMP1 upregulation in the recipient cells. Using our 3D ECM remodelling assay, we evaluated whether TIMP1 upregulation in the recipient fibroblasts promotes ECM remodelling. Inhibition of TIMP1 and its effect on ECM was evaluated by western blotting and 3D ECM-remodelling assays.
Results: IHC analyses of primary colon tumour and liver metastatic tissue samples from patients with CRC revealed higher stromal TIMP1 levels in CRC liver MET than in CRC. The elevated TIMP1 signature in the invasive front was associated with poor progression-free survival in patients with CRC liver MET. Molecular analysis identified TIMP1EV enrichment in CRC-EVs as a major factor in the induction of TIMP1 upregulation in recipient fibroblasts. Mechanistically, our data provide evidence that TIMP1EV-mediated TIMP1 upregulation in recipient fibroblasts induced ECM remodelling, and that TIMP1 inhibition impaired the effects of CRC-EV-mediated ECM remodelling.
Conclusions: These results demonstrate the role of CRC-derived TIMP1EV as ECM modulators, hence suggesting the targeting of TIMP1 as a potential avenue for the prevention of liver metastasis in the future.

Sponsor Presentations

Sponsored Presentations

IZON Science: Stephane Mazlan

Extracellular Vesicles: Automating your Research for Isolation and Accurate Single Particle Characterisation

Efficient isolation and accurate quantification and size determination are imperative in nano biological studies involving extracellular vesicles (EVs). The progression from bench to the bedside, a hallmark of translational research and application, renders both quality and accuracy of EV isolation and measurement increasingly critical. Indeed, advancement in isolation and measurement technology has evolved to meet this need. Accurate measurements can now be done efficiently, rapidly and easily.

Ultracentrifugation has been deemed the gold standard for EV isolation. However, this often results in a reduced yield due to damaged or lost EVs, urging researchers to balance between yield and purity. EVs isolated by Size Exclusion Columns (SEC), however, have been shown to not only preserve EV morphology but also provide higher EV purity attested by a higher level of EV marker detection. Furthermore, the recent launch of our Gen 2 qEV columns allows for a higher EV purity attested by a higher EV-protein ration as well as a higher EV concentration.

In terms of EV measurement, high resolution is an important aspect. It has been shown that optical techniques lack the resolution necessary to for accurate and precise nanoparticle measurements especially when it comes to multimodal samples. This is highly important especially in measuring complex nano biological particles such as EVs where size heterogeneity is an aspect. The level of detail and certainty that Tunable Resistive Pulse Sensing (TRPS) offers is indeed beneficial in the EV field. This technology has demonstrated precision in both size and concentration determination where subpopulations in multimodal samples can be accurately portrayed and distinguished. Capable of measuring EVs as small as 30 nm, our current TRPS instrument, The Exoid, provides not only the accuracy and resolution needed in EV measurements, but it also automises them, contributing to efficiency in data collection.

The EV research community is beginning to make advances in developing EVs as a new class of biological therapeutics. The downstream translation applications range from gene therapy to drug delivery systems. The use of mesenchymal stem cell-derived EVs, for example, are demonstrated to elicit beneficial effects therapeutically and eliminate stem cell transplantation risks. As the potential for EV therapeutics grows, so does the need for precise isolation and measurement of these complex nano biological particles – a challenge that Izon strives to achieve.

More information: www.izon.com

Sponsor Presentations

NanoView: Dan Croucher

ExoView - Next Generation Product Launch - Customizable assays for detecting up to 5 biomarkers on individual EVs and viruses

NanoView recently launched our latest analytical platform for purification-free analysis of extracellular vesicles and viruses. ExoView works by affinity capturing exosomes onto a protein microarray (ExoView chip) via common or custom exosomal markers. Once bound to the surface of the ExoView chip each individual exosome can be sized, counted and characterized in terms of protein expression.

The latest R200 platform adds an additional fluorescent channel and EVs can now be imaged in 4 fluorescent channels (blue, green, red and far-red) meaning that individual exosomes can be phenotyped by up to 5 surface or cargo proteins (4 fluorescent and 1 capture antibody) with single-molecule sensitivities. To complement the new platform, NanoView will also be discussing existing and new assays designed to enable EV and virus research. Standard assays as designed to measure common EV markers from cell culture or plasma samples (with or without purification) and can be extended to detect EV cargo. These assays have been optimized to promote biomarker colocalization on even the smallest EVs and have been extended to include fully user customizable assays that allow users to capture and detect EVs via any custom protein of their choice. We will review newly developed assays for detecting lenti-viruses and their capsids and, lastly, we will provide an overview of assays designed to provide 'Total EV Counts' (not just protein-specific EV counts) as well as exosome concentration assays.

More information: www.nanoviewbio.com

NanoView BIOSCIENCES

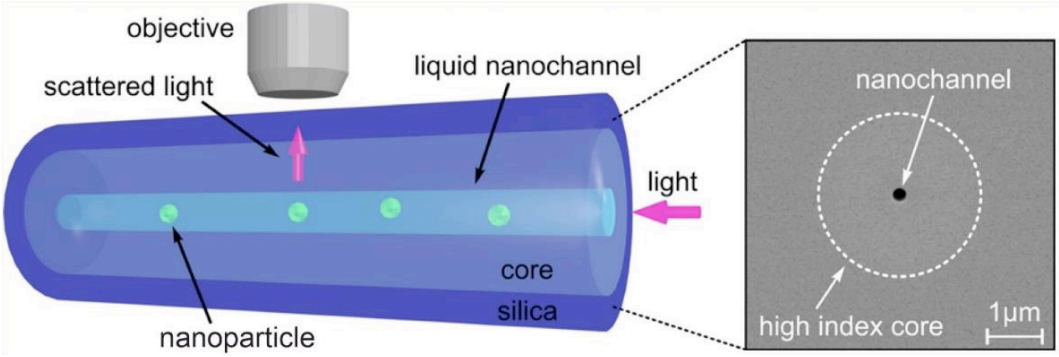
Sponsor Presentations

Dispertech: T. Vazquez Faci

Nanocet: Measure size of single external vesicles (EVs) with high resolution

The NanoCET is a user-friendly tabletop system to measurement the size (20 – 150 nm) and scattering intensity of EVs at high-resolution. Low sample volume (5µl) and no need to label.

The NanoCET technology uses the scattered light of single nanoparticles to measure the scattering intensity and diffusion coefficient of individual nanoparticles as they travel along a nanofluidic channel (see figure). A high level of accuracy is achieved by acquiring data over exceptionally high numbers of frames. Longer time-traces are guaranteed due to the spatial confinement of nanoparticles inside a nanochannel at the centre of an optical fiber. The combination of diffusion and scattering intensity measurements are unique to the NanoCET technology.



More information: www.dispertech.com

Poster Presentations

Poster presentations

NLSEV2021-P1: Jana Ramon

Photoporation for loading of extracellular vesicles with exogenous molecules: a platform technology to study their cell interaction specificity

Jana Ramon* [1], Stephan Stremersch [1], Edward Geeurickx [2], An Hendrix [2], Koen Raemdonck [1], Kevin Braeckmans [1].
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Background: Evidence is growing that EVs are important players in cell-to-cell communication. It is believed that EVs have a unique surface fingerprint that steers their cell tropism. To study this type of intercellular communication, EVs need to be labeled in order to monitor where and how they are taken up by target cells. It remains a challenge, however, to label EVs in a manner that does not interfere with their surface properties. For this purpose, we propose photoporation as pre-formation EV loading method. This technique uses photothermal nanoparticles and pulsed laser light to create transient pores in the cell membrane through which fluorescent labels, are able to passively diffuse directly into the cell's cytosol. We hypothesized that by first delivering labels in the cytosol of parent cells, they would subsequently be internalized in the lumen of EVs during biogenesis.
Methods: As EV producer cell line, we used HEK293T cells transiently transfected with the GAG-EGFP fusion protein. To load EGFP-labeled EVs, labeled dextrans were used at first, which do not specially interact with intracellular structures. In addition, labeled nanobodies were tried, targeted toward EGFP-labeled EVs. Dextrans or nanobodies were first delivered into the cytosol of in vitro-cultured HEK293T GAG-EGFP cells using photoporation. After 48h, EVs were purified from conditioned medium and EV loading efficiency was assessed with a spinning disk microscope. At last, purified EVs were co-incubated with HEK293T recipient cells and uptake of cargo-loaded EVs was evaluated via flow cytometry.
Results: We demonstrated that a fraction of the delivered dextrans or nanobodies was spontaneously passed from the parent cell's cytoplasm into the formed EVs, confirming that EVs indeed obtain their cargo from the cytosol. Furthermore, we were able to track the loaded EVs after uptake by recipient cells in a co-culture experiment. The fraction of recipient cells that had taken up nanobody-loaded EVs was twice as high compared to dextran-loaded EVs indicating an improved EV loading efficiency.
Conclusions: Altogether these results hint toward the potential of photoporation for EV loading, which could facilitate the study of EV behavior under physiologically relevant conditions.

Poster Presentations

NLSEV2021-P2: Joël Beaumont

Metabolic reprograming of the tumor microenvironment by cancer cell derived extracellular vesicles

Joël E.J. Beaumont* [1], Tom G.H. Keulers [1], Marijke I. Zonneveld [1,2], Kasper M.A. Rouschop [1]
**: Presenting author; 1: Department of Radiotherapy, GROW-School for Oncology and Developmental Biology, Maastricht University; 2: Department of Pathology, GROW-School for Oncology and Developmental Biology, Maastricht University*

Introduction: Metabolic reprograming has emerged as one of the hallmarks of cancer. In addition to cancer cell intrinsic metabolic alterations, cancer cells also manipulate their surroundings to provide a growth supporting tumor microenvironment (TME). These metabolic changes support tumor growth by providing nutrients and support tumor progression through the stimulation of angiogenesis and immune suppression. Hypoxia, a common feature of tumors, is associated with a more aggressive disease progression. Here, we aim to elucidate the role of (hypoxic) cancer cell derived extracellular vesicles (EV) in the metabolic reprograming of the TME.
Methods: HT29 (colorectal cancer), U87 (glioblastoma) and MDA-MB-231 (breast cancer) cells were cultured under normoxia, moderate hypoxia (0.2% O2) or severe hypoxia (<0.02% O2) for 24h in medium with 5% EV depleted serum. Subsequently, EV were isolated by size exclusion chromatography. Glucose uptake and lactate secretion of endothelial- and cancer cells, monocytes and fibroblasts were measured after stimulation with 1µg/ml EV for 24 hours. For metabolomics, cells were stimulated with 1µg/ml EV for 24h (cancer cells) or 48h (endothelial cells) in medium supplemented with 13C-labeled glucose, after which metabolites were analysed by mass spectrometry.
Results: Stimulation with HT29/U87/MDA EV increased glucose uptake in endothelial- and cancer cells, monocytes and fibroblasts. Conversely, lactate secretion was not affected in endothelial, HT29, and monocytes stimulated with HT29 EV. Furthermore, lactate secretion was decreased in endothelial- and cancer cells and fibroblasts upon U87/MDA EV exposure. No significant differences were found between EV from either normoxic, moderate hypoxic or severely hypoxic cells. Metabolomic analysis demonstrated increased amino acid uptake by endothelial cells and increased intracellular amino acid levels in cancer cells upon stimulation with EV.
Conclusion: Cancer cells manipulate the TME through the production of EV. Changes in uptake and intracellular concentration of amino acids suggest an effect of EV on the secretome of recipient cells. Additionally, EV increase glucose consumption of target cells and reduce their lactate production, suggesting the (in)activation of specific metabolic pathways. Future experiments will focus on elucidating metabolic pathways affected by EV, the changes in recipient cell secretome and the functional consequences on tumor progression.

Poster Presentations

NLSEV2021-P3: Marieke Roefs

Cardiac progenitor cell-derived extracellular vesicles are retained in the murine heart upon intramyocardial injection

Marieke T. Roefs* [1], Wolf Heusermann [3], Maike Brans [1], Pieter Vader# [1,2], Joost P. G. Sluijter# [1]
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Background: Administration of cardiac progenitor cell-derived extracellular vesicles (CPC-EVs) upon myocardial infarction has the potential to modulate different reparative processes in the heart. In order to develop CPC-EVs as a regenerative treatment, a better understanding of their retention in the heart and in vivo distribution after cardiac administration is required. Here, we investigated if CPC-EVs are retained in the murine heart after intramyocardial (IM) injection using different imaging techniques.
Methods & Results: CPCs were cultured in serum-free medium for 24 hours and EVs were isolated from their conditioned medium using size exclusion chromatography (SEC). EVs were labelled using the AlexaFluorTM 790 or 647 NHS Ester and subsequently separated from quenched free dye using SEC. The concentration and size of EVs was assessed using nanoparticle tracking analysis. Five µL of labelled EVs were either injected IM in the left ventricle or systemically via tail vein of Balb/c mice. Assessment of their in vivo distribution in living animals using the Pearl Imager demonstrated that large amounts of CPC-EVs were retained in the murine heart up to 5 days after administration. Over time, EVs could also be increasingly detected in the liver. Fluorescent analysis of lysates of individual organs confirmed EV presence in the heart and their limited distribution to other organs. In contrast, intravenously injected CPC-EVs could mainly be detected in the liver, already within 15 min after administration. CUBIC tissue clearing and subsequent light sheet fluorescence microscopy demonstrated that CPC-EVs were present in the area of injection but were also found to distribute deeper into the myocardium 15 min post IM injection.
Conclusions: These results demonstrate that CPC-EVs are retained in the heart after IM injection. This is promising for the development of EVs as therapeutics since it suggests that there is no direct need for techniques to increase EV retention.

Poster Presentations

NLSEV2021-P4: Katinka Langedijk

Development of a bioluminescent reporter for EV secretion

Maarten Bebelman [1,2], Caitrin Crudden [1], Catharina J.M. Langedijk* [1], Steven Eleonora [1], Leontine Bosch [1], Frederik Verweij [3], Monique van Eijndhoven [1], Martine Smit [2], Michiel Pegtel [1]
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Background: Extracellular vesicles (EVs) are increasingly recognized for their role in intercellular communication. It has become clear that EV-mediated communication is deregulated in various pathologies, including cancer. Cancer cells have been shown to release increased amounts of EVs with oncogenic cargo and pro-tumorigenic properties. Despite the clear clinical potential of modulating EV-mediated communication, there are currently few pharmacological opportunities for modulating EV secretion. This lack of knowledge and the limited availability of EV-modulating drugs are a direct consequence of the technical difficulties associated with studying EV secretion.
Methods/Results: Currently, the main method of EV quantification is based on the concentration of EVs from culture supernatant by ultracentrifugation, followed by direct particle measurements or western blot against EV marker proteins, such as the tetraspanin CD63. This time-consuming and labor-intensive process that requires large volumes of culture medium, precludes the screening of larger drug libraries. There is still an unmet need for a novel approach that allows for robust, fast and scalable quantification of EV secretion that is compatible with both genetic and pharmacological screens. We have developed HA-NanoLuc-CD63 (HA-NL-CD63) as a bioluminescent reporter for EV secretion that is amenable for high-throughput screening. The generation of endogenous HA-NL-CD63 reporter cells using CRISPR/Cas9-technology enabled us to study EV secretion whilst circumventing the disadvantages associated with CD63 overexpression. Our results indicate that in basal conditions, HEK293 cells secrete the majority of their EVs as microvesicles, independent of exosome-biogenesis machinery. We preformed a broad-spectrum kinase inhibitor screen that identified inhibitors of this process, such as ROCK inhibitors. In parallel, we used the vATPase inhibitor bafilomycin to stimulate (endosome-derived) exosome secretion, and identified a novel lipid kinase. We could validate this model of microvesicle versus exosome secretion preference using CD9, a marker more often associated with plasma membrane-budding microvesicles. The secretion of HA-NL-CD9 knock-in cells showed sensitivity only to those compounds identified in the basal (-bafilomycin) screening conditions.
Conclusion: Overall, this study demonstrates the potential of HA-NL-CD63/CD9 as tools for high-throughput screening for modulators of EV secretion.

Poster Presentations

NLSEV2021-P5: Margarida Viola

Cardiac progenitor cell-derived EVs skew human macrophages towards a pro-inflammatory phenotype

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Background: Repair of damaged heart tissue upon myocardial infarction remains a major challenge. Transplantation of cardiac progenitor cells (CPCs) has been studied as a potential regenerative therapy, but recent studies have shown that the cardioprotective effect of CPCs is mediated by the release of extracellular vesicles (EVs). The benefits of CPC-EVs have mostly been associated with stimulation of angiogenesis and inhibition of cell death. Although macrophages have been suggested to be key for cardiac repair, the effect of CPC-EVs on macrophage polarization is poorly explored.
Methods: EVs were isolated from serum-starved CPCs by ultrafiltration followed by size exclusion chromatography. Human monocytes were isolated from the blood of healthy donors and differentiated into macrophages with M-CSF. Macrophages were subsequently stimulated with LPS + IFNy or with IL4 in order to induce an inflammatory M1 and reparative M2 phenotype, respectively. A third group of macrophages were cultured in media without LPS, IFNy or IL4 to resemble naive M0 macrophages. The obtained macrophages were exposed to CPC-EVs and analyzed by flow cytometry, bulk RNA sequencing and confocal microscopy to assess macrophage phenotype changes.
Results: Stimulation of macrophages with CPC-EVs enhanced the expression of the pro-inflammatory marker CD80, while slightly decreasing the anti-inflammatory marker CD206, in M0 and M2 macrophages. CPC-EV-stimulated macrophages also adopted a morphology that reflects the inflammatory macrophage. In line with these findings, bulk RNA sequencing on M0 and M2 polarized macrophages revealed upregulation of genes involved in inflammatory response for both type I and II interferon signaling when treated with CPC-EVs. In contrast, EV exposure did not induce significant differences in gene expression in M1 polarized macrophages compared with PBS-treated control. Ongoing investigations will provide in-depth insight into the mechanism of action by which CPC-EVs induce this response in macrophages.
Conclusions: Our data suggests that CPC-EVs are able to induce macrophage polarization towards an inflammatory phenotype, which might have implications for CPC-EV treatment after myocardial infarction. This underlines an urgent need to understand the molecular mechanisms underlying the immunomodulatory effect of CPC-EVs before moving into a clinical setting.

Poster Presentations

NLSEV2021-P6: Monique van Eijndhoven

Unbiased and UMI-informed sequencing of plasma EV-associated miRNAs at single-nucleotide resolution

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Background: Recent advances show that apart from 21nt mature miRNAs, miRNAs with non-templated additions (NTAs), called isomiRs have biological relevance that can dramatically change their pre-processing, stability and targetome. Deconvolution of these posttranscriptional modifications is challenging in particular for low-input samples, e.g. plasma extracellular vesicles (pEV) that are considered as a source for minimally-invasive diagnostics. Both for biological studies as well as for diagnostic purposes accurate detection and distinction of mature miRNAs and isomiRs is essential.
Methods: RNA sequencing is currently the only reliable method that can distinguish mature miRNAs from isomiRs with single-nucleotide resolution and that can truthfully reveal isomiR diversity in biological samples. Improvements aside, current small RNA sequencing strategies remain imprecise. We developed “IsoSeek” that diverges from these methods by making use of randomized 5’- and 3’-adapters combined with a 10N unique molecular identifier (UMI).
Results: Using synthetic mature miRNA and isomiR spike-ins in multiple optimization rounds, we show that IsoSeek has reduced ligation and PCR amplification bias leading to improved accuracy in the detection of isomiRs. To investigate the detection of isomiRs in a biological background, we sequenced HEK293T cells in which the terminal uridylyl transferases TUT4 and TUT7 were ablated by CRISPR/Cas9 (TUT4/7 DKO). The overall reduction in uridylation of miRNAs is much more pronounced when using IsoSeek compared to commercial protocols. Looking at individual miRNAs, we characterized the terminal uridylase dependent miRNA uridylome and discovered extensive uridylation of disease-associated miRNAs. When sequencing pEV samples we show that IsoSeek captures the full complexity of isomiRs in comparison with commercial protocols. Finally we tested our IsoSeek protocol by preparing libraries from 188 DLBCL patient pEV samples. Hierarchical clustering of the sequence results before and after UMI correction shows a vast difference. This suggests that when analyzing low-input clinical liquid biopsy samples UMI correction can reduce PCR bias based clustering.
Conclusions: IsoSeek is optimized for qualitative and quantitative miRNA profiling at single-nucleotide resolution for functional and target prediction studies in vitro as well as in ultra low-input biological samples.

Poster Presentations

NLSEV2021-P7: Birke Benedikter

Pro-coagulant extracellular vesicles promote smoking-induced pulmo-vascular inflammation

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Background: Tobacco smoking is a major risk factor for chronic obstructive pulmonary disease (COPD) as well as thrombosis, and both conditions frequently coincide. However, the pathobiological mechanisms linking the two conditions are unclear. We have previously demonstrated that bronchial epithelial cells secrete pro-coagulant, tissue factor (TF)-exposing, extracellular vesicles (EVs) when stimulated with tobacco smoke extract. These EVs TF-dependently promoted thrombin generation when added to human plasma. Here, we explore whether TF-EVs mechanistically link COPD-associated pulmonary inflammation and thrombosis via activation of the coagulation factor receptors PAR1-4.
Methods & Results: Re-analysis of public RNA sequencing data revealed that gene expression of both, TF and PAR-1 is upregulated in whole lung tissue from patients with COPD. Using polarized bronchial epithelial cells cultured on transwell membranes, we demonstrated that TF-exposing EVs are secreted towards the lung lumen, as well as the basolateral side where blood vessels are located. By qPCR of cultured cells and immunohistochemistry of lung tissue, we furthermore showed that bronchial and alveolar epithelial cells, as well as endothelial cells express PAR1 and PAR2 and therefore form potential target cells for TF-EV signaling. Stimulation of human lung endothelial cells with TF-EVs in the presence, but not in absence of 1% citrate plasma, resulted in increased expression of inflammatory markers IL-8 and ICAM-1, suggesting an indirect thrombin-mediated effect. Likewise, thrombin and peptide agonists for PAR1 and PAR2 induced IL-8 and ICAM-1 expression. Further investigation using specific coagulation inhibitors and PAR knockdowns will reveal the signaling pathways resulting in TF-EV mediated endothelial cell activation.
Conclusions: Tobacco smoke induces secretion of TF-EVs by bronchial epithelial cells. These EVs can act on other cell types as demonstrated for endothelial cells. Thereby they promote inflammation and potentially the development of cardiovascular disorders in the context of tobacco smoking and COPD.

Poster Presentations

NLSEV2021-P8: Madalena Monteiro

Extracellular vesicle proteomics reveals exosome biogenesis pathway proteins as potential therapeutic targets in colorectal cancer

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Background: Tumor-derived exosomes play a role in cancer progression, therefore blocking their secretion by cancer cells could halt cancer progression and be a promising novel drug-target. To this end, global insight into the functional players of this process is key for development of a successful therapeutic strategy.
Methods: To identify deregulated pathways in the exosome biogenesis pathway, Mass Spectrometry (MS)-based proteomics was performed on a unique sample set collected from 22 patients, comprising multiple fractions (tissue lysate, soluble secretome and exosomes) of matched normal and colorectal cancer (CRC) tissues (n=18) and adenomas (n=4). An external CRC phosphoproteomics dataset was used to identify potential regulatory kinases via kinase-substrate analysis. Network clustering was performed using ClusterOne and gene ontology using BinGO (cytoscape). CD63-localization and expression was measured using confocal microscopy. Exosome release was assessed by Coomassie stained gels and TSG101 expression using Western blot.
Results: The multi-compartment CRC dataset comprised 3745 exosomal proteins and comparative analysis revealed 455 exosomal proteins with increased abundance in cancer exosomes ($p<0.01$; FC>3). Unbiased functional data mining revealed deregulated pathways that were associated with (intracellular) vesicle trafficking in CRC-tissues compared to healthy tissues. Importantly, the ESCRT-pathway was down-regulated in cancer, while proteins related to endocytosis and exosome release were up-regulated. Targeted data mining to the exosome biogenesis pathway revealed 12 up-regulated proteins and 14 proteins that had increased cellular phosphorylation levels ($p<0.05$). Three upstream kinases were selected for further analysis using drugs in clinical trials or FDA-approved. To this end, CRC cell lines were treated at IC50-correspondent dose for 24h. All three drugs caused a (30-60%) decrease in exosome release after kinase inhibition. Intracellular immunofluorescent staining with CD63 revealed an increased intensity after drug treatment, which suggests an intracellular accumulation of exosomes. We aim to further validate the mechanisms of action underlying the inhibition of kinase-mediated exosome production and release after treatment using (phospho-)proteomics.
Conclusion: In conclusion, we describe a unique multicompartment CRC proteomics dataset and identified potential new drug targets in the exosome-release pathway, which presents a novel therapeutic strategy against CRC.

Poster Presentations

NLSEV2021-P9: Estefanía Lozano-Andrés

Considerations towards the assignment of calibrated fluorescence values to nanoparticles and extracellular vesicles by using MESF beads by flow cytometry

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#: TRAIN-EV Marie Skłodowska-Curie Action-Innovative Training Network, train-ev.eu; ‡: Both authors contributed equally

Background: Flow cytometry is a powerful technique to characterize nanoparticles (NPs) and extracellular vesicles (EVs). However, fluorescence intensity presented in arbitrary units hampers the comparison of results from different laboratories and platforms. Therefore, we investigated the use of calibrated molecules of equivalent soluble fluorophores (MESF)-beads for the assignment of absolute fluorescence units.
Methods: Two FITC-MESF bead sets were evaluated on three platforms (BD Influx, BC CytoFLEX LX, SORP BD FACSCelesta) and two PE-MESF bead sets were used as calibrators on the BD Influx. FITC labeled silica NPs containing unknown fluorescent intensity populations were used as NPs. EVs were isolated from conditioned media of the 4T1 mammary carcinoma cell line, stained with CFSE and labeled with an anti-CD9PE antibody, prior to density gradient floatation. Synthetic NPs and biological EVs were measured on the BD Influx and their respective fluorescent signals were calibrated in standardized units of FITC-MESF, CFSE-ERF and PE-MESF.
Results: Extrapolating bright calibrator beads to dim signals leads to unwanted variations in the dim fluorescence range that vary depending on the MESF-bead set used. By measuring several MESF-bead sets on the BD Influx next to synthetic NPs and biological EVs, we found variations ranging from 27.3% to 76.5% when calibrating different FITC populations from synthetic NPs in FITC-MESF units. Furthermore, we observed a 78.6% variation when calibrating biological EVs in CFSE-ERF units and a 156.9% variation in PE-MESF units from CD9-PE labeled biological EVs.
Conclusions: Our findings show that bright calibrators designed for cellular flow cytometry are far out of fluorescence range from the sub-micron particles to reliably assign absolute fluorescence values to NPs and EVs. Therefore, the use of calibration materials in the lower fluorescence ranges of NPs and EVs and proper calibration reporting are essential.
Funding: European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No [722148].

Poster Presentations

NLSEV2021-P10: Leyla Erozenci

Identification of the cancer extracellular vesicle surface proteome and its application to detect prostate cancer in urine

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Background: Cancer cells secrete extracellular vesicles (EVs), which can be isolated from urine. Urinary EVs might therefore provide a non-invasive diagnosis method not only for proximal cancers such as prostate (PCa), but potentially also for cancers of distant organs. To directly capture and detect cancer-type-specific EVs, an understanding of the EV surface protein constituents and their orientation on the EV membrane is needed.
Methods: EVs were isolated with ultracentrifugation from 17 cell lines representing 5 cancer-types (Prostate-n=5;Colorectal-n=4;Cervical-n=3;Lung-n=3;Bladder-n=2; in duplicate). EV surface proteins were digested with trypsin, after which the surface peptides, luminal and total fractions were quantified using mass spectrometry(MS). EV integrity was evaluated using electron microscopy and flow cytometry. Urinary EVs of prostate(n=15), lung(n=9), colorectal(n=15), cervical(n=15) and bladder cancer patients(n=6), and controls(n = 15) were isolated using the Vn96-peptide-protocol, that precipitates EVs via binding to heat-shock proteins(HSPs), and profiled with MS. 2 PCa-associated EV surface proteins were validated by ELISA in 32 PCa and control urine samples.
Results: EVs keep their structural integrity after trypsinization. Proteomics identified >3500 proteins on the EV surface and >4000 in the remnant fractions. Core EV surface proteome (112 proteins present in all cancer types) was enriched for HSPs, underscoring their utility for pan-cancer EV isolation using the Vn96-protocol. Multiple molecular functions were associated with the EV surface proteome such as vesicle-related and metabolic proteins, but unexpectedly also intracellular DNA/RNA-binding proteins such as histones and ribosomal proteins. Topology analysis mapped 30% of the transmembrane surface proteins with their intracellular/luminal domains on EV surface. To determine whether EV surface proteins can be detected in cancer patients, we isolated urinary EVs from a multi-cancer cohort using the clinically applicable EV-capture kit. Importantly, several PCa-specific EV surface proteins were also highly enriched in the PCa urinary EVs. To further study their clinical applicability, 2 surface candidates were selected ($p<0.05$; fold change>2) for ELISA in 4 ml whole urine and showed increased expression in PCa.
Conclusions: The identified EV surface proteome may have implications in EVs' biological function and provides novel insights that might ultimately translate to a minimally-invasive detection method for PCa, as well as distant cancers.

Poster Presentations

NLSEV2021-P11: Kyra Defourny

Picornavirus security proteins promote the activation of the inflammasome along with the release of extracellular vesicle-enclosed virions

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Background: Extracellular vesicles (EVs) can play an important role in fine-tuning cell-to-cell communication during inflammatory and immune processes. During virus-infection, infected cells can package virus material and host inflammatory mediators in their EVs as a potential mechanism to alert surrounding (immune cells) of their infection status. Inflammasome activation is one of the most potent triggers of proinflammatory communication by virus-infected cells. However, it remains largely unknown whether/how inflammasome activation plays a role in the release and molecular composition of EVs produced in response to virus infection.
Methods: We previously showed that cells infected with the non-enveloped picornaviruses EMCV and CVB3 release EVs that contain entire virions. These EVs can efficiently transfer infections to new cells and have the potential to activate immune cells. Moreover, we discovered that this process depends on the activity of the functionally related viral security proteins CVB3 2A and the EMCV Leader. Here, we used recombinant viruses that loose/gain the expression of these proteins to assess whether there is a correlation between the ability of a virus to promote the release of virus-carrying EVs and its ability to promote inflammasome activation.
Results: Recombinant viruses that lost the capacity to increase EV release and to promote the release of virions within EVs, also lost the ability to induce a pyroptosis-like cell death and the release of cleaved caspase-1, two hallmarks of inflammasome activation. In addition, these deficient viruses lost the ability to induce secretory autophagy, an unconventional secretion pathway triggered by inflammasome activation, which we previously showed to facilitate virus packaging in EVs.
Conclusions: Taken together these findings provide novel insights into the viral factors involved in inflammasome activation during picornavirus infection. Moreover, we demonstrate a putative link between inflammasome activation and the quantity and quality of EVs released by picornavirus infected cells. This opens up new perspectives on how inflammasome activation may enhance virus spread and virus-induced inflammation.

Poster Presentations

NLSEV2021-P12: Celine de Schrijver

Inflammatory MSCs are triggered by extracellular vesicles released by aggressive cancer cells and cause resistance to antimetastatic drugs

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Background: Cancer development and progression heavily rely on the interaction between cancer cells and non-malignant cells in the tumor microenvironment, among which bone marrow mesenchymal stem cells (MSCs) play a key role. In response to tumor-associated signals, MSCs undergo a functional switch, which results in the release of inflammatory factors promoting tumor cell aggressiveness and therapy resistance. We previously demonstrated using an orthotopic xenograft mouse model of osteosarcoma that tumor-secreted extracellular vesicles (EVs) are key triggers for the MSC pro-metastatic behavior. This effect was in part determined by a membrane-bound form of TGFβ on the surface of the vesicles, strongly inducing pro-metastatic IL-6 production in MSCs. In this study we set out to study in depth the tumor EV-induced alterations of MSCs and to identify actionable targets to abrogate the MSC-induced resistance to antimetastatic drugs.
Method: The global tumor EV-induced alterations of the MSC transcriptome were analyzed (in the presence or absence of a TGFβ inhibitor) by RNA-seq. Gene set enrichment analysis (GSEA) was applied to discriminate TGFβ-dependent and -independent pathways. EV RNA-induced MSC alterations were identified by transfecting purified EV-RNA in MSC and by using a selective dsRNA antagonist. We selected candidate targets to block MSC-induced drug resistance and evaluated their effect in an orthotopic xenograft mouse model of osteosarcoma.
Results: EVs from aggressive cancer cell lines unequivocally induced an inflammatory MSC (iMSC) phenotype, characterized by the upregulation of cytokine, chemokine and complement genes, that was not observed with less aggressive cell lines. Apart from IL6, these alterations were mostly independent from TGFβ signaling and related to pattern recognition receptor (PRR) activation. We demonstrate that tumor EV-associated non-coding RNAs trigger TLR3 signaling in MSCs activating an innate immune response leading to high induction of various chemokines, particularly IL8. Importantly, blocking the TGFβ-IL6 signaling decreased metastasis formation in a bone cancer xenograft mouse model, an effect that was counteracted by the injection of iMSCs.
Conclusions: Our work demonstrates that EV-associated TGFβ cooperates with EV-RNA to trigger the development of iMSCs in cancer, and suggests that combination strategies blocking the TGFβ-IL6 and IL8 signaling might abolish iMSC-induced tumor resistance to antimetastatic drugs.

Poster Presentations

NLSEV2021-P13: Marije Kuipers

Time-efficient and high yield purification of EVs from limited sample volumes of parasitic worm secretions

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Background: The parasitic worm Schistosoma mansoni releases extracellular vesicles (EVs) to modulate host immune responses. Studying these EVs is challenging since S. mansoni can only be obtained from living hosts, which limits available material. Furthermore, biophysical properties of EVs differ between life cycle stages and the immunogenic hemoglobin digestion byproduct hemozoin can contaminate EV isolates. Common problems in EV research are the loss of EVs in consecutive isolation/purification steps, which is highly undesirable when working with limited sources. We here propose an optimized isolation protocol to obtain concentrated and highly pure worm EVs while minimizing EV loss, which is transferable to other EV sources.
Methods: Culture medium of S. mansoni adult or juvenile worms was subjected to differential (ultra)centrifugation (UC) or size exclusion chromatography (SEC), followed by density gradient purification using iodixanol or sucrose. EVs and hemozoin were detected by cryo electron microscopy. EV concentrations in fractions from small (1.8ml, 2/13.5h spin), medium (4.5ml, 16h spin), and large (12ml, 16h spin) iodixanol density gradients were quantified using Western blot analysis of Tetraspanin 2.
Results: Adult worm EVs could not be separated from hemozoin by UC or SEC, suggesting that additional purification steps were needed. We compared small-, medium-, and large-volume iodixanol gradients to further purify these EVs. The small-volume iodixanol density gradient allowed separation of EVs from hemozoin within only 2 hours UC. Moreover, this small iodixanol gradient yielded the highest concentration of adult worm EVs within EV-containing fractions compared to medium- and large-sized gradients. EVs from juvenile worms, which differ in composition and morphology from adult worm EVs, could also be efficiently purified using the small-sized iodixanol gradient. Noticeably, longer centrifugation times were needed for adult worm EVs to reach equilibrium in sucrose gradients, while juvenile worm EVs did not float at all in sucrose gradients.
Conclusion: Iodixanol gradients are preferred over sucrose gradients for purification of EVs from different parasitic life stages. We propose the use of small-sized (1.8ml) iodixanol density gradients to simultaneously separate and concentrate EVs from limited sources, like parasites, to increase EV yield while reducing handling time.

Poster Presentations

NLSEV2021-P14: Liang Wu

Imaging flow cytometry, a novel and sensitive tool to quantify and characterize urinary extracellular vesicles with a “isolation-free” protocol

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Background: Isolation or concentration of urinary extracellular vesicles (uEV) is commonly used in EV research though this could induce bias. Recently, we developed a sensitive and isolation-free approach to detect EV in blood plasma using imaging flow cytometry (IFCM). Here, we present an optimized and “isolation-free” protocol for uEV measurement by IFCM.

Method: Cell-free urine from healthy volunteers (n=5) was measured with/without labeling with tetraspanin CD63 (CD63-Alxa488 and CD63-APC). Size-standardized beads ranging from 100 nm to 900 nm and beads with known molecule equivalent soluble fluorochrome (MESF) were primarily used for calibration. Double positive events represented read-out as uEV with strict isotype and detergent controls. The effects of centrifugation, dilution, and DTT on uEV measurement were demonstrated with IFCM using ImageStream.

Result: IFCM precisely distinguished beads with different size by gates of side scatter and bright field, and also arbitrary fluorescent intensities were successfully converted to standardized MESF values in IFCM. In unstained urine, (1.41 ± 0.61) × 106 /ml auto-fluorescent particles were observed which were removed for (98.5 ± 0.9) % by a short run centrifugation (10,000 g & 5 min). In urine stained by CD63-Alxa488 and CD63-APC, there were (4.23 ± 1.46) × 107 /ml double positive uEV and (98.3 ± 0.8) % of them showed lower side scatter than 240 nm beads. These relatively small uEV showed (5060.18 ± 792.68) MESF-APC and (141.91 ± 8.06) MESF- Alexa488. The other uEV with larger scatter showed (52406.59 ± 56491.95) MESF-APC and (1621.00 ± 1645.50) MESF-Alexa488. Dilution (3-fold) of urine enhanced (11.3 ± 4.2) % yield of uEV. Incubation with low-concentration DTT (25 mg/ml) enhanced (8.7 ± 5.7) % yield of uEV.

Conclusion: In summary, here, we demonstrate a protocol how to sensitively, specifically and effectively quantify and characterize uEV by using Imagestream with a simple and isolation-free protocol.

Poster Presentations

NLSEV2021-P15: Ardalan Mansouri

Manipulating the uptake of extracellular vesicle in prostate cancer

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Background: Prostate cancer (PCa), as a common cancer among elderly men, can progress to bone, liver and lymph node metastasis. Cell-cell communication via secreting micro-/macro-molecules in bilayer extracellular vesicles (EVs) might have a major role in tumor growth and metastasis.
As the EVs from cancer cells are released throughout cancer progression, blocking the uptake of EVs by the target cells might subside tumor growth and metastatic process. In the current study, we used the drug repurposing approach to identify compounds that inhibit EV-internalization.
Methods: PNT2C2 normal prostate epithelial cells were treated with >2000 compounds from Prestwick and LOPAC libraries for 16 h. Afterwards, EVs derived from the PCa cell line DU145, isolated via ultracentrifugation and labeled with PKH26, were added to the cells and the EV-uptake was measured by quantitative microscopy after 3 h using the Opera Phenix HCS system. For validation, flow cytometry of PNT2C2 cells incubated with compound and labeled EVs was implemented. Ingenuity pathway analysis (IPA) was employed to explore the mechanism of action of the inhibitory compounds.
Results: Altogether, 173 EV-uptake inhibitors and 220 uptake inducers were identified and the top 15 inhibitors and 10 inducers were considered for the validation steps. From these 25 compounds, 3 toxic and autofluorescent compounds were eliminated. In the validation phase, 4 EV-uptake inhibitors and 4 inducers reproduced the screen results. The inhibitors exhibited a ~3-fold decrease in EV-uptake as compared to the controls, while inducers caused less than a ~1.5 folds increase in EV-uptake.
Besides microscopy, PNT2C2 flow cytometry data confirmed the change in EV-uptake. Interestingly, 2 inhibitors had an almost immediate effect on EV-uptake; within 30 min of incubation. Pathway analysis of the protein targets of the inhibitors, revealed that pathways related to regulation of P glycoproteins and Na/K ATPases subunits are most commonly affected.
Conclusion: Compound screening led to discovery of at least two novel EV-uptake inhibitors, which will be further evaluated as tools to manipulate the EV-uptake to investigate the role of EV cell-cell communication during tumor growth and metastasis.

Poster Presentations

NLSEV2021-P16: Suzy Varderidou-Minasian

Mesenchymal stromal/stem cells-derived extracellular vesicles promote regeneration of motor neurons from amyotrophic lateral sclerosis patients - an insight into molecular mode of action

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Background: Amyotrophic lateral sclerosis (ALS) is a common neurodegenerative disorder characterized by the selective degeneration of motor neurons (MNs) in the brain and spinal cord. AL pathogenesis is incompletely understood, but involves alteration of several important cellular processes. Current therapeutic strategies target one or a few of these alterations, resulting in a minimal effect on ALS patients' survival. Mesenchymal stromal/stem cells (MSC) can support motor neurons and surrounding cells, reduce inflammation and stimulate tissue regeneration. Therefore, MSC are promising candidates to treat ALS. Growing evidence suggests that therapeutic efficacy of MSC depends on their paracrine signalling. Here we investigated the role of bone marrow MSC-derived extracellular vesicles (MSC-EVs), an important component of MSC secretome, in the regeneration of ALS MN.
Methods: We used human iPSC cell lines for WT and the most frequently occurring mutations in ALS (TARDBP, FUS and C9orf72) to model ALS. To generate hiPSC-derived motor neurons (MN) we used highly efficient previously reported protocol. MNs identity was confirmed using MNspecific markers (*ISL1*, *HB9*, *SMI32*) and neuronal marker *TUBB3a.* The proteomic analysis of MSC-EVs treated MN were done using nanoLC ESI MSMS and an Orbitrap Fusion coupled to an Agilent 1290 HPLC system. Data were acquired using Xcalibur software.
Results: MN carrying different ALS mutations produced fewer and shorter neuritis than control cells. This defect was rescued by the treatment with MSC-EVs, suggesting their positive effect on human MN regeneration. To understand the underlying molecular mechanism, we performed proteome analysis of ALS MN and wild type MN treated with MSC-EVs. This revealed strong upregulation of proteins involved in the control of cell death and apoptosis, the effect reversed upon treatment with MSC-EVs. We have also detected a significant number of autophagy and cytoskeleton regulators, which were differentially expressed between ALS cells treated or not with MSC-EVs. Subsequent analysis of autophagy process demonstrated that decreased autophagic flux in ALS MN was upregulated to the control levels by treatment with MSC-EVs.
Conclusions: Our data indicate that MSC-EVs support regeneration of human ALS MN *in vitro* possibly by the control of cell death, autophagy and regulation of cytoskeleton in ALS MN.

Poster Presentations

NLSEV2021-P17: Agustin Enciso-Martinez

Cell-specific labeling of tumor-derived extracellular vesicles, their visualization, enrichment and isolation via synthetic epitopes

Agustin Enciso-Martinez*,# [1], Edwin de Jong# [1], Armagan Kocer [1]
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Background: Extracellular vesicles (EVs) are membrane-bound particles that play a significant role in intercellular communication by carrying biological information between the cells. Deciphering the messages carried by EVs is crucial to understand EV-mediated communication processes under physiological and pathological conditions. The isolation and detection of EVs of interest has thereby become an important first step to study EVs. The presence of other particles, such as lipoproteins or EVs of different origins, which overlap in size and density with the EVs of interest, makes EV isolation challenging with conventional biophysical and biochemical methods.
Methods: We propose incorporating synthetic epitopes into EVs of interest by exploring cells' metabolism. These epitopes, then, serve as specific molecular anchors for covalent attachment of visualization or affinity tags using bio-orthogonal chemistry tools, enabling enriching and visualizing cell-specific EVs with a spatiotemporal resolution.
Results: By employing this strategy, we could selectively label proteins, lipids, and glycoproteins produced in neuroblastoma cancer cells and their EVs in vitro. We could visualize EVs with a higher signal-to-noise ratio than antibody labeling by incorporating fluorescent dye specifically to any of these labelled molecules. We also could selectively enrich and isolate EVs by targeting the synthetic epitopes with an affinity tag.
Conclusion: We believe that the incorporation of synthetic epitopes into EVs for their visualization and isolation will contribute to cell-specific and time-resolved deciphering of intercellular communication in health and disease.

Poster Presentations

NLSEV2021-P18: Philip Askenase (not presented on-site)

Hypothesis for immune training long COVID: Use of convalescent plasma containing suppressive COVID-antigen-specific exosomes

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There have been numerous very disappointing results of Convalescent Plasma Therapy (CPT) in active infections with SARS-CoV-2 virus. This raises a question of how to account for this, given the huge history of seeming benefit of CPT in a variety of infectious and viral diseases over more than a hundred years. We propose the following possible explanation, based on our experimental evidence. In CPT there is a collision between developed desirable viral resistance promoting hyper-immune antibodies and undesirable convalescent exosomes that act antigen (Ag)-specifically to suppress early developing cellular immune responses stimulated by the prior now recovered acute viral disease. These inhibiting exosomes, that can act to suppress Ag presenting cells and anti-COVID-19-Ag-specific effector T cells, are appropriate to convalescence. However, when given early in infection may interfere with endogenous early developing profitable cellular immune antigen-specific anti-viral responses.

To account for the high incidence of the Long Haulers post COVID patients, we postulate that these are due to immune reactivity to Ag remnants of the virus and not residual infection and that positive-acting antigen-specific exosomes may play a role. We propose that convalescent plasma therapy with its content of potential broadly COVID Ag-specific suppressive exosomes be considered for possible effective treatment of the COVID-19 Long Hauler Syndromes. It is additionally possible that present convalescent antibodies to COVID peptides can bind the peptide/MHC surface of Ag-specific exosomes to augment their suppression.

Poster Presentations

NLSEV2021-P19: Melissa Piontek

Lipoprotein particles exhibit distinct mechanical properties

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Samples of extracellular vesicles (EVs) can potentially be contaminated by lipoproteins particles (LPs), as intensively discussed within the EV community. LPs are nano-sized micelle-like structures composed of lipids, apoproteins and cholesterol (ester) and are synthesized by liver cells as well as the small intestine. In contrast to EVs, their outer shell is composed of only a single layer of lipids with their hydrophilic part facing outwards. Consequently, they possess a hydrophobic core. This feature makes LPs a good candidate for the transport of hydrophobic lipids in aqueous solutions. Plasma lipoprotein particles are the most commonly studied subclass of LPs as they are responsible for the deposition of cholesterol ester and other fats in the artery, causing lesions and eventually atherosclerosis. There are various types of plasma lipoprotein particles, which are classified according to their size, density and their composition: high density lipoproteins (HDL), low density lipoproteins (LDL), very low density lipoproteins (VLDL), chylomicrons (CM). Although there are studies available on LP's composition and metabolism, little is known about their mechanical properties. Therefore, we performed a mechanical characterization of these particles using an atomic force microscopy (AFM) nanoindentation approach in order to elucidate the possible effects on EV samples contaminated with LPs. We obtained information about the LP's topography, size, stiffness as well as their deformability. The size distributions of the different types of LPs were confirmed. Demonstrating a qualitatively similar behavior in force indentation curves, some LPs also show a bending modulus of similar range as previously reported for EVs. In particular, the lower density LPs showed a lower bending modulus. The bending modulus of HDL particles could not unambiguously be determined. This is however of little effect on published EV mechanical data determining the bending modulus as various criteria are applied during the nanoindentation measurements, e. g. a minimum particle height after substrate adhesion leading to exclusion of most of the HDL particles from the analysis.

Poster Presentations

NLSEV2021-P20: Nathalie Lak

Extracellular vesicles: a new source of biomarkers in paediatric solid tumours? A systematic review of literature

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Background: Extracellular vesicles (EV) are released by every cell in the body and their cargo reflects the content of the cells of origin. EV are present in many biological fluids, from which they can be isolated by minimally invasive techniques. Particularly in the context of cancer, this provides a promising source of diagnostic, prognostic and predictive biomarkers. Despite the advances as biomarkers in adult cancer, knowledge on EV is lacking for paediatric cancers.
In this review, we provide an overview of in vivo and in vitro studies assessing potential EV-derived biomarkers for paediatric solid tumours.
Method: We performed a systematic literature search and determined to what extent the in vivo findings are substantiated by in vitro data and vice versa. We critically appraised the clinical studies using the GRADE system and evaluated the methods of EV isolation and characterisation for both in vivo and in vitro studies using EV-TRACK and our own PedEV score.
Results: We included 10 in vivo studies and 14 in vitro, on desmoplastic small round cell tumour, hepatoblastoma, neuroblastoma, osteosarcoma and rhabdomyosarcoma. Several identical EV-derived biomarkers were found in multiple studies, within the same tumour entity but also in different tumour types. However, often validation in an independent cohort was absent and most studies on the use of EV in paediatric solid tumours lack standardised reporting of EV isolation and characterisation methods.
Conclusion: There is an urgent need for studies with standardised methodology and reproducible reporting of every step of the isolation and analysis of EV-derived biomarkers from liquid biopsies in paediatric oncology. This will accelerate the validation of EV-based techniques and advance their translation from bench to bedside.

Poster Presentations

NLSEV2021-P21: Onno Arntz

Identification of Functionally and Clinical phenotype related Proteins in Circulating Extracellular Vesicles from Rheumatoid Arthritis Patients that correlate with Patient's Global Assessment of Disease Activity

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Background: Diagnosis and monitoring rheumatoid arthritis (RA) is based on laboratory tests for C-reactive-protein levels (CRP) and erythrocyte-sedimentation-rate (ESR), on the quantitative count of swollen and tender joints (SJC,TJC) by the clinician and patient global assessment of disease activity (PGA-VAS). Some RA-patients are unresponsive to (non)biologics disease-modifying-antirheumatic-drugs therefore additional prognostic biomarkers are needed. This study aims to identify unique proteins of circulating extracellular vesicles in RA patients and examine their relationship to standard disease activity parameters.
Methods: Plasma-EVs (pEVs) from healthy controls (HC;#9), osteoarthritic-(OA:#11) and RA (#9)-patients were isolated by size-exclusion-chromatography. EV amount and size was determined by Nanosight-track-analysis, markers by Western blotting and protein content by microBCA. RA-pEV peptides were identified by mass spectrometry and compared to OA and HC and correlated to their disease activity parameters. Clinical phenotypes of the enriched RA-pEVs proteins were analyzed using Funrich.
Results: Concentration, mode size and protein content of pEVs of RA (9.63 x109 particles/ml, 120nm, 0.30fg protein/particle) OA (9.25x109 particles/ml, 114nm, 0.31fg protein/particle) and HC (9.65x109 particles/ml, 114nm, 0.39fg protein/particle) donors were comparable. Proteomics of RA-pEVs revealed eight significantly enriched and 398 overlapping proteins compared to HC- and OA-pEVs. Remarkable, a higher number of pEV proteins positively correlated to PGA-VAS [#23] than to other disease parameters (TJC[#7],SJC[#8],CRP[#2] and ESR[#3]. Of them, three were also enriched on RA-pEVs whereof Integrin beta and SSC5D were also linked to SJC. Interestingly, Collagen-VI was linked to the subjective health assessment PGA-VAS. Funrich analysis suggests that the RA-pEV proteins correlated PGA-VAS can be linked to muscle and bone disorders.
Conclusions: A small number of proteins were significantly enriched in RA-pEVs as compared to HC and OA, and are potential biomarkers for RA. Interestingly, strong associations of RA-pEV proteins to SJC and in particular to the PGA-VAS score was found which suggests that RA-pEVs could be biomarkers of patient's well-being.

Poster Presentations

NLSEV2021-P22: Josette van Maanen

Characterization of extracellular vesicles from porcine, canine and human notochordal cell-conditioned medium

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Background: Low back pain (LBP) episodes are common and affect everyday life. A major cause for chronic LBP is intervertebral disc degeneration. Notochordal cells (NCs) possess regenerative potential that could be exploited for therapeutic approaches. Extracellular vesicles (EVs) secreted by NCs may mediate this effect. To facilitate proper EV characterization a bead-based western technology was conducted to identify NC-derived EV-associated protein markers.
Methods: NC-conditioned medium (NCCM) was generated by culturing NC-rich tissue of porcine, canine, and human origin. EVs were isolated through differential centrifugation followed by size exclusion chromatography and characterized using DigiWest technology, a high-throughput bead-based multiplex platform.
Results: DigiWest analysis of porcine and canine NCCM-derived EVs revealed the presence of 12 proteins in common. In human NCCM-derived EVs, only 2 proteins were identified, most probably due to technical limitations related to low starting protein quantities. These proteins were also present in porcine/canine NCCM. Other proteins that can be recovered with EVs (co-isolated or contaminant proteins) were not detected, while they were detectable in positive control samples, indicating that they were absent in EV samples.
Conclusions: Altogether, for porcine and canine NCCM-derived EVs, we identified transmembrane, GPI-anchored, and cytosolic proteins that are required for EV characterization according to the standards of the International Society for Extracellular vesicles. Isolated EVs were devoid of contamination based on the absence of non-vesicular components. Based on these results, a panel of 19 proteins was composed for characterization of NC-derived EVs from different species.
F**unding:** This project received funding from the European Union's Horizon 2020 program iPSpine (No. 825925).

Poster Presentations

NLSEV2021-P23: Fang Cheng Wong

Pancreatic cancer-derived extracellular vesicles mediate Schwann cell migration

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Background: Neural invasion (NI) involves the growth and invasion of cancer cells around and into nerves, a process that has been known as a hallmark of pancreatic adenocarcinoma (PDAC) due to its extremely high incidence rate (>90%). NI has been reported to occur at the early stage of PDAC development and glial cells of peripheral nerves, i.e. Schwann cells (SCs) have been shown to be attracted to cancer cells before the onset of cancer invasion. However, the mechanisms leading to NI have not been completely understood.
Method: A 3D coculture assay with SCs confronted to cancer cells was first conducted to evaluate the chemoattraction affinity of SCs towards cancer cells. Next, extracellular vesicles (EVs) were isolated from human cancer cells, fresh tissues, tissue explant model and murine cancer cells by differential ultracentrifugation and characterised by Transmission Electron Microscopy (TEM), Nanoparticle Tracking Analysis (NTA) and western blot according to MISEV2018 guidelines. A novel 3D migration assay incorporating SCs and EVs in a single Matrigel drop was employed to evaluate the migratory ability of SCs in response to EVs, which was further confirmed in the presence of EV uptake inhibitors.
Results: 3D coculture assay revealed that murine SCs (mSCs) exhibited stronger migratory ability when confronted to murine neuroinvasive PDAC cells than to non-neuroinvasive PDAC cells, which could be reversed by EV uptake inhibitors. Interestingly, mSCs confronted to neuroinvasive PDAC cells expressed higher expression levels of protein X than non-neuroinvasive PDAC cells. More importantly, 3D migration assay demonstrated significantly stronger migratory ability of human SCs (hSCs)/mSCs after human PDAC/murine neuroinvasive cancer cell-derived EV treatment than that of the human normal pancreatic epithelial cell-/murine non-neuroinvasive cancer cell-derived EVs, which could be reversed by heparin. At a translational aspect, the increased migration of hSCs could also be observed after treatment of tumor tissue-derived EVs from PDAC patients with neural invasion (Pn1) compared to those diagnosed with no neural invasion (Pn0) pathologically, concomitantly with an increased mRNA level of gene X, indicating the relevance of neuroinvasive cancer derived-EVs in mediating SC migration.
Conclusion: PDAC-derived EVs may be responsible for the migration of SCs toward pancreatic cancer cells. This may underscore the mechanisms underlying the SC carcinotropism in PDAC-associated NI.

Poster Presentations

NLSEV2021-P24: Wouter Woud

Novel avenue of allograft monitoring: Direct measurement of donor-specific extracellular vesicles in human plasma samples

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Background: Extracellular Vesicles (EVs) are tissue-specific, nanosized particles that facilitate cell to cell communication, regulate protein expression and even affect antigen presentation. Recently, we developed a protocol to identify EVs ≤240 nm in complex samples such as plasma without prior isolation of the EVs. Here, we adapted this protocol to identify EVs based on their HLA phenotype as a first step to detect allograft specific EVs in the circulation of kidney transplant recipients.
Methods: EDTA blood samples from kidney transplant donors (HLA-A2+, n=21) and recipients (HLA-A2-, n=33) were collected before transplantation. Platelet-poor plasma (PPP) was generated and samples were diluted in PBS, stained with a donor-specific HLA antibody (HLA-A2) in combination with a common EV marker (tetraspanin CD9) and measured using standardized Imaging Flow Cytometry (IFCM).
Results: Quantification and comparison of CD9+/HLA-A2+ double-positive EVs showed a significant difference between both groups (1.1E7 ± 8.9E6 vs 3.5E5 ± 2.5E5 objects/mL, A2+ vs A2- respectively, p=6.5E-5) with A2- concentrations representing background level of the machine. CV values for inter- and intra-assay variability were 16% and 11%, respectively. Serial dilution of A2+ PPP in A2- PPP (n=5) showed a linear reduction in the numbers of CD9+/HLA-A2+ EVs according to the dilution rate whilst total CD9+ EV levels remained unchanged. The lower limit of detection of IFCM was defined as the dilution at which point CD9+/HLA-A2+ ssEVs dropped below baseline (A2- PPP) and was determined to be 1.5%.
Conclusion: Here we demonstrate for the first time the discriminatory capabilities and lower detection limit of IFCM for identification of specific EV subsets in unprocessed human plasma. Identification, quantification and characterization of donor specific EVs opens up the possibility to monitor these EVs over time after transplantation, and may prove to be a minimally-invasive biomarker.

Poster Presentations

NLSEV2021-P25: Naomi Buntsma

Preventing swarm detection in extracellular vesicle flow cytometry by count rate optimization

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Background: To characterize single extracellular vesicles (EVs) in biological fluids, flow cytometry (FCM) is commonly used. EV FCM, however, is still hampered by various factors, one being the possible occurrence of swarm detection. At physiological concentrations of submicrometer particles in body fluids, multiple EVs can be simultaneously illuminated. Therefore, without appropriate dilution, one event signal is caused by multiple particles, which leads to meaningless signals and an underestimation of the particle concentration. This artefact is named swarm detection. As the concentration of submicrometer particles in body fluids differs orders of magnitude, currently a dilution series is recommended to find the highest count rate (to remain statistical power) without swarm detection. However, a dilution series is laborious and unpractical. The goal of this work is to develop and validate a faster approach, based on the count rate, to optimize the sample dilution and prevent swarm detection.
Methods: We performed FCM (Apogee A60-Micro) measurements on dilution series of plasma from 5 patients suffering an acute myocardial infarction (AFFECT EV study). The total particle concentration of these samples ranges from 1E9 – 1E11/mL for particles exceeding a side scatter cross section of 10 nm2. We used a flow rate of 3,01 uL/min and triggered on side scatter. We systematically explored the relation between the occurrence of swarm detection and the count rate.
Results: Between a count rate of 200 events/s and 4,000 events/s we did not observe swarm detection in any of the samples that were measured. For samples with a total particle concentration of 1E10/mL or higher, swarm detection is prevented when the count rate is below 10,000 events/s.
Conclusions: For EV FCM measurements triggered on side scatter, there is a range of count rates where swarm detection does not occur. Instead of a dilution series, a single diluted sample can be measured to determine the total particle concentration. This offers a single-step approach to determine the dilution factor that leads to an optimized count rate, thereby remaining statistical power, while preventing swarm detection in plasma samples.

Poster Presentations

NLSEV2021-P26: Elena Martens-Uzunova

Androgens alter the heterogeneity of small extracellular vesicles and the small RNA cargo in prostate cancer

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Proliferation and survival of prostate cancer cells are driven by the androgen receptor (AR) upon binding to androgen steroid hormones. Manipulating the AR signaling axis is the focus for prostate cancer therapy. Therefore it is crucial to understand the role of androgens and AR on extracellular vesicle (EV) secretion and cargo. In this study, we report that plasma-derived circulating vesicles consisting of CD9 and double-positive for CD9 and Prostate Specific Membrane Antigen (PSMA) are increased in patients with advanced metastatic prostate cancer, whereas double positives for CD9 and CD63 small extracellular vesicles (S-EVs) are significantly higher in patients with localized prostate cancer. Androgen manipulation by dihydrotestosterone (DHT) and the clinical antagonist enzalutamide (ENZ) altered the heterogeneity and size of CD9 positive S-EVs in AR-expressing prostate cancer cells, while assessment of the total number and protein cargo of total S-EVs was unaltered across different treatment groups. Furthermore, hormone stimulation caused strong and specific effects on the small RNA cargo of S-EVs. A total of 543 small RNAs were found to be regulated by androgens including miR-19-3p and miR-361-5p. Analysis of S-EVs heterogeneity and small RNA cargo may provide clinical utility for prostate cancer and be informative to understand further the mechanism of resistance to androgen targeted therapy in castration-resistant prostate cancer.

Poster Presentations

NLSEV2021-P27: Arjan Wiskerke

Optimizing cytometry for extracellular vesicles characterization

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Introduction: We have improved the limit of detection (LOD) of a customized flow cytometer 30-fold to realize detection of single extracellular vesicles (EVs) with a diameter of 91-nm. Next, we aim to improve the detection speed. The detection speed is limited by the time and the intensity of the illumination of an EV. Slowing down the EV flow will increase the magnitude of the integrated optical signals, and thus improves the LOD, but at the expense of detection speed. Currently, we are able to characterize 5,000 EVs/s, but we are aiming to increase this count rate to 50,000 EVs/s by parallelization.
Methods: We use a customized BD FACSCanto flow cytometer of which the optics have been placed on an optical bench. We started with parallelization of the detection process by replacing the photomultiplier tubes with a CMOS imaging device, followed by slowing down the flow. We have developed custom image analysis software in LabVIEW to process signals from single polystyrene beads.
Results: Preliminary measurements using a simple inspection camera were done. Polystyrene beads of 140 nm were well detectable and the coefficient of variation (CV) of the 380 nm beads were 8%.
Conclusions: We have developed a flow cytometry imaging approach for simultaneous detection of EVs with good quality in terms of CV and detection limit. Further improvements of the flow system are the implementation of a homogeneous flow speed through the illuminated area.

Poster Presentations

NLSEV2021-P28: Chantal Scheepbrouwer

Re-assessment of the small RNA composition in EVs by innovative sequencing

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Background: Extracellular vesicles (EVs) including exosomes have shown to play a role in various biological processes and diseases. Their content reflects in large extent that of the donor cell and includes proteins, RNAs, and DNA. Over the last years conflicting findings on the RNA cargo of EVs have been published, and there is ongoing debate on the functional delivery of this RNA content. With the application of our recently developed ALL-tRNAseq we aim to re-evaluate the RNA composition and the functional contribution of the RNA machinery in EVs.
Methods: EVs and non-EV fractions were isolated from MDA and RN cell lines using Size Exclusion Chromatography (SEC). To address the full small RNA composition in cells, EVs and the extracellular non-EV fractions, we applied several chemical treatments to both EVs and RNA to help determine their RNA composition. Additional knockout cell lines were used to gain a deeper understanding of several RNA maturation processes. Small RNA sequencing data was generated using a custom-made library preparation method on the Illumina platform.
Results: Our ALL-tRNAseq approach allowed us to characterize several different aspects of the RNA composition in EVs. Interestingly, we observed various highly structured RNA species inside EVs that are underrepresented in other sequencing-based studies. We found that transfer RNAs (tRNAs) are the most dominant small noncoding RNA class in EVs, consisting mostly of mature full-length tRNA classes. Moreover, ALL-tRNAseq detected pre-miRNAs in several cell lines, but in contrast to previous reports, we could not observe pre-miRNA expression inside EVs of cancer-derived cell lines.
Conclusion: Overall, ALL-tRNAseq highlights an important, underrepresented aspect of RNA content in EVs and advances our knowledge of the small noncoding RNA composition in EVs, providing a new reference for future investigations.