NLSEV
Netherlands Society for Extracellular Vesicles

Fifth annual meeting of the

Netherlands Society for Extracellular Vesicles (NLSEV)

Thursday and Friday 17th and 18th November 2022

Crowne Plaza Maastricht, Ruiterij 1, 6221 EW

Maastricht

Sponsors


nanofcm


BECKMAN
COULTER
Life Sciences


DISPERTECH


NanoView
BIOSCIENCES


Distrilab
particle technology


bio-techne
protein simple


IZON


cytiva


sysmex
Lighting the way
with diagnostics


ThermoFisher
SCIENTIFIC


Luminex
A DiaSorin Company


MH&NS
school for
mental health
and neuroscience


NUTRIM


Universiteitsfonds Limburg
| SWOL |


CARIM
School for
Cardiovascular
Diseases


Maastricht University

NLSEV
Netherlands Society for Extracellular Vesicles

Executive board of NLSEV

Esther Nolte ’t Hoen
President

Rienk Nieuwland
Vice President

Rubina Baglio
Secretary General

Kasper Rouschop
Treasurer

Michiel Pegtel

Pieter Vader

Chiefs Science & Meetings

Fons van de Loo

Martijn van Herwijnen

Chiefs Communication, Sponsors, Outreach

Edwin van der Pol
Chief Digital Commun.

Marije Kuipers

Birke Benedikter

Social media managers

NLSEV
Netherlands Society for
Extracellular Vesicles

NLSEV-2022 Local Organising Committee

Paula Da Costa Martins

Kasper Rouschop

Frank Stassen

Rory Koenen

Leon Schurgers

Birke Benedikter

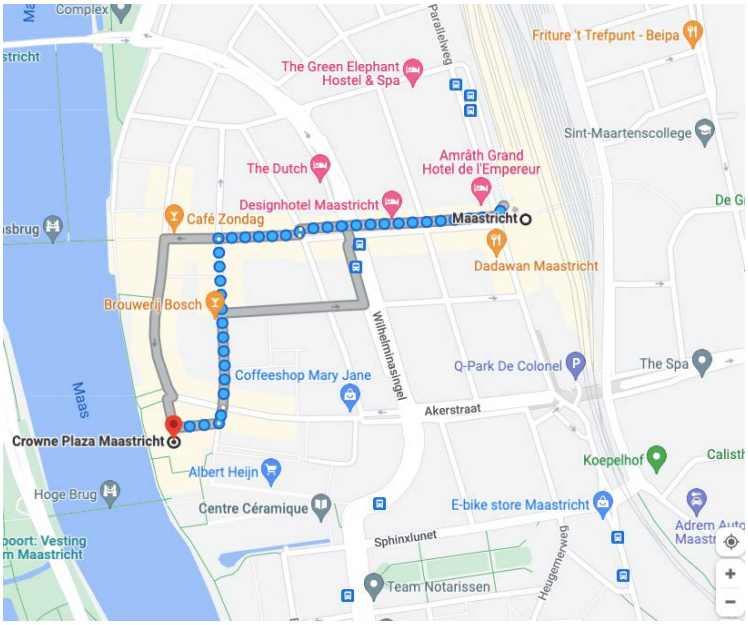
Tom Keulers

Britta Bettin

Jordy Kocken

Practical information

Route to Crowne Plaza Maastricht by public transport (recommended):



From Maastricht Station:
A short (10 min) walk to Ruiterij 1

Route to Crowne Plaza by car:

Follow the A2 highway towards Maastricht. Take exit to the N2 and continue until you take exit 53 towards Maastricht-Centrum Noord

Keep left towards Berg en Terblijt.

Turn left onto Terblijterweg and after roughly 50 m turn right onto Doctor Schaepmanstraat and continue slightly left onto Burgemeester Bauduinstraat. Take a right turn onto President Rooseveltlaan and continue onto Koningsplein. Enter the roundabout and take the 1st exit onto Scharnerweg and continue onto Akerstraat. Continue onto Hoogbrugplein and turn left

Turn left followed by a right turn onto Hoogbrugstraat. Make a sharp left onto Ruiterij and you arrive at the Crowne Plaza.

Programme

Thursday, November 17th

12.30 - 13.30 Registration & Poster Mounting

13.30 - 13.45 Welcome by Esther Nolte-'t Hoen and the Local Organising Committee

13.45 - 14.30 Keynote lecture
Chairs: Esther Nolte-'t Hoen and Tom Driedonks

Jayanta Debnath *University of California, San Francisco, USA*
Secretory Autophagy and Cargo Specification In Extracellular Vesicles and Particles

14.30 - 15.00 Selected presentations: EV biogenesis and composition
Chairs: Esther Nolte-'t Hoen and Tom Driedonks

Pilar Martinez-Martinez *Maastricht University, Maastricht, The Netherlands*
Function of ceramide transfer protein for biogenesis and sphingolipid composition of extracellular vesicles

Nicolas Hense *RWTH Aachen, Aachen, Germany*
PIKfyve inhibition reduces the calcification potential of vascular smooth muscle cell-derived extracellular vesicles

15.00 – 15.30 Coffee break & Poster viewing

15.30 – 16.15 Selected presentations: EV detection technology
Chairs: Pieter Vader and Wouter Woud

Sander Kooijmans *Utrecht University, Utrecht, The Netherlands*
A novel EV subpopulation isolation platform allows discovery of differences in cargo delivery capacity between EV subpopulations

Josette van Maanen *Utrecht University, Utrecht, The Netherlands*
Proteomic profiling of extracellular vesicles from notochordal cells

Agustin Enciso-Martinez *Leiden University Medical Center, Leiden, The Netherlands*
Label-free detection and characterization of single sub-micrometer particles in plasma of prostate cancer patients

Programme

16.15 - 16.35 Sponsored talks

Sponsored presentation: Distrilab
Colocalization with F-NTA

Sponsored presentation: NanoFCM
NanoFCM brings flow cytometry capabilities to the nanoscale

16.35 - 17.00 Coffee break & Poster viewing

17.00 - 17.15 Updates from the NLSEV board

17.15 – 18.00 Selected presentations: EVs in cancer
Chairs: Michiel Pegtel and Britta Bettin

Joel Beaumont *Maastricht University, Maastricht, The Netherlands*
Cancer derived extracellular vesicles modulate endothelial cell metabolism

Steven Wang *Amsterdam UMC, Amsterdam, The Netherlands*
Plasma Extracellular Vesicle- microRNAs for Early Outcome Prediction in Patients with Aggressive B-Cell Lymphoma

Crescenzo Massaro *Amsterdam UMC, Amsterdam, The Netherlands*
Cancer-released EVs counter drug efficacy by triggering inflammatory Mesenchymal Stem Cells

18.00 – 20.00 Poster Meet & Greet with Drinks & Snacks
18.00 – 19.00 Presentation & jury assessment odd poster number
19.00 - 20.00 Presentation & jury assessment even poster numbers

Note: no full dinner is provided. There are many restaurants in the area to make your own dinner arrangements after the poster session.

Programme

Friday, November 18th

09.00 - 09.30 Arrival/Registration and coffee

09.30 - 10.15 Keynote lecture
Chairs: Paula Da Costa Martins and Naomi Buntsma

Peter Peters *Maastricht University, Maastricht, The Netherlands*
Applications of Nanoscopy in EV Biology

10.15 - 10.25 Sponsored talks

Sponsored presentation: Beckman Coulter
BEC LS Solutions for EV research - an update

10.25 - 10.55 Coffee break & Poster viewing

10.55 -11.40 Selected presentations: Cell type specific EV release and composition
Chairs: Kasper Rouschop and Agustin Enciso Martinez

Kiana Buttiens *KU Leuven, Leuven, Belgium*
Ultrasensitive in vivo BLI tool for non-invasive detection of tumor cells receiving genetic information via Extracellular Vesicles

Kyra Defourny *Utrecht University, Utrecht, The Netherlands*
A comparison of extracellular vesicle release induced by picornavirus infection in different cell model systems including iPSC-derived cardiomyocytes

Suzy Varderidou *University Medical Center Utrecht, Utrecht, The Netherlands*
Amyotrophic Lateral Sclerosis Proteomic Signature And Treatment With Mesenchymal Stem Cell-derived Extracellular Vesicles

11.40 - 12.00 Sponsored talks

Sponsored presentation: Dispertech
To be announced

Sponsored presentation: Nanoview Biosciences
Purification-Free Characterization and Sizing of Exosomes & Viruses with ExoView Technology

Programme

12.00-13.00 Lunch break & Poster Viewing

13.00-13.45 Keynote Lecture
Chairs: Rubina Baglio and Pablo Lara

Eva Rohde *Paracelsus Medical University, Salzburg, Austria*
EV-based Therapeutics

13.45 – 14.30 Selected presentations: EV uptake and cargo delivery
Chairs: Fons van de Loo and Sander Kooijmans

Ardalan Mansouri *Erasmus Medical Center, Rotterdam, The Netherlands*
Manipulating the uptake of extracellular vesicle in prostate cancer

Vivian Hegemann *Utrecht University, Utrecht, The Netherlands*
Modulating binding affinity of aptamer-based loading constructs for efficient EV-mediated CRISPR/Cas9 delivery

Omnia Elsharkasy *University Medical Center Utrecht, Utrecht, The Netherlands*
A role for integrin beta 1 in extracellular vesicle-mediated functional RNA delivery

14.30 - 15.00 Coffee break & Poster Viewing

15.00 - 15.45 Selected presentations: Immune-related functions of EVs
Chairs: Leon Schurgers and Martijn van Herwijnen

Pablo Lara *Leiden University Medical Center, Leiden, The Netherlands*
M1-derived extracellular vesicles enhance photodynamic therapy and promote immunological memory in preclinical models of colon cancer

Margarida Viola *Utrecht University, Utrecht, The Netherlands*
Human iPSC-derived cardiomyocytes release different EV-subpopulations in both normoxia and hypoxia conditions and trigger macrophage activation

Xinyi Pei *Utrecht University, Utrecht, The Netherlands*
Extracellular vesicles released by picornavirus-infected cells trigger an antiviral response in immune cells

Programme

15.45 - 16.30 Panel Discussion
Chairs: Michiel Pegtel and Pieter Vader

16.30 - 17.00 NLSEV awards and closing

Oral Presentations

Oral presentations

Keynote Speaker 1:

Prof. Jayanta Debnath

Department of Pathology University of California, San Francisco, USA

Dr. Jayanta (Jay) Debnath is Distinguished Professor and Chair of Pathology at the University of California, San Francisco and a member of the UCSF Helen Diller Family Comprehensive Cancer Center, Bakar Immuno-X and Bakar Aging Research Institute. His laboratory is internationally recognized for its expertise on the diverse cell biological roles of autophagy during cancer progression and metastasis.

Dr. Debnath is a board certified pathologist, who received his M.D., magna cum laude, from Harvard Medical School, followed by clinical training in pathology at the Brigham and Women's Hospital. He completed post-doctoral training at the Harvard Medical School Department of Cell Biology with Prof. Joan Brugge, where he became widely known for his studies of oncogene regulation of cell death and autophagy using three-dimensional (3D) organotypic culture systems. He started his independent laboratory as an Assistant Professor at UCSF in 2005, where he has risen to the rank of Distinguished Professor and Chair. His research program focuses on two broad areas: 1) delineate the multifaceted roles of autophagy in adhesion independent survival, breast cancer progression and metastatic disease; and 2) dissect the biochemical and in vivo physiological functions of the molecules controlling autophagy (called ATGs) to ultimately exploit this process for therapeutic benefit. Recently, he has been illuminating how the autophagy pathway orchestrates secretory and exocytic functions distinct from its long-recognized roles in lysosomal catabolism.

Dr. Debnath currently serves as Cancer Section Chief Editor of Autophagy, Editor of the Annual Reviews of Pathology and on the editorial board of Genes and Development. He has previously served as Chair of the Programmatic Review Panel for the Department of Defense Breast Cancer Research Program (2018) and Chair of the Tumor Cell Biology Study Section for NIH (2016-18). His major honors include: HHMI Early Career Award for Physician Scientists (2006), DOD Breast Cancer Research Program Era of Hope Scholar Award (2011), elected membership into the American Society of Clinical Investigation (2013), American Society of Cell Biology Keith Porter Mid-Career Investigator Award (2016), Ramzi Cotran Memorial Lectureship from Brigham and Women's Hospital, Harvard Medical School (2019), and American Society of Investigative Pathology Outstanding Investigator Award (2021).

Oral Presentations

Keynote Speaker 2:

Prof. Eva Rohde

Department of Transfusion Medicine, Paracelsus Medical University of Salzburg, Austria

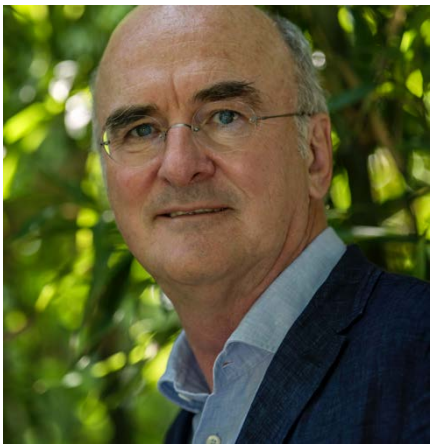
Eva Rohde is Head of the Department of Transfusion Medicine at the University Hospital of the Paracelsus Medical University in Salzburg, Austria. She received her M.D. from the Karl-Franzens University of Graz, Austria in 1994, finished training in Transfusion Medicine in 2005 and spent several years in stem cell research as a postdoctoral fellow. Her research focuses on the application of mesenchymal stromal cell-based therapies with special emphasis on their extracellular vesicles (MSC-EVs).

Eva Rohde is Founding Member of the Spinal Cord Injury and Tissue Regeneration Center Salzburg (SCI-TRECS), Austria and Director and Qualified Person of the SCI-TReCS GMP Unit. The GMP Unit in Salzburg has achieved the pharmaceutical manufacturing authorization for MSC and MSC-EVs in 2015 and participated a clinical trial testing MSCs in multiple sclerosis. Recent effort is directed towards the preclinical characterization and the regulatory requirements for the clinical assessment and future application of stem-cell based products, including stromal cell-derived extracellular vesicles (EVs). In vitro and in vivo potency assays to define the immunomodulatory, neuroprotective, neuroregenerative and scarless healing support of MSC-EVs are currently developed.

Eva Rohde is involved in the scientific and organizational management of the Transfer Center EV-TT (Extracellular Vesicles Theralytic Technologies, 2019-2022). EVTT is funded by the European Union and the state of Salzburg and treads on new grounds towards the development of so-called Exosome Therapies. It has pioneered the focused activities on theralytic technologies for Nanovesicles, and the international visibility and acceptance of these efforts are outstanding. The overarching topic is the development of novel cell-free therapies in conjunction with scientifically solid application-oriented analytical technologies.

Oral Presentations

Local keynote Speaker 3:



Prof. Peter Peters

The Maastricht Multimodal Molecular Imaging Institute

Throughout his career, he has made important technological breakthroughs in EM that have made the study of biomolecular processes down to the nanoscale level possible. For example, he was instrumental with a team in Utrecht in establishing cryo-immunogold EM, which has since then become the gold standard for ultrastructural detection of different proteins at the subcellular level. He has improved sample-preparation techniques for improved cryo-EM analysis. In 2018 he co-founded CryoSol-World, a company moving cryo-EM sample preparation including extra cellular vesicles into the new era of automation and high-throughput. More details: https://cryosol-world.com

He has applied this technical expertise and breakthroughs mostly to the field of cellular immunology. Due to the nature of his expertise, this was often done in close collaboration with other renowned scientists. Early in his career, he discovered the MHC class II compartment (MIIC), where MHC II molecules load their antigenic peptides to modulate immune responses. He established that secretory granules of cytotoxic T cells are of lysosomal nature that release vesicles from them that interacted with the target membrane. It was the first papers for the role of secreted extracellular vesicles in communicating in a receptor specific manner with other cells. At the US National Institutes of Health, he identified ARF6 as a regulator for endocytosis. More recently, Professor Peters discovered that pathogenic mycobacteria causing tuberculosis to move from the phagosome into the cytosol of dendritic cells and macrophages, whereas nonpathogenic mycobacteria do not. This paper is one of the top 10 cited in tuberculosis in the last 10 years. As another example of his groundbreaking work, he participated in establishing the human organoid culture system as close collaborator of Hans Clevers.

His forefront position in EM led him to initiate the establishment of the €18-million Netherlands Centre for Electron Nanoscopy that opened in 2011 and is now part of the EU roadmap of large research infrastructure. Since 2014, he has been a distinguished university professor (one of only four) at Maastricht University, where he led the creation of and now co-directs the Maastricht Multimodal Molecular Imaging Institute, which was established under the €21-million investment programme and specialises in advanced macromolecular imaging. Two years ago, he participated as one of the three PI's in a €100 million grant to establish state of the art EM infrastructure in nearby Julich.

Oral Presentations

Academic/research appointments

1991–1994: Postdoctoral scholar, Laboratory of Richard Klausner, National Institutes of Health, Bethesda, MD, USA

1994–1998: Junior Principal Investigator, Utrecht University, Utrecht, The Netherlands

1998–2008: Professor of Cell Biology, Free University, Amsterdam, The Netherlands

1998–2013: Group Leader, Division of Cell Biology, Netherlands Cancer Institute, Amsterdam, The Netherlands

1999–2013: Dean, Postdoc Career Development Initiative, The Netherlands

2009–2013: Professor of Nanobiology, Kavli Institute of Nanoscience, Delft University of Technology

2014–present: Distinghuised University Professor of Nanobiology, Maastricht University

2018-present: CSO of CryoSol-World

Oral Presentations

NLSEV2022-01 Pilar Martinez-Martinez

Function of ceramide transfer protein for biogenesis and sphingolipid composition of extracellular vesicles

Simone M. Crivelli1,2, Caterina Giovagnoni3, Zhihui Zhu1, Priyanka Tripathi1,2, Ahmed Elsherbini1, Zainuddin Quadri1, Jian Pu4, Liping Zhang1,2, Branislav Ferko5, Dusan Berkes5, Stefka D. Spassieva1, Pilar Martinez-Martinez*3#, Erhard Bieberich1,2#

**: Presenting author; 1Department of Physiology, University of Kentucky, Lexington, KY 40506, United States 2Veterans Affairs Medical Center, Lexington, KY 40502, United States 3Maastricht University, Department of Psychiatry and Neuropsychology, School for Mental Health and Neuroscience, Maastricht, the Netherlands 4Department of Surgery, University of Kentucky, Lexington, KY 40536, United States 5Department of Organic Chemistry, Slovak University of Technology, Radlinského 9, 81237, Bratislava, Slovak Republic*

#Shared senior authorship

The formation of extracellular vesicles (EVs) is induced by the sphingolipid ceramide. How this pathway is regulated is not entirely understood. Here, we report that the ceramide transport protein (CERT) mediates a non-vesicular transport of ceramide between the endoplasmic reticulum and the multivesicular endosome at contact sites. The process depends on the interaction of CERT's PH domain with PI4P generated by PI4KIIα at endosomes. Furthermore, a complex is formed between the START domain of CERT, which carries ceramide, and the Tsg101 protein, which is part of the endosomal sorting complex required for transport (ESCRT-I). Inhibition of ceramide biosynthesis reduces CERT-Tsg101 complex formation. Overexpression of CERT increases EV secretion while their inhibition reduces EV formation and the concentration of ceramides and sphingomyelins in EVs. In conclusion, we discovered a function of CERT in regulating the sphingolipid composition and biogenesis of EVs, which links ceramide to the ESCRT-dependent pathway.

Oral Presentations

NLSEV2022-O2: Nicolas Hense

O28 PIKfyve inhibition reduces the calcification potential of vascular smooth muscle cell-derived extracellular vesicles

Nicolas Hense*, Bilal Mir, Nikolaus Marx, Claudia Goettsch.

** presenting author. all: Department of Medical Clinic I – Cardiology, Medical Faculty, RWTH Aachen University*

Background: Cardiovascular calcification is a predictor and contributor to cardiovascular disease. Especially microcalcifications might cause atherosclerotic plaque rupture, leading to myocardial infarction. Previously we demonstrated that extracellular vesicles (EVs) released from calcifying vascular smooth muscle cells (SMCs) contribute to microcalcification formation. Tissue non-specific alkaline phosphatase (TNAP) is a characteristic cargo of EVs with high calcification potential. The molecular machinery that functionally regulates the specific loading of calcification prone proteins into EVs is unknown. FYVE-Type Zinc Finger Containing Phosphoinositide Kinase (PIKfyve), a lipid kinase, plays a role in the endolysosomal maturation. We hypothesize that alterations in endomembrane homeostasis will modulate EV cargo and SMC calcification.

Methods and Results: Immunohistochemical staining revealed the presence of PIKfyve in human calcified carotid arteries. In calcifying SMCs, inhibition of PIKfyve by the small molecule Apilimod caused a dose-dependent increase of EV release assessed by nanoparticle tracking analysis (up to 5.2 fold, $p<0.01$). Apilimod-induced EVs exhibited reduced mineralization potential and TNAP activity (-58%±14%, $p<0.01$) assessed by osteosense-based flow cytometry, turbidity assays, and enzymatic assays, respectively. The EV size pattern did not alter. Analysis of the EV cargo demonstrated that Apilimod reduces TNAP protein and promotes the accumulation of the autophagosomal marker Lc3b-II. On a cellular level, PIKfyve inhibition by Apilimod or siRNA inhibited TNAP mRNA, protein, and activity in calcifying SMCs. Further, Apilimod reduced matrix mineralization and collagen-enriched extracellular matrix in calcifying SMCs. Transcriptome analyses revealed that the anti-calcifying effect of PIKfyve inhibition is accompanied by an increased expression of adipogenic transcription factors and genes of the cholesterol and fatty acid metabolism pathways. Fluorescence-based assays showed increased fatty acid and disrupted low-density lipoprotein (LDL) uptake in Apilimod-treated SMCs. Finally, in Ldlr-deficient mice fed a high-fat, high-cholesterol diet for 15 weeks, Apilimod application for 5 weeks increased PI3P levels and decreased plaque TNAP activity in the aortic arch, while plaque size or collagen content did not alter.

Conclusion: Disrupting endolysosomal maturation with Apilimod promotes the release of EVs with reduced calcification potential and induces a phenotypic adaption towards adipocyte-like SMCs, causing reduced SMC calcification. Anti-calcific effects of Apilimod in vivo remain to be further investigated.

Oral Presentations

NLSEV2022-O3: Sander A.A. Kooijmans

A novel EV subpopulation isolation platform allows discovery of differences in cargo delivery capacity between EV subpopulations

Casper Triesscheijn[1], Bella Monica[1], Rowan Frunt [1], Ioanna Paspali [1], Mark Tielemans [1], Raymond M. Schiffelers [1], Olivier G. de Jong [2], Pieter Vader [1,3], Sander A.A. Kooijmans* [1].
**presenting author, 1: CDL Research, University Medical Center Utrecht; 2: Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University; 3: Department of Experimental Cardiology, University Medical Center Utrecht*

Background: The molecular content of extracellular vesicles (EVs) is highly heterogeneous. As EV surface molecules determine interactions with their environment, EV functionality likely varies between EV subpopulations with different surface markers. However, it remains challenging to test this hypothesis as methods to isolate intact EV subpopulations based on surface markers are lacking. Here, we used statistical modelling to optimize a novel method to isolate intact and functional EV subpopulations using antibody-coated magnetic beads, and compared their capacity to functionally deliver actively loaded Cas9/single guide RNA (sgRNA) complexes.
Methods: EVs from HEK293T, MDA-MB-231 and HeLa cells were isolated using tangential flow filtration and size exclusion chromatography (SEC). EV subpopulations were captured on antibody-coated magnetic beads targeting tetraspanins or phosphatidylserine (PS). Design-of-experiments (DoE)-based statistical modeling was applied to optimize an elution buffer to release EVs from the beads. Integrity of released EVs was characterized by transmission electron microscopy (TEM), Nanoparticle Tracking Analysis (NTA) and western blotting. EV donor cells were engineered to secrete EVs loaded with Cas9/sgRNA complexes and cargo delivery capacity was compared using the CROSS-FIRE reporter assay (de Jong et al, Nat Commun, 2020).
Results: A DoE-based library of elution conditions was applied to magnetic beads which had captured MDA-MB-231 EVs via CD9 or CD81 antibodies. pH of the elution buffer was identified as the most critical factor for both EV release from the beads and for integrity of released EVs. In a second optimization round, optimal elution conditions were established to release EV subpopulations enriched for CD9, CD63, CD81 or PS. Released EVs appeared intact by TEM and NTA and lacked typical contaminants observed in the original EV isolates. Furthermore, EVs engineered with CD9- or CD63- anchored Cas9/sgRNA complexes and VSV-G could functionally deliver their content to reporter cells after elution from beads. Interestingly, in these assays, CD9+ and CD81+ EV subpopulations showed distinct delivery capacity compared to CD63+ or PS+ EVs.
Conclusions: Using DoE methodology, we developed a novel platform to isolate intact, functional EV subpopulations based on their surface marker expression. This universal capture-and-release platform can be used to study EV surface-functionality relationships, and holds the potential to enrich EVs with desirable characteristics for therapeutic purposes.

Oral Presentations

NLSEV2022-O4: Josette C. van Maanen

Proteomic profiling of extracellular vesicles from notochordal cells

J.C. van Maanen*[1], F.C. Bach[1], F.M. Riemers[1], H.R. Vos[2], M.H.M. Wauben[3], M.A. Tryfonidou[1]
**presenting author; 1 Department of Clinical Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands; 2 Molecular Cancer Research, Center for Molecular Medicine, University Medical Center Utrecht, Utrecht University, Oncode Institute, Utrecht, The Netherlands; 3 Department of Biomolecular Health Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands"*

Background: Intervertebral disc degeneration is a major cause of chronic low back pain, a considerable burden to society. Notochordal cells (NCs), juvenile disc cells which are present in healthy discs, secrete extracellular vesicles (EVs) with regenerative properties that could be exploited for therapeutic approaches. It is unknown, however, which biologically active molecules are responsible for the EV-mediated effects. Therefore, this study comprehensively analyzed the protein composition of NC-derived EVs.
Methods: Conditioned medium was generated from pig NC-rich tissue. EVs were isolated through differential centrifugation, size exclusion chromatography, and sucrose density gradients. After size exclusion, EV-containing fractions were selected based on protein quantity, pooled, and ultra-centrifugated (100.000g). Thereafter, the pellet was floated in a linear sucrose gradient to collect purified EVs, whereas the supernatant was concentrated using filtration, yielding primarily soluble proteins (SOL). Proteomic analysis was performed using mass spectrometry. Data was analyzed using Perseus (v.2.0.3.1).
Results: Preliminary analysis (n=2) identified 1362 proteins in total, of which 941 were present in the EV samples, with a high correlation between donors (r=0.96). There was a considerable overlap in proteins detected in EV and SOL samples (630 proteins), while 311 proteins were uniquely identified in EV samples. These unique EV proteins contained well-known EV markers, e.g. tetraspanins (CD9, CD81) and Flotillin-1. Of the overlapping proteins, 115 proteins were up to ten fold enriched in EV samples; 23 these proteins are present in the Vesiclepedia top 100. In contrast, commonly detected proteins in SOL or uniquely present in SOL samples contained amongst others extracellular matrix proteins (Aggrecan, Collagen).
Conclusions: This is the first study that reveals the proteome of NC-derived EVs. EV isolation methods used in this study successfully enriched for EV markers. Proteins detected in both EV and SOL samples might be proteins that are loosely attached to NC-EVs, leading to co-isolation. Further analysis of 3 additional donors is currently performed and may indicate which proteins are involved in the biological functions of NC-EVs.

This project is funded by European Union’s Horizon 2020 program iPSpine (No.825925)."

Oral Presentations

NLSEV2022-O5: Agustin Enciso-Martinez

Label-free detection and characterization of single sub-micrometer particles in plasma of prostate cancer patients

Agustin Enciso-Martinez (1)*, Edwin van der Pol (3,4,5), Chi M. Hau (4,5), Theo M. de Reijke (6), Rienk Nieuwland (4,5), Leon W.M.M. Terstappen (2), Peter ten Dijke (1) and Cees Otto (2).
** presenting author*

Background: The presence of large (> 1 µm) tumor-derived extracellular vesicles (tdEVs) in the blood cell fraction of metastatic castration-resistant prostate cancer (mCRPC) patients has proven to be prognostic. However, the majority of tdEVs are expected in the cell-free plasma fraction. Little is known about the chemical composition of single tdEVs and other sub-micrometer particles in the plasma of mCRPC patients. In this pilot study, we interrogated individual particles in the plasma of mCRPC patients and healthy donors in a label-free manner and directly in suspension.
Methods: Platelet-poor blood plasma from mCRPC patients (N=5) and healthy donors (N=5) was prepared. Single particle analysis was achieved by optical trapping and simultaneous acquisition of both Rayleigh and Raman scattering (OT-sRRs) to detect single trapping events and disclose their corresponding chemical composition. Multivariate analysis was performed to identify and compare the chemical fingerprint of individual particles.
Results: We identified various types of lipoprotein particles as well as other particle types in a label-free manner. Interestingly, lipidic particles from some of the patients showed a high contribution of nucleic acids to their biomolecular profile, unlike particles from healthy donors. Using OT-sRRs, we further investigated the potential interaction between lipoprotein particles and tdEVs, and our preliminary data suggests that both particle types might associate with each other.
Conclusions: Further studies on a larger cohort of donors are necessary to expand the diagnostic potential of the proposed methods. However, the current results support the feasibility of OT-sRRs for single particle analysis of blood plasma. Furthermore, implications concerning the interaction between lipoproteins and EVs should be taken into account when using circulating tdEVs as biomarkers.

Oral Presentations

NLSEV2022-O6: Joel E.J. Beaumont

Cancer derived extracellular vesicles modulate endothelial cell metabolism

Joel E.J. Beaumont* (1), Lydie M.O. Barbeau (1), Kim G. Savelkouls (1), Kim R. Kampen (1), Marijke I. Zonneveld (1), Tom G.H. Keulers (1), Kasper M.A. Rouschop (1).

** presenting author, 1: Department of Radiotherapy, GROW—School for Oncology and Reproduction, Maastricht University Medical Center+, 6229 HX Maastricht, The Netherlands*

Introduction

Background: Cancer cells secrete EV to corrupt cells in their surrounding into providing a growth supportive environment, for example by inducing angiogenesis for increased delivery of oxygen and nutrients. Induction of angiogenesis is associated with a metabolic switch in endothelial cells (EC), yet mechanisms driving this switch are relatively unknown. Hypoxia is a common feature of solid tumors, associated with an aggressive disease. It induces angiogenesis and alters the molecular cargo of EV. Here, we evaluate the effects of (hypoxic) cancer cell derived EV on the metabolic reprogramming of the tumor microenvironment (TME).
Methods: Colorectal cancer- (HT29), glioblastoma- (U87), and breast cancer- (MDA-MB-231) cells were cultured under normoxia, moderate hypoxia (0.2% O2) or severe hypoxia (<0.02% O2) for 24h with 5% EV depleted serum. EV were isolated by size exclusion chromatography and analysed using mass spectrometry. After labelling of EV with CFSE, uptake was analysed by FACS and confocal imaging. Glucose uptake and lactate secretion were measured after 24h EV stimulation. For metabolomics, EC were cultured in medium supplemented with 13C-labeled glucose and stimulated with EV for 48h. Afterwards, (un)labelled metabolites in cells and medium were analysed. De novo protein synthesis was assessed by click-it chemistry.
Results: Confocal and flow cytometric analysis demonstrated the uptake of EV by recipient cells irrespective of producer cell oxygenation status. Proteomic analysis identified the presence of various (glycolytic) enzymes within HT29, U87 and MDA derived EV. In line, these EV increase glucose uptake in EC, cancer cells, monocytes and fibroblasts. However, lactate production did not increase. Metabolic tracer analysis of EC shows increased synthesis of amino acids (AA) after stimulation with normoxic and moderate hypoxic HT29 and MDA derived EV. Furthermore, AA uptake was increased upon EV stimulation, which was dependent on EV producer cell oxygenation status. Despite the increase in AA synthesis and uptake, intracellular AA levels remain unaltered, suggesting increased usage. In line, EC demonstrate increased de novo protein synthesis upon stimulation with (hypoxic) EV.
Conclusion: Depending on the specific needs of a tumor and its environmental conditions (oxygenation), cancer cells manipulate the metabolism of TME cells to support tumor growth.

Oral Presentations

NLSEV2022-O7: Steven Wang

Plasma Extracellular Vesicle- microRNAs for Early Outcome Prediction in Patients with Aggressive B-Cell Lymphoma

Steven Wang* [1,3], Esther E.E. Drees* [2,3]*, Cristina Gómez-Martín [2,3], A. Vera de Jonge [1], Monique van Eijndhoven [2], Nils Groenewegen [2,4], Sandra Veldt-Verkuijlen [2], Marcel Nijland [5], Marjolein van der Poel [6], Yorick Sandberg [7], Rozemarijn van Rijn [8, Pim Mutsaers [9], Vibeke K.J. Vergote [10], Marie José Kersten [11], Yavuz M. Bilgin[12], Wendy B.C. Stevens[13], Marc Durian [14], Tjeerd Snijders [15], Daphne de Jong [2], Josée Zijlstra [1,3], Martine E.D. Chamuleau# [1,3], Dirk Michiel Pegtel# [2,3] *Co-first authors | #Co-senior authors

** presenting author, [1] Amsterdam UMC Location Vrije Universiteit Amsterdam, Hematology, Amsterdam [2] Amsterdam UMC Location Vrije Universiteit Amsterdam, Pathology, Amsterdam [3] Cancer Center Amsterdam, Imaging and Biomarkers, Amsterdam [4] ExBiome BV, Amsterdam [5]University Medical Center Groningen, Groningen [6] Maastricht University Medical Center, Maastricht [7] Maasstad Hospital, Rotterdam [8] Medical Center Leeuwarden, Leeuwarden [9] Erasmus University Medical Center, Rotterdam [10] University Hospitals Leuven, Leuven, Belgium [11] Amsterdam UMC Location University of Amsterdam, Department of Hematology, Cancer Center Amsterdam, Amsterdam [12] Adrz, Goes [13] Radboud University Medical Center, Nijmegen [14] Elisabeth-TweeSteden Hospital, Tilburg, The Netherlands [15] Medical Spectrum Twente, Enschede*

Background: Early and accurate outcome prediction is essential in the clinical management of high-grade B-cell lymphoma (HGBL). Plasma EV-microRNAs (pEV-miRNAs) are considered promising biomarkers. We applied small RNA sequencing of pEV-miRNAs and built signatures for early response prediction for lymphoma patients.
Methods: We analyzed PAXgene ccfDNA plasma samples from 38 of the 97 patients included in the HOVON-152 trial (NCT03620578), a prospective, multicenter, non-randomized phase II trial. In this trial, patients receive one cycle of R-CHOP followed by five cycles of DA-EPOCH-R. Responders were defined as complete metabolic response (CMR, Deauville score 1-3) on EOI PET/CT, other non-CMR responses were classified as non-responders. 20 responders and 18 non-responders were analyzed. EVs were isolated with SEC as confirmed with transmission electron microscopy (TEM), tunable resistive pulse sensing (TRPS), and western blot (WB). Library preparation was done according to IsoSeek (van Eijndhoven et al., 2021. bioRxiv) and sequenced on the NovaSeq 6000 (Illumina). We applied machine learning (ML) to build models with EV-miRNAs for early response prediction. The model was internally validated and tested with bootstrapping (1000x) and over optimism estimate as well as the adjusted AUC was calculated.
Results: TEM after one cycle of R-CHOP revealed abundant particles < 200 nm and TRPS measured significantly higher particle concentration in EV-enriched fractions. pEVs were positive for CD63, CD81, flotillin-1, syntenin, HSP70, and negative for calnexin as determined by WB in accordance with the 2018 MISEV Criteria. The most optimal model was an Elastic Net regression (a = 0.4) model consisting of 199 miRNAs with an area under the curve (AUC) of 0.95 (Confidence Interval (CI): 0.90 – 0.99) [Sensitivity: 88%, Specificity: 90%; Positive Predictive Value (PPV): 90%, Negative Predictive Value (NPV): 87%]. After taking into account of over optimism estimate of 0.14, the adjusted AUC was 0.81 (CI: 0.64-0.96), which is higher than the performance of interim PET/CT [Sensitivity: 33-87%, Specificity: 49-94%; PPV: 20-74%, NPV: 64-95%].

Oral Presentations

Conclusion: ML models using pEV-miRNAs yielded a robust signature that can predict EOI response already after one cycle of R-CHOP. If validated in independent cohorts, pEV-miRNAs could potentially guide risk-adapted treatment strategies in aggressive lymphomas.

Oral Presentations

NLSEV2022-O8: Crescenzo Massaro

Cancer-released EVs counter drug efficacy by triggering inflammatory Mesenchymal Stem Cells

Crescenzo Massaro* [1], Celine De Schrijver [1], Tonny Lagerweij [1], Juan Simon Nieto [1], Cristina Gomez Martin [1], Leontien Bosch [1], Cristina Giorgio [2], Laura Brandolini [2], Marcello Allegretti [2], D. Michiel Pegtel [1], S. Rubina Baglio [1].

** presenting author, 1: Department of Pathology, Cancer Center Amsterdam, Amsterdam UMC, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands. 2: Dompè farmaceutici S.p.a., Milan, Italy.*

Background: Mesenchymal stem cells (MSCs) play a key role in the progression of bone cancers. We and others previously showed that in response to tumor-associated signals, MSCs acquire an inflammatory phenotype promoting tumor cell aggressiveness, though the underlying the mechanisms remained incompletely understood. In this study we set out to define how tumor EVs induce inflammatory MSC (iMSC) development and to identify combination therapies that can counteract MSC-induced drug resistance.

Methods: Tumor EV-induced alterations of the MSC transcriptome were analyzed by RNA-seq and compared with single cell RNA-seq data of patient-derived iMSCs. Gene set enrichment analysis (GSEA) was applied to discriminate TGFβ-dependent and independent pathways upon TGFβ inhibition or knockdown. EV RNA-induced expression changes were identified by transfecting purified EV-RNA in MSCs 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 model of OS. Ladarixin (an allosteric inhibitor of the IL8 receptors CXCR1 and CXCR2) and tocilizumab (anti-IL6 receptor antibody) were administered starting from day one until the experimental endpoint. Metastasis were quantified by histological examination.

Results: EVs from aggressive cancer cell lines induced an inflammatory MSC phenotype highly similar to that of patient bone marrow-derived iMSCS. Such phenotype is characterized by increased expression of chemokines, including IL8 and IL6 as the most upregulated. Apart from IL-6, these alterations were mostly independent from TGFβ signaling and related to pattern recognition receptor (PRR) activation. We demonstrated that tumor EV-associated non-coding RNAs trigger TLR3 signaling in MSCs activating an innate immune response leading to high induction of IL8 and other chemokines. Ladarixin and tocilizumab combination significantly reduced metastasis formation in a spontaneous metastasis model and overcame iMSC-induced resistance observed with single antimetastatic treatments.

Conclusions: EV-associated TGFβ together with EV-RNA induce iMSCs development in OS. Ladarixin in combination with tocilizumab reduced metastasis formation in a xenograft mouse model of OS, and, importantly, may prevent the occurrence of iMSC-induced tumor resistance to antimetastatic drugs.

Oral Presentations

NLSEV2022-O9: Kiana Buttiens

Ultrasensitive in vivo dual-color BLI tool for non-invasive detection of tumor cells receiving genetic information via Extracellular Vesicles

Kiana Buttiens*[1], Cláudio Pinheiro [2,3], Bella B. Manshian [4,5], An Hendrix [2,3], Stefaan J. Soenen [4,5,6]*
**presenting author, 1 NanoHealth and Optical Imaging Group, Department of Imaging and Pathology, KULeuven, Belgium 2 Laboratory of Experimental Cancer Research, Department of Human Structure and Repair, Gent University, Ghent, Belgium; 3 Cancer Research Institute Ghent (CRIG), Ghent, Belgium; 4 Leuven Cancer Institute, KULeuven, Belgium; 5 Translational Cell and Tissue Research Unit, Department of Imaging and Pathology, KULeuven, Belgium; 6 KU Leuven Institute of Physics-based Modeling for In Silico Health, KULeuven, Belgium*

Background: Studies revealed significant effects of nanomaterial(NM)-mediated inflammation on tumor malignancy. Also, NMs distinctly affect tumor cell migration and metastasis, based on NM and tumor type. For a further understanding on how relevant NMs affect the TME, many questions remain unanswered, mainly related to NM delivery to the tumor site and intratumoral localization. Objective: We aimed to look into key players potentially explaining these earlier found differences in cellular behavior and metastasis formation in distinct tumor types upon NM exposure. Extracellular vesicles (EVs) sparked our interest for their known key role in TME transformation and metastatic processes.
Methods: We first studied the effect of relevant NMs (Au, Ag, Si and Fe3O4) on EV generation in cancer cells in vitro with image-based flow cytometry. Further, we developed a non-invasive preclinical in vivo BLI method based on a custom lentiviral vectorsystem, enabling us to detect low numbers of cells receiving genetic info (e.g. via EVs) by switching from constitutively expressed green luciferase (CBG99) to Cre-induced red luciferase (PpyRE9) expression. To validate our tool, we isolated EVs from Cre+ MDA-MB-231 media using density-gradient ultracentrifugation (DG-UC), characterized according to MISEV guidelines (WB & NTA), and injected them (IT or IV) in an NSG mouse model with MDA-MB-231 tumor reporter cells transduced with our custom vectorsystem.
Results: Significant upregulation of EV generation upon Au NP administration in MDA-MB-231 cells is detected in vitro. Further, our newly developed non-invasive imaging tool confirmed transfer of genetic information in MDA-MB-231 reporter cells both in vitro and in vivo.
Conclusion: Our novel imaging tool successfully detects uptake of genetic information in tumor cells non-invasively. Further, it enables us to study the impact of NMs on tumor malignancy in vivo and the role of intercellular communication (e.g. via EVs) in these processes in future experiments.

Oral Presentations

NLSEV2022-O10: Kyra A.Y. Defourny

A comparison of extracellular vesicle release induced by picornavirus infection in different cell model systems including iPSC-derived cardiomyocytes

Kyra A.Y. Defourny(1)*, Margarida Viola(2)#, Xinyi Pei(1)#, Charlotte Nurmohamed(1), J.P.G. Sluijter(2), F.J.M. Van Kuppeveld(3), Esther Nolte-'t Hoen(1).

**presenting author, 1) Division of Cell Biology, Metabolism & Cancer, Department of Biomolecular Health Sciences, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 2, 3584 CM Utrecht, The Netherlands. 2) Department of Cardiology, Experimental Cardiology Laboratory, University Medical Center Utrecht, 3584 CX Utrecht, Netherlands; 3) Virology Section, Division Infectious Diseases & Immunology, Department of Biomolecular Health Sciences, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands. # these authors contributed equally*

Background: Extracellular vesicles (EVs) from virus infected cells can contribute to infection spread by transmitting infectious virus particles. Currently, large efforts are being performed to study the composition, release, and functional properties of EV-enclosed virions. However, few studies have addressed whether viruses induce similar EV release in cells of different tissue origin, and whether the composition and functional properties of EV-associated viruses are cell type-dependent. To address this issue, we compared the impact of encephalomyocarditis virus (EMCV) and coxsackievirus B3 (CVB3) infection, two causative agents of viral myocarditis, on EV release in commonly used (tumor) cell lines of different tissue origins, as well as human induced pluripotent stem cell-derived cardiomyocytes.
Methods: EVs were isolated by differential ultracentrifugation and iodixanol density gradient purification. The impact of infection on EV release was assessed quantitatively and qualitatively by high resolution flow cytometry and western blot analysis. Viral content of EVs was monitored by end-point dilution assay.
Results: We show that EMCV and CVB3 infection increased EV production in all cells studied. In addition, we show that all cells released EV-associated virus particles. However, cell-type dependent differences in the protein composition of virus-induced EVs were observed, along with the light scattering properties of virus-induced EVs during flow cytometric analysis.
Conclusions: Overall, the data highlight the existence of cell-type dependent and independent features of EV release in response to infection, and provide first insight into EVs potentially released during viral myocarditis.

Oral Presentations

NLSEV2022-O11: Suzy Varderidou-Minasian

Amyotrophic Lateral Sclerosis Proteomic Signature And Treatment With Mesenchymal Stem Cell-derived Extracellular Vesicles

Suzy Varderidou-Minasian*[1], Svetlana Pasteuning-Vuhman[2], Annabel Timmers[1], Maarten Altelaar[3,4], Magdalena J Lorenowicz[5,6], R Jeroen Pasterkamp[1].

**presenting author, 1 Department of Translational Neuroscience, UMC Utrecht Brain Center, University Medical Center Utrecht, Utrecht University, 3584 CG Utrecht, The Netherlands; 2 VectorY. Matrix Innovation Center, Science Park 408, 1098 XH Amsterdam; 3 Biomolecular Mass Spectrometry and Proteomics, Bijvoet Center for Biomolecular Research and Utrecht Institute for Pharmaceutical Sciences, University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands.; 4 Netherlands Proteomics Center, Padualaan 8, 3584 CH Utrecht, The Netherlands; 5 Center for Molecular Medicine, University Medical Center Utrecht, Universiteitsweg 100, 3584 CG, Utrecht, The Netherlands; 6 Regenerative Medicine Center, Uppsalalaan 8, 3584 CT, Utrecht, The Netherlands.*

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder with a lifetime risk of 1:400, primarily affecting upper and lower motor neurons. Unfortunately, there are only two drugs approved to treat ALS, which increase patient survival only by a few months. This highlights the urgent need for developments of new ALS modifying therapies, which have been hampered by high failure rate of new drug candidates during clinical trials.

Stem cell therapy is one of the promising approaches that is in development for treating ALS. The beneficial effects of stem cell therapy rely on their paracrine signaling, suggesting extracellular vesicles (EVs) as possible non-cell based therapy. In particular, mesenchymal stem cells (MSCs) contribute to reparative process.

To better translate and assess therapeutic potential of MSC-EVs, we combined state of the art induced pluripotent stem cell (iPSC) technologies to obtain patient-derived spinal low motor neurons and a comprehensive proteomic analysis to decipher the proteomic signature.

To investigate the key molecular perturbations underlying disease pathology of ALS, a comprehensive proteomic analysis was performed showing mutation-specific and common ALS-specific changes. Mutation-specific effects in ALS motor neurons with hexanucleotide expansion in C9ORF72 exhibit dysregulation in proteins targeting to ER and cytoplasmic translation. In FUS-ALS motor neurons, iron ion homeostasis and cellular response to stress processes are affected. In ALS-TDP-43 motor neurons protein transport and localization are affected. Common ALS mechanisms such as mRNA splicing, proteasomal ubiquitin and mitochondrial associated processes are dysregulated.

Furthermore, we demonstrated these underlying dysregulated mechanisms after treatment with MSC-EVs. More importantly, MSC-EV treatment can restore the protein expression levels impaired in ALS suggesting for future therapeutic potential for ALS.

Oral Presentations

NLSEV2022-O12: Ardalan Mansouri

Manipulating the uptake of extracellular vesicle in prostate cancer

Ardalan Mansouri*[1], Pedro Matos Filipe[2], Natasja Dits[1], Andrea Sacchetti[3], Judith Farrés[2], Guido Jenster[1], Martin van Royen[3].

**presenting author, 1: Department of Urology, Erasmus MC, Rotterdam, The Netherlands, 2: Information Technology Department, Anaxomics Biotech, Barcelona, Spain, 3: Department of Pathology, Erasmus MC, Rotterdam, The Netherlands.*

Background: Prostate cancer (PCa), as a common cancer among elderly men, can metastasize to bone, liver and lymph node. Cell-cell communication via secreting and uptake of extracellular vesicles (EVs) has a major role in tumor growth and metastasis. Accordingly, manipulating the uptake of EVs by the target cells might subside tumor growth and metastatic process. In the current study, we used drug repurposing and an artificial neural network (ANN)-based approach to identify compounds that inhibit EV-internalization.
Methods: DU145 PCa cells, were treated with more than 2000 compounds from Prestwick and LOPAC libraries for 16 hours. EVs from the same cell line, isolated via ultracentrifugation and labeled with PKH26, were added to the cells and the EV-uptake was measured 3 hours later using the Opera Phenix HCS system. EV uptake inhibition was validated with flow cytometry. Ingenuity pathway analysis (IPA) and ANN were used to elucidate the mechanism of action of the EV-uptake 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. In the validation phase, three EV-uptake inhibitors and four 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. Flow cytometry data confirmed the change in EV-uptake. Pathway analysis of the protein targets of the inhibitors using IPA, revealed that pathways related to regulation of P glycoproteins and ATPases are most commonly affected. Compared to the validated compounds, ANN results suggested a synergic effect of number of compounds from the above-mentioned libraries on EV-uptake.
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. Further analysis using ANN alluded to the possible effect of a combination of compounds on EV-uptake. Moreover, ANN is being utilized to investigate any influence of the compounds on cell proliferation, cell cycle and cell death.

Oral Presentations

NLSEV2022-O13: Charlotte V. Hegeman

Modulating binding affinity of aptamer-based loading constructs for efficient EV-mediated CRISPR/Cas9 delivery

Charlotte V. Hegeman [1*], Omnia M. Elsharkasy [2], Olaf L. Cotugno [2], Pieter Vader [2, 3], Olivier G. de Jong* [1, 2].

**presenting author, 1: Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, The Netherlands. 2: CDL Research, University Medical Center Utrecht, The Netherlands. 3: Department of Experimental Cardiology, University Medical Center Utrecht, The Netherlands.*

Background: The CRISPR/Cas9 toolbox consists of modular nucleases that can be employed to efficiently modify genomic sequences with high specificity. Hence, these systems open new avenues in the development of gene-editing therapies. However, targeted delivery of the large Cas9-sgRNA ribonucleoprotein (RNP) complexes remains challenging due to immunogenicity, negative charge, and rapid degradation. An approach to potentially overcome most of these limitations is the use of extracellular vesicles (EVs) as intercellular delivery vehicles. EVs exhibit the natural ability to carry RNA and proteins across biological membranes and can be engineered to load biotherapeutic molecules and target specific tissues.
Methods: To load Cas9-sgRNA complexes into EVs, sgRNAs containing MS2 aptamers and a fusion protein of CD63 and tandem MS2 coat proteins were expressed alongside Cas9 and VSV-G in HEK293T cells. To study the effect of binding affinity on Cas9-sgRNA delivery, both the interacting sgRNA MS2-hairpin and the RNA-binding domain of the MS2 coat protein were mutated. To separately study the effects of affinity on cargo loading and release, a UV-sensitive photocleavable protein (PhoCl) was included in the MS2-CD63 construct to maximize cargo release. We used a previously published fluorescent stoplight reporter system that can be activated by delivered Cas9-sgRNA complexes to measure functional Cas9-sgRNA delivery (De Jong et al., Nat Commun. 2020).
Results: We confirmed that adaptation of the sgRNAs did not adversely affect their functionality. Comparing Cas9-sgRNA delivery for the different modulated sgRNAs revealed that adapting binding affinity highly affects functional delivery (0.5% to 22.2%). A similar effect on functional delivery was seen after adaptation of the affinity of the RNA-binding domain of the MS2 coat protein. After UV-treatment, photocleavable MS2-PhoCl-CD63 fusion proteins revealed similar Cas9 delivery for most sgRNAs with varying affinities, indicating that cargo release, and not loading, was a limiting factor in aptamer-mediated Cas9 delivery.
Conclusions: Here, we describe a novel way to optimize EV-mediated loading and delivery of Cas9-sgRNA complexes. Our results demonstrate that EVs are capable of functional Cas9-sgRNA complex delivery and that modulation of binding affinity can be used to facilitate efficient Cas9 delivery.

Oral Presentations

NLSEV2022-O14: Omnia M. Elsharkasy

A role for integrin beta 1 in extracellular vesicle-mediated functional RNA delivery

Authors: Omnia M. Elsharkasy* [1], Willemijn S. de Voogt [1], Leanne van der Werff [1], Raymond M. Schiffelers [1], Olivier G. de Jong [1,2], Pieter Vader [1,3]

** presenting author. 1: CDL Research, University Medical Center Utrecht, The Netherlands. 2: Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, The Netherlands. 3: Department of Experimental Cardiology, University Medical Center Utrecht, The Netherlands.*

Background: Various uptake mechanisms for extracellular vesicles (EVs) have been widely explored, but the underlying molecular mechanisms through which EVs are able to provide functional RNA transfer to recipient cells are not fully understood. Here, we demonstrate a role for integrin beta 1 (ITGB1) in recipient cells in EV-mediated uptake and RNA delivery.

Methods: To study RNA transfer we used a fluorescent stoplight reporter system, which is activated by Cas9 upon functional sgRNA delivery (De Jong et al., Nat Commun. 2020). HEK293T reporter cells were transfected with ITGB1 siRNA or non-coding siRNA as a control. ITGB1 knockdown efficiency was determined using RT-qPCR. EVs from MDA-MB-231 cells expressing a targeting sgRNA were isolated by tangential flow filtration followed by size exclusion chromatography and were then added to HEK293T reporter cells. Functional RNA transfer was quantified by flow cytometry. For uptake experiments, EVs were labelled using MemGlow640. Additionally, intracellular EV trafficking was tracked in live cells using a Nanoimager-S (ONI).

Results: ITGB1 mRNA knockdown efficiency in recipient cells was ~ 85%. EVs containing targeting sgRNA showed functional RNA delivery in cells transfected with control siRNA. In cells transfected with ITGB1 siRNA functional delivery was strongly reduced. This was observed both in a co culture setting and by adding isolated EVs. Complementarily, knockdown of ITGB1 or ITGB1-blockage using neutralizing antibodies resulted in a significant decrease in EV uptake in recipient cells. Moreover, using live imaging we observed co-localization of MDA-MB-231 EVs with ITGB1 and their trafficking together inside recipient HEK293T cells.

Conclusions: MDA-MB-231-EV uptake and EV-mediated functional RNA transfer into HEK293T recipient cells is mediated by ITGB1. In-depth understanding of the molecular mechanisms underlying EV-mediated RNA transfer could contribute to achieving more efficacious EV-mediated RNA delivery and improved RNA therapeutics.

Funding: The work of O.M.E, L.v.d.W, R.M.S. and P.V. is supported by the European Union's Horizon 2020 Research and Innovation Programme under grant agreement No. 825828. W.S.d.V. and P.V. are supported by the European Research Council (ERC) Starting grant OBSERVE (# 851936). O.G.d.J. is supported by a VENI Fellowship from the Netherlands Organisation for Scientific Research (NWO) VI.Veni.192.174.

Oral Presentations

NLSEV2022-O15: Pablo Lara

M1-derived extracellular vesicles enhance photodynamic therapy and promote immunological memory in preclinical models of colon cancer

Pablo Lara*,1 Ruben V. Huis in 't Veld,1 Martine J. Jager,2 Roman I. Koning,4 Ferry Ossendorp,3 Luis J. Cruz1,
** presenting author. 1 Department of Radiology, Leiden University Medical Centre (LUMC), Leiden, The Netherlands;2 Department of Ophthalmology, Leiden University Medical Centre (LUMC), Leiden, The Netherlands;3 Department of Immunology, Leiden University Medical Centre (LUMC), Leiden, The Netherlands;4 Department of Cell and Chemical Biology, Section Electron Microscopy, Leiden University Medical Centre (LUMC), Leiden, The Netherlands;*

Extracellular vesicles (EVs) are promising drug carriers of photosensitizers for photodynamic therapy (PDT) in cancer treatment, due to their ability to circulate in blood and enter cells efficiently. The therapeutic potential of EVs has been suggested to depend on the type and physiological state of their cell of origin. However, the effects of deriving EVs from various cells in different physiological states on their antitumor capacity are rarely evaluated. We compared the antitumor efficacy of EV-mediated PDT by incorporating the photosensitizer Zinc Phthalocyanine (ZnPc) into EVs from multiple cells sources. ZnPc was incorporated by a direct incubation strategy into EVs derived from immune cells (M1-like macrophages and M2-like macrophages), cancer cells (B16F10 melanoma cancer cells) and external sources (milk). Our data show that all EVs are suitable carriers for ZnPc and enable efficient PDT in vitro in co-culture models and in vivo. We observed that EV-mediated PDT initiates immunogenic cell death through the release and exposure of damage associated molecular patterns (DAMPs) on cancer cells, which subsequently induced dendritic cell (DC) maturation. Importantly, of all ZnPc-EVs tested, in absence of light only M1-ZnPc displayed toxicity to MC38, but not to DC, in monoculture and in co-culture, indicating specificity for cancer over immune cells. In MC38 tumor-bearing mice, only M1-ZnPc induced a tumor growth delay compared to control in absence of light. Interestingly, M1- but not M2-mediated PDT, induced complete responses against MC38 tumors in murine models (100% versus 38% of cases, respectively), with survival of all animals up to at least 60 days post inoculation. Finally, we show that all cured animals are protected from a rechallenge with MC38 cells, suggesting the induction of immunological memory after EV-mediated PDT. Together, our data show the importance of the cell type from which the EVs are obtained and highlight the impact of the immunological state of these cells on the antitumor efficacy of EV-mediated PDT.

Oral Presentations

NLSEV2022-O16: Margarida Viola

Human iPSC-derived cardiomyocytes release different EV-subpopulations in both normoxia and hypoxia conditions and trigger macrophage activation

Margarida Viola *[1], Renee Maas [1], Maarten Bebelman [2], Michiel Pegtel [2], Pieter Vader [1,3], Joost P.G. Sluijter [1]

** presenting author. 1: Experimental Cardiology Laboratory, University Medical Center Utrecht, The Netherlands, 2: Exosome research group, Amsterdam UMC, The Netherlands, 3: CDL Research, University Medical Center Utrecht, The Netherlands.*

Background: A strong inflammation takes place in the heart after a myocardial infarction (MI). Cardiac resident macrophages and monocyte-derived macrophages coordinate this response as well as the subsequent wound healing. Extracellular vesicles (EVs) released by cardiac cells after MI have been described to shape inflammation in a mouse model, however, it is unknown which EV-subpopulations contribute to macrophage response and how this happens. Likewise, there is a gap in how EVs are produced in cardiomyocytes and how EV biogenesis changes under ischemic conditions. To address these questions, we studied the effect of hypoxia on EV release and content from human induced pluripotent stem cells derived-cardiomyocytes (iPSC-CMs). We further investigated the effect of hypoxia on EV functionality on human macrophages.

Methods: EV subpopulations were obtained by differential ultracentrifugation. Exophers were pelleted at 2000g, large EVs at 10000g, and small EVs at 100000g. EV characterization was performed by western blot, NTA, and electron microscopy. iPSC-CMs were transfected with CD63-pHluorin and imaged with live cell TIRF microscopy. iPSC-CMs were transduced with CD63-NanoLuc to robustly quantify EV release. Hypoxia was induced in iPSC-CMs with BD Pouch System. Human macrophages were differentiated from primary monocytes acquired from the blood of healthy donors. The functional effect of EVs on macrophages was assessed by RT-PCR.

Results: We demonstrated that iPSC-CMs secrete different EV subpopulations during both normoxia and hypoxia. EV subpopulations differ in protein content and their ability to stimulate macrophages. CD63-pHluorin shows that at least part of the cardiomyocyte-derived EVs are exosomes, originating from multivesicular bodies. Furthermore, nutrient starvation decreased cardiomyocyte EV secretion. Finally, preliminary data suggested that EVs secreted under hypoxic conditions can stimulate macrophages.

Conclusions: Our data suggest that human cardiomyocytes can release EV-subpopulations different in protein content and capacity to stimulate macrophages. This highlights the role of cardiomyocyte-derived EVs in communication with their microenvironment, namely with macrophages, after ischemic injury.

Oral Presentations

NLSEV2022-O17: Xinyi Pei

Extracellular vesicles released by picornavirus-infected cells trigger an antiviral response in immune cells

Xinyi Pei* 1, Kyra A.Y. Defourny 1, Frank J.M. van Kuppeveld 2, Esther N.M. Nolte-'t Hoen 1

** presenting author. Division of Cell Biology, Metabolism & Cancer, Department of Biomolecular Health Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands*

2 Virology Section, Division Infectious Diseases & Immunology, Department of Biomolecular Health Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

Background: Naked viruses of the picornaviridae family commonly spread via lysis of infected cells. However, recent studies indicate that, prior to cell lysis, naked viruses can be packaged and released inside extracellular vesicles (EVs). The EV membrane protects enclosed virus particles against neutralizing antibodies, thereby facilitating virus spread. However, it is unknown how immune cells respond to virus-induced EVs. Previously, we demonstrated that the picornavirus Encephalomyocarditis virus (EMCV) induces the release of various virus-carrying and other EV subsets. Here, we investigated how virus-induced EVs affect the antiviral response of immune cells.

Methods: EVs were isolated and purified from the supernatant of EMCV-infected cells, via differential ultracentrifugation and Optiprep density gradient. EVs were characterized by western blot, high resolution flow cytometry and TCID50 assay. Human peripheral blood mononuclear cells (PBMCs) were incubated with EVs from (non-)infected cells or with naked virus, after which cytokine production was assessed by RT-qPCR, LEGENDplex Multiplex assay, and flow cytometry.

Results: EVs from EMCV-infected cells, but not those from non-infected cells, stimulated the immune cells to produce the antiviral cytokines IFN type I, II, III IP-10 and inflammatory cytokines IL-6, IL-8 and TNF-a. Intriguingly, we observed that EVs from infected cells, of which a subset contained naked virus, were more potent in triggering overall IFN type I production and IP-10 production in PBMCs than a similar amount of naked virus. The virus-induced EV uptake experiments indicated that the monocytes are the most targeted immune cell type in PBMCs. The isolated monocytes are also demonstrated to be activated by EMCV-induced EVs and produce type I IFN and IP-10.

Conclusions: Although EVs can function as pro-viral factor by protecting enclosed naked virus particles from neutralizing antibodies, our data indicates that virus-induced EVs may also alarm the immune system by triggering the production of antiviral/inflammatory cytokines. These findings shed light on the diverse effects of virus-induced EVs on the antiviral immune response

Sponsor Presentations

Sponsored Presentations

Distrilab: Christina Klasen

Colocalization with F-NTA

In the last twenty years, the number of publications dealing with extracellular vesicles (EVs) has increased drastically. Since EVs play a pivotal role in many different diseases such as cancer, diabetes, cardiovascular, neurogenerative and respiratory diseases, it is not surprising that research interest in these subcellular structures is already high and continues to expand. The challenge lies in their small size of only about 100 nm. Because of this particular size, many methods are not suitable for their characterization. The Nano Particle Tracking (NTA) technique provides a method to precisely analyze the size and concentration of EVs on a single particle basis. Moreover, the ZetaView® is an NTA system that can analyze up to four fluorescent channels, enabling phenotyping and analysis of subpopulations. The new x30 generation is additionally capable of performing colocalization studies.

More information www.distrilab.nl

Sponsor Presentations

Nanofcm: Natalia Gebara

NanoFCM brings flow cytometry capabilities to the nanoscale

Conventional flow cytometers often struggle to meet the sensitivity requirements for the analysis of nanoscale particles, such as exosomes, nanomedicine, and viruses. To meet this challenge, NanoFCM has developed the NanoAnalyzer, a dedicated nano-flow cytometry platform, which offers a flexible and high-throughput solution for sub-micron analysis. By using the NanoAnalyzer, single-particle characterization can be achieved which simultaneously measures the side scatter (40 -1000nm) and fluorescent properties of particles.

The size detection of the NanoAnalyzer favourably compares to electron microscopy and covers the entire size range of EVs, offering a detailed analysis of size, concentration and biochemical properties by direct correlation of the physical and phenotypic data. It is by combination of all these properties that the NanoAnalyzer is an ideal next-generation technique/ instrument for the analysis of EVs

More information: www.nanofcm.com

Sponsor Presentations

Beckman Coulter: Lutz Ehrhardt

BEC LS Solutions for EV research - an update.

More information: www.beckmancoulter.com

Sponsor Presentations

Dispertech:

More information: www.dispertech.com

Sponsor Presentations

NanoView: Jan Brants

Purification-Free Characterization and Sizing of Exosomes & Viruses with ExoView Technology

Comprehensively characterizing your EVs can be a struggle even in a highly purified cell culture sample. Throw in complex biofluids, limited sample volume and rare EV subpopulations and that struggle can become a nightmare. Thankfully the ExoView R200 provides the solution!

We will discuss how our proprietary ExoView chips utilize the power of affinity capture to isolate EVs from starting materials ranging from cell culture media to murine cerebral spinal fluid. All without the need for prior sample purification. Once EVs are bound to the surface of the chip, the ExoView platform can size, count and phenotype vesicles on a single particle basis, in one sample run. The R200 features 4 fluorescent channels (blue, green, red and far red) allowing individual EVs to be phenotyped by up to 5 surface or cargo proteins with single molecule sensitivity.

We will cover the use of ExoView Tetraspanin kits for providing EV characterization on the basis of common exosome markers and highlight how ExoFlex chips allow full user customization to enable capture and detection of EVs via specific cell or disease markers.

More information: www.nanoviewbio.com

NanoView BIOSCIENCES

Poster Presentations

Poster presentations

NLSEV2022-P1: Bart Snieder

Understanding the role of PI4KIIIβ in bafilomycin-induced exosome secretion

Maarten P. Bebelman*1,2, Caitrin C. Crudden*1, Bart Snieder1, Katinka Langedijk1, Steven Eleonora1,Urszula Baginska1, Olaf Cotugno1, Jan Paul M. Bebelman2, Monique A.J. van Eijndhoven1, Leontien Bosch1, Martine J. Smit2, Guillaume van Niel3, Frederik J. Verweij4, D. Michiel Pegtel1

Background: Exosomes are membrane-limited extracellular vesicles (EVs) stemming from multivesicular bodies, which have been shown to play a role in several pro-tumorigenic processes. Because a large portion of the exosome biogenesis is unknown, there are no good inhibitors known that specifically target exosome secretion.
Methods: In this project we generated with CRISPR-CAS knock-in technology HEK293 cells with a nanoluciferase moiety in the endogenous CD63 gene. We consistently measured a striking 6-8 fold increase in CD63-EV release upon bafilomycin (Baf) treatment. This corresponded well with elevated MVB-PM fusion rates as measured with CD63-pHluorin suggesting that at least a proportion of the secreted EVs are from acidic internal compartments and could be considered exosomes.
Results: We next performed a small molecule drug screen and identified PI4KIIIβ as critical mediator of Baf-induced EV-release. We pharmacologically inhibited different isoforms of PI4K and PIP5K1C and have shown that PI4KIIIβ is the only PI4K isoform that is involved in the baf-induced exosome biogenesis. When we knocked-out PI4KIIIβ using CRISPR Cas9, we observed a 50% decrease in exosome secretion, validating the effects of chemical inhibition. Interestingly, when knocking-out Snap 23, we observed the same decrease as with PI4KIIIβ. However, when we inhibited both molecules simultaneously, the decrease was around 90 %, suggesting separate roles in EV biogenesis and release. Additional experiments with chemical inhibitors and siRNAs suggest a model in which PI4KIIIβ drives exosomes biogenesis that are released independently of Snap 23.
Conclusion: We suggest that PI4KIIIβ plays a part in the baf-induced exosome biogenesis pathway that acts independent of Snap 23. Further investigation into their role within the exosome biogenesis of cancer cell lines is needed to explore potential therapeutic implications of our findings.

Poster Presentations

NLSEV2022-P2 Jinzhe Ju

The role of GABARAPL1 in the biogenesis of EVs

Jinzhe Ju, Tom G. Keulers, Marc van Zandvoort, Kasper Rouschop

Background: Extracellular vesicles (EVs) are mediators of intercellular communication. Their content, including mRNA and protein, alters the phenotype of receiving cells. In the context of cancer progression, EVs contribute to processes including angiogenesis, metastasis and dampening of immune responses. Previously, we showed that the LC3/GABARAP protein family member, GABARAPL1, is required for endosomal maturation, cargo sorting and EV secretion in periods of poor oxygenation (hypoxia). In the current study, we aim to explore the mechanistic function of GABARAPL1 in EV biogenesis.
Methods: Fluorescently labelled GABARAPL1 and the ESCRT-dependent and -independent pathway proteins (VTA-1 Syn-1 TSG101) were expressed in U87 cells. Endogenous proteins were assessed by immunofluorescent labeling. The cells were exposed to normoxia and severe hypoxia (O2<0.02%), followed by conventional confocal microscopy and stimulated emission depletion (STED) confocal microscopy. Knockdown cell models were utilized to address GABARAPL1 dependent effects on EV biogenesis.
Results: Interestingly, GABARAPL1 knockdown results in changes in EV composition as illustrated by density changes and abundance of CD63 and CD81 in different sucrose fractions. No co-expression of GABARAPL1 with VTA-1 and/or TSG101 was observed. In contrast, GABARAPL1 colocalized in cells with ESCRT-independent pathway marker protein, Syntenin-1, and CD63 during normal and hypoxic conditions. Colocalization was observed in areas non-related to endoplasmic reticulum (ER), Golgi or lysosomes. In the absence of GABARAPL1, recruitment of syntenin-1 to CD63-positive structures is prevented.
Conclusion: GABARAPL1 is important for EV biogenesis and determines recruitment of syntenin-1 to CD63 positive structures.

Poster Presentations

NLSEV2022-P3: Lisa Mennens

Adipocyte-derived extracellular vesicles in obesity: the quest for a proper isolation protocol

Lisa Mennens* [1,2,3], Baharak Hosseinkhani [2], Johan Jocken [3], Kenneth Verboven [1,2]
**Presenting author; 1: REVAL - Rehabilitation Research Center, Hasselt University, Diepenbeek, Belgium; 2: BIOMED - Biomedical Research Center, Hasselt University, Diepenbeek, Belgium; 3: Department of Human Biology, NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University Medical Center, P.O. Box 616, 6200 MD Maastricht, the Netherlands.*

Background: In individuals with obesity, an excess of adipose tissue gradually leads to ectopic lipid deposition, dysfunctional interorgan crosstalk and peripheral muscle and liver insulin. Recently, adipocyte-derived extracellular vesicles (adEVs) came on the scene because of their ability to transport important signaling molecules (including protein and miRNAs). Based on a broad interest yet large heterogeneity in methodology and lack of prior optimization studies, there is a clear need for a uniform adEV-isolation protocol. Therefore, our study aims to provide a standard approach for adEV isolation in future mechanistic studies.
Methods: adEVs were isolated from differentiated human multipotent adipose-derived stem cell (hMADs) cultures and characterized following MISEV18 guidelines, including TEM, NTA and western blot. A reliability experiment was set out to check reproducibility (in terms of adEV yield) following ultracentrifugation (UC) or UC combined with size-exclusion chromatography (SEC). The effects of adipocyte differentiation and different incubation conditions on adEV yield were also determined.
Results: Using SEC after UC, isolated adEVs were nearly eliminated from samples as adEV yield was below detection limit. UC alone showed more consistent results (average yield: 2,8e8 ± 2,1e7; mean size: 246nm ± 15nm), indicating higher reproducibility. During differentiation, the highest adEV yield was observed at day 6, and decreasing towards full differentiation (day 10-14). As lipids and fatty acids further accumulate during adipocyte differentiation, these data might indicate the inefficiency of UC to isolate floating lipid loaded adEVs. Moreover, incubation of differentiated adipocytes with serum-free medium inhibits adEV release as mean adEV yields were similar to background signals, suggesting the need for appropriate culture conditions to trigger in vitro EV release.
Conclusion: Further optimization of isolation protocols is required to solve several challenges with respect to various types of adEVs.

Poster Presentations

NLSEV2022-P4: Olivier de Jong

Extracellular vesicle-mediated delivery of CRISPR/Cas9 via aptamer-based loading and inducible cargo release strategies

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.

Conclusion: 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.

Poster Presentations

NLSEV2022-P5: Carmen López-Iglesias,

How can electron microscopy contribute to the EVs characterization? The Maastricht Microscopy CORE Lab EVs workflow.

Kèvin Knoops, Hans Duimel, Willine van de Wetering, Helma Kuijpers, Chris Lewis, Carmen López-Iglesias
Microscopy CORE Lab, FHML-M4I, Maastricht University

The morphological characterization of extracellular vesicles (EVs) has been under debate continuously. The right choice of method has been subjected more to the technical possibilities regarding instrumentation and expertise rather than to the quality and information it provided. For years, EVs were analyzed by negative staining electron microscopy because of its simplicity; however, it also hampered the biological interpretation seriously and thus resulted in quite some wrong conclusions in published literature. In order to preserve the ultrastructure better, new hybrid room temperature-cryo techniques like cryo-sectioning and freeze-substitution were introduced to the field allowing further immunogold labeling. Now, in the recent past years, cryo-TEM was proposed as the best method in order to (1) avoid artifacts from the sample preparation, (2) reach high resolution and (3) localize proteins of interest in the best unperturbed state.

Here, the Maastricht Microscopy CORE Lab (MCL) presents how combinations of different conventional and cryo-methods can be applied in different workflows in order to optimize structural and morphological analysis. We compare different TEM preparation methods like negative staining, chemical fixation followed by cryo-sectioning (Tokuyasu method), high-pressure freezing followed by freeze-substitution and low temperature acrylic resins embedding, as well as cryo-TEM. We demonstrate that cryo-TEM retains a superior preservation of membranes and proteins allowing for high-resolution analysis of the lipid bilayer environment. In that regard, cryo-sectioning and freeze-fracturing are complementary methods, albeit at lower resolution. Immunolabeling is applied in order to localize antigen: if epitopes are facing the external side of the vesicles, labeling with gold-conjugated antibodies is applied in solution, however, if epitopes are facing the internal side of the EVs, labeling is best applied after (cryo-)sectioning of pelleted EVs. Furthermore, we demonstrate the large-scale scanning of TEM grids which facilitates EV detection in samples with low EV-concentrations and how (cryo-)electron tomography elucidates the 3-dimensional structure of EVs at unprecedented resolution.

Poster Presentations

NLSEV2022-P6: Sajitha Sasidharan

Impact of Normoxic and Hypoxic Conditions in EV Biophysics: An AFM-based Imaging Approach

Sajitha Sasidharan*[1], Melissa C. Piontek [1], Elizabeth Orozco Garcia [2], Martin C. Harmsen [2], Wouter H. Roos [1]

1: Moleculaire Biofysica, Zernike Instituut, Rijksuniversiteit Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands, 2: University Medical Centre Groningen, University of Groningen, Department of Pathology and Medical Biology, Hanzeplein 1, 9713 AV Groningen, The Netherlands

Background: Extracellular vesicles (EVs), i.e. membranous release from cells, are molecular signatures of the parent cells and they can reflect the cellular adaptations upon changes in the microenvironment. Also, EVs mediate short and long range communication for tumour progression, metastasis, and angiogenesis. Though a few reports link the physical properties of the EVs to metastasis, the impact of normoxic and hypoxic oxygen conditions on the tissues and their influence on the secretion and the physical properties of EVs remains elusive. As an example of physical properties, it is feasible that membrane mechanical properties affect the propensity of EVs to adhere to and enter target cells. To address the missing link, we studied the effect of the hypoxic and normoxic conditions in the production and the physical properties of the EVs secreted by HeLa and SiHa cell lines.

Methods: An AFM-based imaging and indentation approach, which is a label-free technique, was utilized to probe the distribution, morphology and biomechanical properties of the EVs secreted by cultured tumour cells. The physical properties of the surface adhered particles, such as size and deformation upon substrate binding, were evaluated for the vesicles secreted by HeLa and SiHa cells in normoxia and hypoxia conditions.

Results: SiHa cells under hypoxic conditions released more EVs than under normoxic conditions, whereas in HeLa cells a similar concentration of EVs was observed irrespective of conditions. Also, the average size of the vesicles produced under normoxic and hypoxic conditions by both cell lines was similar. However, the deformation pattern revealed that both HeLa and SiHa normoxic EVs deformed more (i.e. are softer) than their respective hypoxic EVs.

Conclusion: We have shown that how under hypoxia conditions the rigidity of EVs increases. These results are of relevance for diagnostics, as the increased rigidity of EVs under hypoxia could potentially be used as a mechanical indicator for the presence of tumours. Our approach can easily be extended with studies on EVs from other cancer cells and is expected to find wider applicability.

Poster Presentations

NLSEV2021-P7: Jari Verbunt

Bacterial Membrane Vesicles in Metabolic Health

Jari Verbunt* [1,2], Johan Jocken [2], Ellen Blaak [2], Paul Savelkoul [1,3] and Frank Stassen [1].
**Presenting author, 1: Department of Medical Microbiology, infectious diseases & Infection prevention, School of Nutrition and Translational Research in Metabolism (NUTRIM), Maastricht University Medical Center+, 6229 ER Maastricht, The Netherlands, 2: Department of Human Biology, School of Nutrition and Translational Research in Metabolism (NUTRIM), Maastricht University Medical Center+, Universiteitssingel 50, 6229 ER Maastricht, The Netherlands, 3: Department of Medical Microbiology and Infection Control, Amsterdam University Medical Centers, Amsterdam, The Netherlands*

Background: The intestinal microbiota play a pivotal role in human metabolic health and diseases. Microbiota functionalities include energy production, immune priming, and regulation of host energy metabolism. In metabolic diseases like obesity the composition and functionality of the gut microbiota is often altered, with profound implications for the human host. Importantly, gut bacteria produce bacterial membrane vesicles (bMVs) containing bacterial metabolites. Effects of bMVs on the host in the context of metabolic disease, are uncharacterized. In this work we present an initial analysis of the fecal bacterial composition (total bacteria) and bMV repertoire produced by these bacteria in lean and overweight/obese subjects.
Methods: Fecal samples (12 lean, 12 overweight/obese) were collected and stored at −80°C. DNA was obtained from feces-derived bacterial pellet and from purified bMVs. Obtaining purified bMVs was performed through consecutive (ultra)centrifugation and size exclusion chromatography steps. Column-purified DNA was subjected to 16S rRNA variable region amplification and Illumina sequencing. Sequencing data was used to compare DNA abundancies between groups.
Results: Surprisingly, in both groups marked differences were found in the Firmicutes/Bacteroidetes (FB) ratio between the bacterial and the bMV fractions (lean: 23.2 (bact) vs 0.6 (bMVs), overweight/obese: 21.1 (bact) vs 0.5 (bMV)). Furthermore, on average Actinobacteriota account for approximately 9% of ASVs (amplicon sequence variants) detected in bacterial analyses whereas in bMVs this is <0.01%. In both the lean and overweight/obese groups, there is significantly decreased ASV richness and alpha diversity at the species level for bMV DNA compared to bacterial DNA. When comparing lean versus overweight/obese subjects, no significant differences in composition, richness, diversity or bacterial abundance could be distinguished in vesicles or bacteria.
Conclusion: In both lean and overweight/obese subjects, the FB ratio in bacteria is diametrically opposed to the FB ratio in bMVs. The paramount finding is thus that the most prevalent bacteria in the intestine seem not to be the most profuse vesicle producers, indicating an additional layer of complexity in gut-host interaction warranting further investigation. When comparing lean and overweight/obese study populations, we could not identify significant differences in bacteria or bMV repertoire, possibly due to small sample sizes.

Poster Presentations

NLSEV2022-P8: Maaike Dekker

Optimization of urine processing for quantitative miRNA profiling by NGS

M.H.S. Dekker 1,2, D. Harbers1, N.J. Groenewegen3, M.A.J. van Eijndhoven2, C. Gómez-Martín2, D.M. Pegtel2, J. de Rooij1

1You2Yourself B.V; Enschede, the Netherlands, 2Department of Pathology, Exosomes Research Group, VU University Medical Center, Amsterdam, the Netherlands, 3ExBiome B.V; Amsterdam, the Netherlands

Background: To improve public health and patient wellbeing, as well as to reduce healthcare costs, improved prevention and early detection of disease is necessary. We are investigating the potential of microRNA profiles in urine for such early detection of e.g. cancer, cardiovascular and neurodegenerative diseases.
A complication in studying early disease biomarkers is the lack of sample cohorts as patients mostly present themselves at the hospital, where samples are taken for research, at a later stage of a disease when physical symptoms have become apparent. To solve that problem, we are conducting the Urimon study in which a large cohort of healthy people is periodically donating blood and urine that is processed and stored in the Stibion biobank. A percentage of these people become sick during participation and their samples then truly capture the early stages of disease.
Obtaining periodic sample series that catch the onset of disease also offers the opportunity to personalize biomarker measurements by taking personal baseline values at health into account. Our final envisioned product is a periodically performed personalized multi-disease early detection test.
Methods: Although there is already a lot of literature on microRNA analysis from urine, there is no consensus on the optimal laboratory procedures. Therefore, my first aim is to optimize urine processing and storage, microRNA isolation and library preparation, in order to achieve robust and reproducible microRNA profiling. Furthermore, we are investigating the importance of extracellular vesicles in determining urinary microRNA profiles. We use the previously established IsoSeek method from the Pegtel lab (van Eijndhoven et al., 2021) as a starting point for our research.
Results & discussion: The outline and progress of the Urimon study and results from the optimization research will be presented and discussed.

Poster Presentations

NLSEV2022-P9: Carla Jorquera-Cordero

Extracellular Vesicles from M1-Polarized Macrophages Combined with Hyaluronic Acid and a B-Blocker Potentiate Doxorubicin's Antitumor Activity by Downregulating Tumor-Associated Macrophages in Breast Cancer

Carla Jorquera-Cordero 1,2, Pablo Lara 2,3, Luis J. Cruz 3, Timo Schomann 2,3 , Anna van Hofslot 3, Thaís Gomes de Carvalho 2,4,5 , Paulo Marcos Da Matta Guedes 6 , Laura Creemers 1 , Roman I. Koning 7 , Alan B. Chan 1,2 and Raimundo Fernandes de Araujo Junior 2,3,4,5

1 Department of Orthopedics, University Medical Center Utrecht, 3584 CX Utrecht, The Netherlands; 2 Percuros B.V., 2333 CL Leiden, The Netherlands; 3 Translational Nanobiomaterials and Imaging (TNI) Group, Radiology Department, Leiden University MedicalCenter, Albinusdreef 2, 2333 ZA Leiden, The Netherlands; 4 Postgraduate Program in Health Science, Health Science Department, Federal University of Rio Grande do Norte (UFRN), Natal, RN 59078 970, Brazil 5 Cancer and Inflammation Research Laboratory, Department of Morphology, Federal University of Rio Grande do Norte, Natal, RN 59078 970, Brazil 6 Department of Microbiology and Parasitology, Federal University of Rio Grande do Norte, Natal, RN 59078 970, Brazil 7 Electron Microscopy, Cell and Chemical Biology, Leiden University Medical Center, 2300 RC Leiden, The Netherlands

Background: One of the main reasons for cancer's low clinical response to chemotherapeutics is the highly immunosuppressive tumor microenvironment (TME). Tumor-associated M2 macrophages (M2-TAMs) orchestrate the immunosuppression, which favors tumor progression. Extracellular vesicles (EVs) have shown great potential for targeted therapies as, depending on their biological origin, they can present different therapeutic properties, such as enhanced accumulation in the target tissue or modulation of the immune system.

Methods: In the current study, EVs were isolated from M1-macrophages (M1-EVs) pre-treated with hyaluronic acid (HA) and the B-blocker carvedilol (CV). The resulting modulated-M1 EVs (MM1-EVs) were further loaded with doxorubicin (MM1-DOX) to assess their effect in a mouse model of metastatic tumor growth. The cell death and cell migration profile were evaluated in vitro in 4T1 cells. The polarization of the RAW264.7 murine macrophage cell line was also analyzed to evaluate the effects on the TME. Tumors were investigated by qRT-PCR and immunohistochemistry.

Results: MM1-DOX reduced the primary tumor size and metastases. NF-KB was the major gene downregulated by MM1-DOX. Furthermore, MM1-DOX reduced the expression of M2-TAM (CD-163) in tumors, which resulted in increased apoptosis (FADD) as well as decreased expression of MMP-2 and TGF-B. These results suggest a direct effect in tumors and an upregulation in the TME immunomodulation, which corroborate with our in vitro data that showed increased apoptosis, modulation of macrophage polarization, and reduced cell migration after treatment with M1-EVs combined with HA and CV.

Conclusion: Our results indicate that the M1-EVs enhanced the antitumor effects of DOX, especially if combined with HA and CV in an animal model of metastatic cancer.

Poster Presentations

NLSEV2022-P10: Willemijn S. de Voogt

Tracking extracellular vesicles and their cargo in recipient cells

Willemijn S. de Voogt* [1], Kevin Harrijvan [1], Daniel E. Murphy [1], Pieter Vader [1,2].
**presenting author, 1: CDL Research, University Medical Center Utrecht, 2: Department of Experimental Cardiology, University Medical Center Utrecht.*

Background: Extracellular vesicles (EVs) are promising carrier systems for the delivery of therapeutic RNA by virtue of their low immunogenicity and high efficiency in cargo delivery. However, the fundamental mechanisms underlying EV-mediated functional RNA transfer remain poorly understood. RNA delivery efficiency may be dependent on EV donor and recipient cell type, the pathway of EV uptake, as well as the route of intracellular trafficking. Understanding the fate of EVs and their cargo in recipient cells is therefore crucial in order to exploit the potential of EVs as RNA delivery vehicles. Here, we demonstrate that the intracellular trafficking pathways of EVs and their cargo can be tracked using single particle tracking (SPT), colocalization analysis, and single-molecule fluorescence in situ hybridization (smFISH).
Methods: Isolated EVs were labelled with MemGlow 640 and incubated with HeLa recipient cells at 4°C. Recipient cells transiently expressed endosomal markers fused to a fluorescent protein. Next, unbound EVs were washed away and EV uptake was chased at 37°C. Specific RNAs were labelled in donor or recipient cells using smFISH. Fixed and live cell images were acquired with a Nanoimager-S (ONI). SPT analysis was performed on time-lapse videos. Fixed cell images were processed and Manders colocalization coefficients were calculated using ImageJ.
Results: Colocalization of EVs and early endosome marker Early Endosomal Antigen 1 (EEA-1) and late endosome marker Lysosomal-associated membrane protein-1 (LAMP-1) strongly increased within 40 minutes after uptake. Afterwards, colocalization with EEA1 decreased, whereas LAMP-1 colocalization reached a plateau. Colocalization of EVs and EEA1 or LAMP-1 was confirmed by two-color SPT. smFISH allowed imaging of specific mRNA sequences in EV donor cells and in recipient cells upon internalization of EVs.
Conclusion: Using colocalization analysis and SPT, we found that EVs are internalized by HeLa cells and are subsequently trafficked from early to late endosomes. Furthermore, smFISH analysis shows potential to image and track specific cargo in recipient cells. This may increase our understanding of the mechanisms underlying functional RNA transfer by EVs.

Poster Presentations

NLSEV2022-P11: Nils J. Groenewegen

EV-miRNA Biomarker discovery service: From EV isolation to data analysis of Small RNA sequencing

Nils J. Groenewegen1,2, Monique A.J van Eijndhoven2, Cristina Gómez-Martín2, Michael Hackenberg1,3, Johan de Rooij1,4, D. Michiel Pegtel1,2.

1Exbiome B.V., The Netherlands. 2Exosomes Research Group, Department of Pathology, VU University Medical Center Amsterdam, The Netherlands. 3University of Granada, Spain. 4You2Yourself BV, The Netherlands.

Exbiome offers a service for the identification and detection of biomarkers in Extracellular Vesicles (EVs) from liquid biopsies like urine, blood, and a range of tissue-derived fluids. Biomarkers from liquid biopsies can have diagnostic and/or prognostic value. They could also lead to the discovery of molecular subtypes of diseases and thereby improve treatment choices. Moreover, liquid biopsy methods are cost-effective compared to standard of care and minimally invasive.

Key to our platform is the isolation of EVs from bio-fluids by Size Exclusion Chromatography under gravity-mediated flow. This delivers pure and intact EVs that allow the discovery of disease signatures by purification and sequencing of miRNAs. Our sequencing and analysis pipeline identifies mature miRNAs as well their modified versions called isomiRs in an unbiased matter (M.A.J van Eijndhoven, et al. BioRxiv 2021). These isomiRs add additional depth of information and enhance diagnostic potential of small RNA profiles.

As a proof of principle we have used our platform to discover panels of miRNA biomarkers in plasma for 1) detection of activity of Classical Hodgkins Lymphoma (M.A.J van Eijndhoven, et al. JCI Insight 2016) 2) detection and treatment response prediction in Diffuse large B-Cell Lymphoma 3) Detection, treatment response assessment and relapse detection in Multiple Myeloma. In urine, our platform generated a prostate cancer miRNA/isomiR panel that shows superior diagnostic potential than the standard of care prostate specific antigen (PSA) (Koppers-Lalic D, et al. Oncotarget, 2016). These cases evidence the effectiveness of this platform in the discovery of small RNA biomarker panels with diagnostic potential.

Typically, our platform detects 600 to 800 unique microRNAs (not including isomiRs) in EVs isolated from 1-2 ml of plasma. Currently we are expanding the application of our methodology to tissue supernatants and peritoneal fluids.

Here, we show an overview of the EV-contained small RNA sequencing platform that we offer as a service and examples of our reporting layout

Poster Presentations

NLSEV2022-P12: Marije Kuipers

Comparative study design to investigate bone-specific targeting of cancer cell-derived extracellular vesicles

Marije Kuipers* [1], Richard Wubbolts [1], Joaquin Seras-Franzoso [2], Ibane Abasolo [2], Esther Nolte-'t Hoen [1]

*presenting author, 1: Division of Cell Biology, Metabolism & Cancer, Department of Biomolecular Health Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

2: Drug Delivery & Targeting CIBBIM-Nanomedicine, Vall d'Hebron Institute of Research Universitat Autònoma de Barcelona, Barcelona, Spain

Background: There is a strong need to identify cancer extracellular vesicle (EV) proteins that determine tissue-specific binding and uptake. Bone is a metastatic site for various cancers, including prostate cancer. Prostate cancer-derived EVs can affect osteoblasts and osteoclasts, but data on how efficient these EVs target different bone cells versus other potential target cells are limited. Comparative analysis of EV binding/uptake by different cell types with substantial difference in cell size and morphology is challenging. Working with multinuclear osteoclasts, for example, refutes strategies where equal amount of EVs are added to equal cell numbers. We reasoned that equalizing cell surface area was a better approach, and designed an experimental strategy using cell coverage area in culture systems as normalization factor for comparing EV-targeting to bone cells and monocytes.

Methods: EVs from PC3 cells cultured in EV-depleted medium were enriched using differential centrifugation, labeled with PKH26, and purified by iodixanol density gradient centrifugation. Osteoblasts (SAOS-2), osteoclasts (differentiated from THP-1 using PMA, M-CSF and RANKL), monocytes (THP-1), and prostate cancer cells (PC3) were seeded in various amounts per well. Light microscopic images of these cultures were used for calculation of cell coverage area using ImageJ. Wells with similar coverage area were used for EV incubation. PKH26 labeled EVs or dye control were incubated with the four cell types for different timepoints. Interaction of EVs with cells was measured by flow cytometry.

Results: We will present data demonstrating that THP-1 cells can be differentiated into osteoclast-like cells based on Tartrate Resistant Acid Phosphatase (TRAP) staining and visible multinuclear cells. Different numbers of the target cell types needed to be seeded to obtain equal cell coverage per well. We point out possibilities and limitations of assessing cell coverage area, and show preliminary data on differences in the efficiency with which PC3 EVs are bound/taken up by different target cell types.

Conclusion: Optimized strategies to compare EV binding/uptake to different target cell types can help to identify EV-subsets targeting specific tissues and molecules involved in this process. This may also guide the development of EV-mimics for tissue-specific drug delivery.

Poster Presentations

NLSEV2022-P13: Chao Li

TGF-β inhibits the biogenesis of small extracellular vesicles by downregulating RAB27A/B expression.

Chao Li* [1], Agustin Enciso Martinez [1], Peter Ten Dijke [1].
**presenting author, 1: Oncode Institute and Department of Cell and Chemical Biology, Leiden University Medical Center, Leiden, Netherlands.*

Background: Small extracellular vesicles (sEVs) are important messengers that carry heterogeneous cargos and play a key role in intercellular communication. Exosomes are a subtype of sEVs which are derived from endosomes. The fusion of multivesicular bodies (MVBs) with the plasma membrane and extracellular release of intraluminal vesicles (ILVs) as exosomes is the last step of exosome secretion. RAB27A and RAB27B, which are two Rab GTPases, have been proved to be key regulators of MVB docking and fusion with the plasma membrane. In this study, we aimed to explore how TGF-β affects secretion and composition of sEVs by regulating RAB27A and RAB27B.
Methods: Cells were treated with or without TGF-β. SEVs were collected from conditioned medium via different isolations methods, including ultracentrifugation (UF) and size exclusion chromatography, and their concentration was estimated using nanoparticle tracking analysis. Super-resolution fluorescence microscopy was used for comparing the cellular localization and relative amount of CD63-positive organelles. The differences in sEV size distribution and morphology between treated and non-treated samples were observed by cryogenic electron microscopy. Luciferase Reporter Assay, real-time PCR, western blot and RNA-sequencing were conducted to find out the specific mechanism of how TGF-β-regulated expression of RAB27A/B.
Results: The particle number of sEVs released by MDA-MB-231 cells and 19TT cancer-associated fibroblasts (CAFs) can be downregulated by TGF-β. TGF-β negatively regulated the transcription of RAB27A and RAB27B, and therefore downregulated the mRNA and protein level of RAB27A and RAB27B. TGF-β also had an accumulation effect on the CD63-positive organelles in HeLa cells which was caused by the downregulation of RAB27A and RAB27B, resulting in the reduction of sEVs. Because RAB27A and RAB27B are involved in the exosome secretion pathway, our results suggest that the decrease of sEVs in TGF-β-treated cells was particularly due to a decrease of exosomes.
Conclusion: TGF-β can inhibit the docking of CD63 positive MVBs to the plasma membrane by downregulating the RAB27A and RAB27B protein level, leading to a reduced number of exosomes released by cells.

Poster Presentations

NLSEV2022-P14: Celine De Schrijver

Tumor-secreted EVs in bone cancer-induced immune suppression

Celine De Schrijver1, Tonny Lagerweij1, Crescenzo Massaro1, Alberta G.A. Paul2, D. Michiel Pegtel1, Juan Garcia Vallejo3, S. Rubina Baglio1

1Department of Pathology, Cancer Center Amsterdam, Amsterdam UMC, VU University Medical Center, Amsterdam, The Netherlands; 2 Application Department, Cytek Biosciences, Inc., Fremont, California, USA

3MCBI, Cancer Center Amsterdam, Amsterdam UMC, VU University Medical Center, Amsterdam, The Netherlands.

Background: Cancer progression critically depends on the ability of tumor cells to suppress the immune response to avoid immune destruction. We previously demonstrated that bone cancer tumor-secreted extracellular vesicles (EVs) trigger an inflammatory loop in bone marrow stromal cells leading to enhanced tumor growth and metastasis formation in vivo. Here we set out to study in detail the bone marrow immune alterations induced by tumor cells and identify which of these changes are determined by cancer EVs.

Method: To evaluate how bone tumor cells alter the bone marrow immune environment in vivo, we developed a high dimensional immune profiling methodology based on spectral flow cytometry. This method enables determination of all relevant lymphoid and myeloid immune cell subsets of murine bone marrow and tumor tissue from small amount (30-50µl) of input material. To study how tumor EVs contribute to immune suppression, we generated stable bone cancer cell lines with constitutive and inducible expression of shRNAs against Syntenin, Rab11b and Rab35. The impact of silencing the selected proteins was evaluated by TRPS, Western blot and CD63-nanoluc assay.

Results: Tumor-induced alterations of the bone marrow immune environment were assessed in an orthotopic osteosarcoma immunocompetent model. We uncovered striking differences in immune composition and phenotypic traits in tumor-bearing vs control tibias with consistent enrichment of suppressive PD-L1+ macrophages, PD-L1+ monocytes, N2 neutrophils, PD-1+ CD8 T cells and Foxp3+ regulatory T cells. Notably, the immune profile of the tumor-bearing bone marrow reflected that of the tumor infiltrate, indicative of a strong local effect. We successfully generated stable human and murine cell lines with constitutive and inducible knockdowns for Rab11b, Rab35 and Syntenin. All knockdowns led to a decrease in EV release by TRPS and CD63 nanoluc assay.

Conclusion: Bone tumor cells orthotopically inoculated in fully immunocompetent mice dramatically alter composition and phenotype of bone marrow immune cells to generate an immunosuppressive environment. Future in vivo studies with cancer cells impaired in EV release will shed light on the tumor EV-mediated alterations of the bone marrow immune environment."

Poster Presentations

NLSEV2022-P15: Anna E. George

ER membrane contact sites support endosomal small GTPase conversion for exosome secretion

Frederik J. Verweij[1,2,3], Maarten P. Bebelman[1,5,6], Anna E. George[2,3]*, Mickael Couty[1], Anaïs Bécot[1], Roberta Palmulli[1], Xavier Heiligenstein[4], Julia Sirés-Campos[4], Graça Raposo[4], Dirk Michiel Pegtel[5], Guillaume van Niel[1,7]

**Presenting Author 1 : Institute for Psychiatry and Neurosciences of Paris, Hopital Saint-Anne, Université de Paris, Institut national de la santé et de la recherche médicale, U1266, Paris, France 2: Department of Cell Biology, Neurobiology and Biophysics, Utrecht University, Utrecht 3: Centre for Living Technologies, Alliance Eindhoven University of Technology, Wageningen University & Research, Utrecht University, University Medical Center Utrecht 4 : Institut Curie, Paris Sciences & Lettres Research University, CNRS, UMR144, Paris, France 5 : Department of Pathology, Cancer Center Amsterdam, Amsterdam University Medical Center, Amsterdam 6: Division of Medicinal Chemistry, Amsterdam Institute for Molecules Medicines and Systems, VU University, Amsterdam 7: Groupe Hospitalier Universitaire Paris Psychiatrie et Neurosciences, Hôpital Sainte Anne, Paris*

Background: Exosomes are lipid bound extra-cellular vesicles that are formed within so called Multi Vesicular Bodies (MVBs) by inward budding of the limiting membrane. These MVBs undergo endosomal maturation and release exosomes when these MVBs fuse with the Plasma Membrane (PM) instead of being targeted for lysosomal degradation. The exact mechanism by which these endosomes are generated and rendered competent to fuse with the PM to release their exosomal cargo is still unclear. Besides the inter-endosomal hetero and homotypic interactions, interplay with the other organelles is also known to be involved in endosomal maturation. In this study, we gain insight into the molecular identity of the PM fusing CD63-positive MVBs and the role dynamic membrane contact sites (MCS) with the Endoplasmic Reticulum (ER) play in this process.

Methods: To visualize and characterize MVBs fusing with the PM, we used a previously developed dual-color TIRF-microscopy approach, exploiting a CD63-based pH-sensitive optical EV reporter. These live-imaging studies were complemented with fluorescent imaging of fixed cells and biochemical analysis.

Results: Here, we reveal that CD63 positive MVBs rendered competent for PM fusion are derived from a subclass of late endosomes (LE)/MVBs at pre-endolysosomal stage that are catalytically non-active. These MVBs undergo a Rab7a/Arl8b/Rab27 small-GTPAse switching cascade, rendering them competent for PM fusion. Inter organellar interaction with the ER through dynamic ER-LE MCS has a stimulatory effect on exosome secretion. We found that Rab7-effector ORP1L, a previously identified ER-LE MCS mediator, facilitates the dynein mediated centripetal movement of MVBs and small GTPase-switching from Rab7a to Arl8b. The switch to Arl8b allows kinesin mediated movement towards the cell periphery, followed by a switch to Rab27a and subsequent targeting, docking and fusion with the PM.

Conclusion: We identify exosome secretion as a multi-step process, where non-catalytic pre-endolysosomal MVBs are rendered fusion competent with the PM. This process is tightly regulated by dynamic ER-LE MCS through ORP1L, impacting endosomal maturation and GTPase switching. Altogether, this study highlights a novel role for the ER in exosome secretion.

Poster Presentations

NLSEV2021-P16: Elham Zonoobi

Characterization of tumour markers on colorectal carcinoma extracellular vesicles

Elham Zonoobi* [1,2], Ana Matoshi [2,3], Stephan Scheffer [2], Alexander L. Vahrmeijer [2], Peter J.K. Kuppen [2], Luuk J.A.C. Hawinkels [4], Cornelis F.M. Sier [2,3]

**Presenting author, 1: Edinburgh Molecular Imaging Ltd., E, UK, 2: Department of Surgery, Leiden University Medical Centre, Netherlands, 3: Percuros B.V., Leiden, Netherlands, 4: Department of Gastroenterology, Leiden University Medical Centre, Netherlands*

Background: Colorectal carcinoma (CRC) is globally the third most prevalent cancer, with the second highest mortality rate, especially due to liver metastases. Curative treatment consists of resection of the malignant tissue in combination with (neo)adjuvant chemo/radiation, depending on the stage of the disease. Resection by endoscopy or surgery and follow-up of the patients are sometimes challenging, because discrimination of malignant tissue from normal tissue is not always feasible by the naked eye and palpation. Image-guided endoscopy or surgery are relatively novel tools facilitating the detection of malignant tissue in real time, based on near infrared fluorescent (NIRF) tracers in combination with a dedicated camera system. The tracers consist of a fluorescent dye which is conjugated to a detecting determinant like peptides, aptamers, or monoclonal antibodies, with affinity for proteins overexpressed on the membrane of the tumour cells. If the membranes of extracellular vesicles (EV), which are shed from tumour cells, represent a similar protein repertoire as the original cells, there might be several applications, like EV-based personalized selection of tracers, or monitoring of disease after therapy. This study aims to isolate EVs from various colorectal cancer cell lines and compare the protein content of EVs and cells for membranous tumour markers c-MET, CEA, EpCAM, and IGF1R.

Methods: EV isolation by differential ultracentrifugation is performed on culture media of a panel of colon carcinoma cell lines: Caco2, Colo320, HCT116, HT29, RKO, and SW480. Transmission electron microscopy and immunofluorescence microscopy are used to verify and characterize the EVs. The presence of the tumour markers on the membranes of the parental tumour cells and their respected tumour-derived extracellular vesicles are compared with flow cytometry, cytospot/cytospin, western blotting, and confocal immuno-fluorescence microscopy.

Results: All 6 CRC cell lines produce EVs and all tumour markers showed a high correlation between parental tumour cell lines and their respective EVs.

Conclusion: The similarities of membrane-associated protein distribution between EVs and their parent cells suggest a possible clinical use for EVs. Non-invasive analysis of EVs from blood of cancer patients might be applicable for personalized selection of NIRF-tracers for image-guided resection of cancer and for monitoring of disease recurrence afterwards.

Poster Presentations

NLSEV2022-P18: Cristina Gómez-Martín

Accurate isomiR composition reassessment by small RNA sequencing and its application in plasma EV samples

Cristina Gómez-Martín*[1,2], Ernesto Aparicio-Puerta[3], Monique van Eijndhoven[1,2], José M. Medina[4,5], Michael Hackenberg[4,5,6,7], Michiel Pegtel[1,2]

**presenting author; [1] Amsterdam UMC Location Vrije Universiteit Amsterdam, Pathology, De Boelelaan 1117, Amsterdam, The Netherlands [2] Cancer Center Amsterdam, Imaging and Biomarkers, Amsterdam, The Netherlands [3] Chair for Clinical Bioinformatics, Saarland University, 66123 Saarbrücken, Germany [4] Department of Genetics, Faculty of Science, University of Granada, 18071 Granada, Spain [5] Bioinformatics Laboratory, Biotechnology Institute, Centro de Investigación Biomédica, PTS, Avda. del Conocimiento s/n, 18100-Granada. Spain [6] Instituto de Investigación Biosanitaria ibs.GRANADA, University of Granada, 18071 Granada, Spain [7] Excellence Research Unit ""Modelling Nature"" (MNat), University of Granada, 18071 Granada, Spain*

Background: MiRNAs are small non-coding RNA transcripts that control translational repression. Next-generation sequencing revealed minor sequence variants (isomiRs) generated by post-transcriptional modification thought to dramatically change their pre-processing, stability and targetome[1]. Deconvolution of these 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[2]. Because library preparation procedures introduce artifacts and bias, current protocols employ randomized sequence adapters. How these compare to classic protocols in terms of isomiR calling remained unclear.

Methods: We performed a comparative analysis of 8 fixed and randomized (2-5N) adapter small-RNAseq protocols, analysing samples coming from a, theoretically, isomiR-free pool of synthetic miRNAs, HEK293T cell lines and pEV samples. sRNAbench[3] pipeline was used.

Results: Data from the isomiR-free pool revealed that that less than 5% of miRNA reads can be attributed to library-preparation artifacts except for two protocols. On average non-templated additions (NTAs) that shouldn't be present in the pool amass to 5% of the miRNA reads for each class. However, in HEK293T cells 4 and 5N bias-reducing protocols detect far fewer (<1%) NTA-C and NTA-G compared to NTA-A and -U (10%), showing their accuracy in the detection of this highly biologically relevant isomiRs. We also show considerable accuracy and concordance between protocols in detecting NTA-U isomiRs using terminal RNA uridyl transferases TUT4/TUT7 knock-out HEK293T cells. This was confirmed also using pEV samples.

Conclusion: Our data reveals that 6 protocols generally accurately detect true isomiRs while 2 suffer from false isomiR generation. This analysis is of high relevance as an accurate isomiR quantification is essential for understanding their physiological role and for their potential role as biomarkers.

[1] Gebert, L. F. R. et al. Regulation of microRNA function in animals. Nat Rev Mol Cell Biol 20, 21–37 (2019).

[2] Drees, E. E. E. et al. Extracellular vesicle miRNA predict FDG-PET status in patients with classical Hodgkin Lymphoma. Journal of Extracellular Vesicles 10, e12121 (2021).

[3] Aparicio-Puerta, E. et al. sRNAbench and sRNAtoolbox 2022 update: accurate miRNA and sncRNA profiling for model and non-model organisms. Nucleic Acids Research 50, W710–W717 (2022)."

Poster Presentations

NLSEV2022-P19: Jillian W.P. Bracht

Platelet removal from human blood plasma improves detection of extracellular vesicle-associated miRNA

Jillian W.P. Bracht* [1], Mandy Los [1], Monique A.J. van Eijndhoven [2], Britta Bettin [1,3], Edwin van der Pol [1,3], D. Michiel Pegtel [2], Rienk Nieuwland [1,3].

1: Amsterdam UMC location University of Amsterdam, Vesicle Observation Centre, Laboratory of Experimental Clinical Chemistry, Department of Clinical Chemistry, Amsterdam, The Netherlands; 2: Amsterdam UMC location Vrije Universiteit Amsterdam, Department of Pathology, Amsterdam, The Netherlands; 3: Amsterdam UMC location University of Amsterdam, Department of Biomedical Engineering and Physics, Amsterdam, The Netherlands.

Background: Human blood plasma prepared by centrifugation contains not only extracellular vesicles (EVs) but also platelets and erythrocyte ghosts (ery-ghosts). Because platelets and ery-ghosts co-migrate with EVs during isolation, their presence can interfere with downstream analysis of EVs. Here we studied whether analysis of miRNA associated with plasma EVs (EV-miRNA) is affected by the presence of platelets and ery-ghosts.
Methods: EDTA blood was collected from healthy donors (n=3), and plasma was prepared by the centrifugation protocol recommended by the International Society on Thrombosis and Haemostasis (ISTH), and by a centrifugation protocol from an EV-miRNA expert lab (non-ISTH protocol). EVs were isolated from plasma by size-exclusion chromatography CL-2B (SEC2B), and concentrations of platelets, ery-ghosts, and EVs (150-1,000 nm) were measured by calibrated flow cytometry (Apogee A60-Micro and BD FACSCanto). Three miRNAs, let7a-5p, miR-21-5p, and miR-223-3p, were measured by Taqman qRT-PCR. Measurements were performed with and without filtration using 0.8 µm track-etched filters to remove platelets and ery-ghosts from plasma and EV-enriched SEC fractions.
Results: Plasma prepared by both centrifugation protocols contained platelets and ery-ghosts (~106-107 mL-1), of which a proportion (105 mL-1) co-migrated with EVs into the EV-enriched SEC2B fractions. Filtration removed platelets and ery-ghosts (>97%; $p<0.05$) and did not affect the EV concentrations ($p>0.70$). The miRNA concentrations were 2-4-fold overestimated due to the presence of platelets but not ery-ghosts.
Conclusion: In the present study we demonstrate that human plasma prepared by different centrifugation protocols contains platelets and ery-ghosts, which co-migrate with plasma EVs during isolation by SEC2B. The presence of platelets leads to overestimation of the EV-miRNA concentration, but platelets can be efficiently removed by filtration. Thus, filtration of human plasma is expected to improve comparability and reproducibility of quantitative EV-miRNA studies. Therefore, we recommend to measure and report the plasma concentration of platelets for EV-miRNA studies, and to filter plasma before downstream analyses or storage in biobanks.

Funded by Health Holland (AQrate project).

Poster Presentations

NLSEV2022-P20: Wouter W. Woud

Size and Fluorescence Calibrated Imaging Flow Cytometry: from Arbitrary to Standard Units

Wouter W. Woud* [1], Haley R. Pugsley [2], Britta Bettin [3,4], Zoltan Varga [5], Edwin van der Pol [3,4].
** Presenting Author, 1: Erasmus MC Transplant Institute, Department of Internal Medicine, University Medical Center Rotterdam, Rotterdam, The Netherlands; 2: Luminex Corporation A DiaSorin Company, USA*
3: Department of Biomedical Engineering and Physics, Amsterdam UMC location University of Amsterdam, Amsterdam, The Netherlands; 4: Department of Clinical Chemistry, Amsterdam UMC location University of Amsterdam, Amsterdam, The Netherlands; 5: Biological Nanochemistry Research Group, Institute of Materials and Environmental Chemistry, Research Centre for Natural Sciences, Budapest, Hungary

Background: Imaging flow cytometry (IFCM) is a technique that can detect, size, and phenotype Extracellular Vesicles (EVs) at high throughput (thousands/minute) in complex biofluids without prior EV isolation. However, the generated signals are expressed in arbitrary units, which hinders data interpretation and comparison of measurement results between instruments and labs. While fluorescence calibration can be readily achieved, calibration of side scatter (SSC) signals presents an ungoing challenge for IFCM. Here, we present an approach to relate the SSC signals to particle size for IFCM, and perform a comparability study between three different IFCMs using plasma EV test samples (PEVTES).
Method: SSC signals for different sizes of polystyrene (PS) and hollow organosilica beads (HOBs) were acquired with a 405-nm 120-mW laser and without a notch filter before detection. Mie theory was applied to relate scatter intensities to particle size. Fluorescent calibration was accomplished with 2-µm APC, FITC, and PE MESF beads (custom-order, BD). Size and fluorescence calibration was performed for three IFCMs in two labs. GFP-labelled exosomes (SAE0193, SigmaAldrich) and APC and PE labelled plasma EV test samples (PEVTES, METVESII project) were used as EV samples. EV concentrations were compared between instruments within an EV size range of 180-800 nm and a fluorescence intensity range of x-y MESF.
Results: 82-nm PS beads could be readily discerned from background signals based on their SSC intensity. Fitting of the obtained PS bead SSC intensities with Mie theory resulted in a coefficient of determination of 0.993 between theory and data. The measurements of HOBs, fluorescence calibration beads, and test samples, as well as the interlaboratory comparison analysis are scheduled for early October.
Conclusion: Here we demonstrate - for the first time – scatter calibration of an IFCM. The quality of the scatter to diameter relation and scatter sensitivity of the IFCMs are similar to state-of-the-art flow cytometers. This development will support the reliability of EV research with IFCM by providing robust standardization and reproducibility, which are pre-requisites for understanding the biological significance of EVs.

Poster Presentations

NLSEV2022-P21: Liang Wu

Undetectable donor-HLA-A2 specific urinary extracellular vesicles reflect the absence of HLA-class I positive vesicles in urine

Liang Wu *[1], Karin Boer[1], Martijn van Heugten[1], Dennis A. Hesselink[1], Carla C. Baan[1]
**Presenting author, 1:Department of Internal Medicine, Division of Nephrology and Transplantation, Erasmus MC Transplantant Institute, University Medical Center Rotterdam, Rotterdam, The Netherlands.*

Background: Kidney transplantation is the treatment of choice for patients with end-stage kidney disease. After transplantation, urinary extracellular vesicles might reflect the kidney allograft status. Here, we aim to identify donor uEV based on human leucocyte antigen (HLA) mismatching with the recipient to identify transplant-derived uEV and selectively detect changes in the kidney graft.
Methods & Results: Urine samples were collected from 12 pairs of HLA-class I A2+ healthy donors and HLA-A2- kidney transplant recipients (KTR) before transplantation. Using an isolation-free imaging flow cytometry (IFCM) protocol, CD9 and HLA-A2 double-positive (CD9+/HLA-A2+) uEVs were quantified. IFCM readouts were corrected for isotype control staining and detergent lysis to ensure reliable EV detection. In kidney donor and recipient urine, concentrations of CD9+/HLA-A2+ uEVs were comparable, 0.6. ± 1.8 × 107/ml and 1.0 ± 0.3 × 107/ml respectively. This false positive detection of HLA-A2+ uEVs in HLA-A2- recipient urine was not redressed by substituting an alternative HLA-A2 antibody or protocol optimizations such as urine or antibody dilution and using a high gain model of IFCM to improve the detection sensitivity. In order to further explore the physiology of HLA-class-I in uEV, 10 unprocessed urine and plasma samples from healthy volunteers were analyzed using an anti-HLA-class-I (heavy chain) antibody not specific to only HLA-A2. To exclude a possible matrix-effect from urine, uEVs were purified from these samples using ultracentrifugation (UC), and a spike-in of B cell-derived EVs into urine was performed. CD9+/HLA-class-I+ uEV could not be identified in whole urine or UC isolates. However, 8 hours after spike-in at 37°C revealed detectable CD9+/HLA-class-I+ uEVs (3.5 × 106/ml before, 3.1 × 106/ml after spike-in, dilution adjusted).
Conclusion: Donor-specific HLA class I cannot be used to identify donor kidney-derived EV in urine. Contrary to plasma, no HLA class I+ EV could be recognized in urine, while spike-in of HLA class I+ EV showed no influence of urine on EV quantification and abundance. This suggests that previous proteomics results identifying HLA- class I in uEV studies may not reflect membrane-associated HLA-class-I. Identifying donor-specific uEVs requires further advances to recognize transplant-specific uEV and associated markers in urine.

Poster Presentations

NLSEV2022-P22: Naomi C. Buntsma

Impaired release of platelet-derived EVs in patients with low platelet concentrations contributes to bleeding tendency

Naomi C. Buntsma* [1,2,3], Aleksandra Gasecka [2,4], Sylwester Rogula [4], Sytske Talsma [2], Jagoda Rytel [5], Krzysztof Mądry [5], Grzegorz Basak [5], Edwin van der Pol [2,3], Rienk Nieuwland [2]

1: Department of Neurology, Amsterdam UMC, the Netherlands; 2: Laboratory of Experimental Clinical Chemistry, Amsterdam UMC, the Netherlands; 3: Biomedical Engineering, Amsterdam UMC, the Netherlands; 4: 1st Chair and Department of Cardiology, Medical University of Warsaw, Poland; 5: Department of Hematology, Oncology and Internal Medicine, Medical University of Warsaw, Poland

Background: Platelet aggregation and the release of procoagulant platelet-derived extracellular vesicles (PEVs) are essential in hemostasis. It is yet unknown why patients have a bleeding tendency below a critical platelet concentration of 5·10ˆ7 mL-1. We hypothesize that below this critical platelet concentration not only platelet aggregation is inhibited, but also the release of PEVs due to reduced interaction between platelets.
Methods: Citrate-anticoagulated blood was collected from healthy individuals (n=3) and platelets were activated by different concentrations of thrombin-receptor activating peptide (TRAP) in undiluted or 10-fold diluted whole blood samples. PEV concentrations and single platelet activation were determined using calibrated flow cytometry (Apogee A60-Micro, EV diameter ≥160 nm). Blood was also collected from idiopathic thrombocytopenia purpura (ITP) patients (n=3) and healthy controls (n=3). Platelet aggregation was stimulated by addition of TRAP (30 µM), using saline as control. After aggregation was completed, the release of PEVs was measured with flow cytometry after platelet removal.
Results: TRAP activated up to 84% of platelets in both undiluted and 10-fold diluted blood. The PEV concentration increased 12-fold in undiluted blood and 3-fold in diluted blood. TRAP-induced platelet aggregation was 4-fold increased in healthy controls compared to ITP patients. The PEV concentration increased 1.6-fold in healthy volunteers (to 3.1·10ˆ8 mL-1) upon stimulation with TRAP but was unaffected in ITP patients (mean concentration 3.0·10ˆ7 mL-1 in both saline and TRAP-treated samples).
Conclusion: Platelet activation below a critical platelet concentration, either in diluted blood from healthy controls or in blood from ITP patients, inhibits both platelet aggregation and the release of PEVs. Thus, both platelet activation and platelet-platelet interaction are prerequisites to PEV release. PEVs offer an important and essential surface for coagulation and hence our findings support the hypothesis that the bleeding risk of patients with low platelet concentration is the combined effect of impaired platelet aggregation and impaired release of PEVs.

Poster Presentations

NLSEV2022-P23: Britta Bettin

Off-the-shelf, stable biological test samples to validate calibration procedures for extracellular vesicle measurements

Britta Bettin [1,2,3], Chi. Hau [1,3], Edwin van der Pol [1,2,3], Rienk Nieuwland [1,2]
1 Laboratory Experimental Clinical Chemistry; 2 Biomedical Engineering & Physics; 3 Vesicle Observation Center; Amsterdam UMC, Location University of Amsterdam, Amsterdam, the Netherlands

Background: Concentrations of extracellular vesicles (EVs) in body fluids are being explored as disease biomarkers. To measure EV concentrations, most laboratories use flow cytometers (FCMs), but concentrations are incomparable between FCMs. To improve comparability, the METVES II consortium develops reference materials and methods to calibrate FCMs, which require validation by test samples containing EVs. To minimize variation introduced by the test samples itself, we developed off-the-shelf, stable and flow cytometry compatible plasma-derived EV test samples (PEVTES).
Methods: Plasma was collected and prepared from healthy donors and pooled. EVs were double-labeled with CD61-APC and CD235a-PE or lactadherin-FITC, isolated by size-exclusion chromatography to reduce swarm detection, diluted in cryopreservation agent (trehalose), frozen in liquid nitrogen, and stored at -80°C. After thawing the EV concentrations were measured directly, and after 1, 3, 6, and 12 months, on a calibrated FCM (Apogee A60-Micro).
Results: Compared to the fresh starting material, the concentration of CD61+ (platelet) and CD235a+ (erythrocyte) EVs decreased 10% (p=0.74, p=0.34), and 30% for lactadherin+ EVs (p=0.01) after 1 month. After 12 months of storage, the concentration of CD61+ EVs decreased 36% (p=0.07) compared to 1 month of storage, but the concentration of CD235a+ and lactadherin+ EVs were stable (p=0.76, p=0.95).
Conclusions: PEVTES can be stably stored in trehalose for up to 12 months at -80°C. The PEVTES will be key to validate newly developed reference materials and methods in a global comparison study involving 25 laboratories. Furthermore, the PEVTES can be used as a quality control sample for EV flow cytometry measurements.

Poster Presentations

NLSEV2022-P24: Jordy M.M. Kocken

Living Myocardial Slices as a method for Right Ventricle Specific Extracellular Vesicle Research

Jordy M.M. Kocken*[1,2], S. Marisa Oliveira [3], I. Falcão Pires[3], P. A. da Costa Martins[1,2,3]
** presenting author; 1: Department of Molecular Genetics, Faculty of Sciences and Engineering, Maastricht University, 6229 ER Maastricht, The Netherlands. 2: CARIM School for Cardiovascular Diseases, Faculty of Health, Medicine and Life Sciences, Maastricht University, 6229 ER Maastricht, The Netherlands. 3: UnIC@RISE, Department of Surgery and Physiology, Faculty of Medicine, Cardiovascular Research and Development Centre, University of Porto, Al. Prof. Hernâni Monteiro, 4200-319 Porto, Portugal*

Background: Right ventricle failure (RVF) is the leading cause of death in people with pulmonary hypertension (PH). Current treatment therapies target endothelial dysfunction to lower pulmonary artery (PA) pressures and maintain cardiac output. Strategies to study molecular pathways or therapies for RVF are either in vitro models that are not RV specific, and usually limited to one cell type, or animal models that do not translate well to the human situation.
Methods: Living myocardial slices (LMS) represents a method with the advantage of combining the in vitro and in vivo models. This method cuts a specific myocardial part derived from an animal model or human donor into slices used for functional characterization ex vivo. To improve our understanding of the RV remodeling, we used the RV free wall from the monocrotaline (MCT) rat model to assess the possibility of using LMS to provide more in-depth data regarding the remodeling of the RV. Seven weeks old Wistar rats were injected with monocrotaline (MCT, n=18) or vehicle (Control, n=12). Twenty-four days later, as soon as the animals started to show signs of weight loss, we assessed hemodynamic in vivo RV function (PV-loops) and harvested the RV. The RV was then sliced into 300 □m thick slices, placed in a culture chamber and electrically stimulated (1Hz, 3mA, 5 ms) for 48 h. After this, the slices were measured, weighed and snap frozen for molecular biology analysis The contractions of the slices were recorded and later analyzed.
Results: Analysis of the contraction data showed that RV slices of MCT-rats presented increased peak force developed during contraction (1100 vs 255 □N/mm2), and a lower time to peak (50 vs 100 ms), resembling in vivo RV hypertrophic remodeling during adaption towards increasing PA pressures. Culture media was collected at 24 and 48 h and extracellular vesicles were isolated using ExoQuick and quantified using nano tracking analysis.
Conclusions: While deeper characterization of the EVs and RV tissue is ongoing, RNA was extracted and send for miRNA sequencing, since miRNAs are highly conserved, to find new pathways involved in RV remodeling and communication.

Poster Presentations

NLSEV2022-P25: Simon van de Wakker

Size matters: Functional differences of small extracellular vesicle subpopulations

van de Wakker SI* [1], Bauzá-Martinez J [2], Rios Arceo C [1], Manjikian H [1], Willms EF [3], de Jong OG [2], Wu W [2], Sluijter JPG [1], Vader P [1].
* Presenting author; 1: UMC Utrecht, The Netherlands; 2: Utrecht University, The Netherlands; 3: La Trobe University, Melbourne, Australia

Background: Increasing evidence indicates that small extracellular vesicles (sEVs) are present as heterogeneous populations. sEV heterogeneity represents a major challenge in the field, in particular related to the understanding of differences in function. In this project, functional aspects of cardiac progenitor cell (CPC)-derived EV subpopulations are studied. CPC-derived sEVs have shown great potential to stimulate cardiac repair. Studying sEV heterogeneity could provide new insights into contributing therapeutic mechanisms underlying sEV-mediated cardiac repair.
Methods: CPC- and mesenchymal stromal cell (MSC)-derived sEVs were purified by binding chromatography followed by separation using size-exclusion chromatography (SEC) or asymmetric flow field flow fractionation (A4F) for fractionation of different sEV-subfractions. sEVs were characterized using western blot, nanoparticle tracking analysis, bicinchoninic acid protein assay, transmission electron microscopy and mass spectrometry. Functional differences were studied using different cellular assays to determine AKT phosphorylation, wound healing, angiogenesis and cardiomyocyte survival.
Results: SEC or A4F were used to separate three distinct subpopulations of CPC and MSC-derived sEVs which were identified based on differential expression of sEV marker proteins. These sEV subpopulations differed in size, appearance, proteomic composition and function. Mass spectrometry analysis confirmed the differences in expression levels of sEV marker proteins, as well as annexins, rab proteins, integrins, histones and proteasomal proteins. Furthermore, gene ontology cellular component analysis revealed differences in cellular origin. Larger sEVs were more enriched for proteins derived from the plasma membrane, medium sized sEVs were enriched for markers involved in endosomal trafficking and smaller sEVs were enriched for proteins derived from the endoplasmic reticulum and the proteasome complex. sEV subpopulations exerted clear functional differences upon exposure to recipient endothelial cells and cardiomyocytes.
Conclusions: SEC and AF4 allow for isolation and in-depth study of the functional heterogeneity of sEVs. In our study, we observed the existence of different subpopulations based on size, differential composition and biological function. Increasing knowledge of sEV heterogeneity will contribute to a better understanding of the mechanisms of action of sEVs and will improve translation to the clinic and potentially an off-the-shelf approach to stimulate cardiac repair."

Poster Presentations

NLSEV2022-P26: Jillian W.P. Bracht

Pore size, degree of cross-linking, column length, resin combination and re-using size-exclusion chromatography columns affect plasma EV yield and purity

Jillian W.P. Bracht* [1], Mandy Los [1], Edwin van der Pol [1,2], Rienk Nieuwland [1,2].
1: Amsterdam UMC location University of Amsterdam, Vesicle Observation Centre, Laboratory of Experimental Clinical Chemistry, Department of Clinical Chemistry, Amsterdam, The Netherlands; 2: Amsterdam UMC location University of Amsterdam, Department of Biomedical Engineering and Physics, Amsterdam, The Netherlands.

Background: Size-exclusion chromatography (SEC) is a size-based separation method. When blood plasma is used as a starting material, (lipo)proteins co-migrate with EVs and thus may affect downstream analyses. This study investigates the yield and purity of EVs isolated with SEC columns that vary in pore size, agarose cross-linking degree, column length and resin combination. In addition, we studied the effect of re-using SEC columns on EV yield and purity.
Methods: EVs were isolated from platelet-free human pooled plasma using SEC columns with differences in pore size (35 nm/SEC4B, 70 nm/SEC2B), degree of agarose cross-linking (Gen1, Gen2), column length (10, 14 mL) and resin combination (agarose, agarose+fractogel). For each column, three EV-enriched fractions (each 0.5 mL) were identified by flow cytometry (Apogee A60; size detection range >150 nm), pooled, and used to measure EV yield (flow cytometry) and purity (Bradford protein assay, ApoB and ApoA ELISAs). The effect of re-using SEC2B Gen2 columns on EV yield and purity was also tested.
Results: EV yield increased when using: i) smaller pore sizes (1.5-fold higher for SEC4B versus SEC2B), ii) agarose with lower cross-linking degrees (1.4-fold higher for Gen1 versus Gen2), and iii) a single resin type (3.6-fold higher for agarose versus agarose+fractogel). Column length or re-use did not affect EV yield. However, increased EV yields also led to increased (lipo)proteins, and thus low EV purity. The concentration of (lipo)proteins reduced when using i) columns with larger pore sizes (37-fold lower for SEC2B versus SEC4B), ii) agarose with higher cross-linking degrees (36-fold lower for Gen2 versus Gen1), iii) longer columns (2-fold lower for 14 mL versus 10 mL), and iv) columns with combined resin types (38-fold lower for agarose+fractogel versus agarose). Moreover, re-using columns increased the concentration of (lipo)proteins up to 5-fold.
Conclusions: The most appropriate EV isolation column depends on the effect of contaminants on downstream analyses. High EV yield is related to low EV purity and vice versa. For high EV yields, SEC4B Gen1 columns are recommended. For high EV purity, SEC2B Gen2 14 mL columns should be used. We do not recommend to re-use columns.

Funded by Health Holland (AQrate project).

Poster Presentations

NLSEV2022-P27: Onno J. Arntz

Protein Profiling of Plasma-derived Extracellular Vesicles from Systemic Sclerosis Patients to Find Potential Biomarkers of this Disease.

Onno J. Arntz, Sander I. van Leuven, Peter m. van der Kraan, Madelon C. Vonk, Fons A.J. van de Loo.
Department of Rheumatology, Radboud University Medical Center, NIJMEGEN, NETHERLANDS

Systemic sclerosis (SSc) is an autoimmune disease characterized by vasculopathy and tissue fibrosis of the skin and internal organs. Interstitial lung disease (ILD) is a frequent complication and is the leading cause of death in SSc patients. The pathophysiology is complex and not completely understood, but it is speculated that extracellular vesicles (EVs) play a role[1]. EVs are cell-derived membrane vesicles and part of intercellular communication. The EVs content reflects the cells from which they were released, and the proteomic analysis of EVs could yield diagnostic biomarkers but also serve as indicators of disease improvement or progression.

In this study we analyzed the proteins of plasma EVs (pEVs) of 20 SSc patients (10 with and 10 without interstitial lung disease (ILD)), and 10 age and sex matched healthy donors (HC), by mass spectrophotometry. Observed protein levels were correlated to laboratory and clinical parameters of their disease to discover potential biomarkers.

Concentration, size, and protein content of pEVs from SSc patients (1.62*1010 particles/ml, 109nm, 3.20fg protein/particle) and HC (2.61*1010 particles/ml, 118nm, 2.36fg protein/particle) were comparable. Proteomic analysis revealed 605 (>99%) overlapping proteins in SSc-pEVs, compared to HC-pEVs. Levels of nine proteins were significantly enriched while 15 proteins were significantly diminished in SSc-pEVs, compared to HC-pEVs. After protein-protein interaction analysis, the blood clotting factors vWF and F8 were observed as central proteins of the significantly enriched proteins and heat shock protein HSP90AA1 was central for the diminished proteins. Interestingly, pEVs from SSc patients with ILD showed significantly lower levels of 11 proteins with also HSP90AA1 as central protein. Epithelium factors MUC5B and SEPP1 were both significantly diminished in SSc+ILD donors, and correlated with lung capacity parameters suggesting that these pEV markers have the potential to detect lung pathology in SSc patients.

In summary, this study found protein markers on SSc-pEVs that could serve as potential diagnostic biomarkers to identify different phenotypes of this disease.

1. Role of extracellular vesicles in autoimmune pathogenesis. Wu et al. Front. Immunol. 2020

Poster Presentations

NLSEV2022-P28: Mona Shahsavari

Protocol to size extracellular vesicles with microfluidic resistive pulse sensing

Mona Shahsavari* [1,2], Rienk Nieuwland [1], Ton van Leeuwen [2,3,4], Edwin van der Pol [1,2].
** presenting author, 1: Amsterdam UMC location University of Amsterdam, Laboratory of Experimental Clinical Chemistry, Department of Clinical Chemistry, Vesicle Observation Centre, Amsterdam, The Netherlands, 2: Amsterdam UMC location University of Amsterdam, Department of Biomedical Engineering and Physics, Amsterdam, The Netherlands, 3: Amsterdam Cardiovascular Sciences, Atherosclerosis and Ischemic Syndromes, Amsterdam, the Netherlands, 4: Cancer Center Amsterdam, Imaging and Biomarkers, Amsterdam, the Netherlands"*

Background: Microfluidic resistive pulse sensing (MRPS; nCS1, Spectradyne) is a technology to size single particles in suspension. To wet the interior of the microfluidic chip, a wetting agent is required to reduce the surface tension of samples. Before, we used bovine serum albumin (BSA). BSA leaves extracellular vesicles (EVs) intact but insufficiently decreases the surface tension, thereby impeding measurements. Here, we aimed to develop a new protocol for MRPS measurements of EVs by introducing Poloxamer-188 as a wetting agent.
Methods: A cell-free erythrocyte concentrate sample was used as an EV test sample. To find the percentage of labeled EVs out of all particles, EVs were labeled with CD235a and measured with flow cytometry (FCM; A60-Micro, Apogee; lower detection limit ~145 nm). To study the effect of wetting agents on EVs, the unstained sample was diluted in filtered Dulbecco's phosphate-buffered saline (DPBS), or DPBS containing 0.1% BSA (w/v), 0.05% Poloxamer-188 (v/v), 0.1% Triton X-100 (v/v), or 1% Tween 20 (v/v), and measured with FCM. Furthermore, the accuracy of MRPS in determination of sample volume, which is proportional to the average time particles transit through the pore, was investigated.
Results: FCM results confirm that the majority of particles in the EV test sample are EVs, because 76% and 93% of all particles were CD235a+ and susceptible to detergent lysis with Triton X-100, respectively. Whereas the total concentration of EVs was unaffected by the presence of Poloxamer-188 or Tween 20, the size distribution in Tween 20 differed from the other wetting agents. The concentration of EVs <235 nm increased by 54% and of EVs >235 nm decreased by 34% in Tween 20, compared to positive controls (DPBS, and DPBS containing BSA). MRPS measurements show that poloxamer-188 improves the accuracy in determination of sample volume compared to BSA, because the interquartile range of particles' transit time is 11 µs, which is lower than 56 µs for BSA and 16 µs for Tween 20.
Conclusions: Poloxamer-188 is a more suitable wetting agent than Tween 20 and BSA for MRPS measurements of EVs.

Poster Presentations

NLSEV2022-P29: Kees Vlak (Sponsor poster)

Characterization of EVs isolated by a novel magnetic bead-based method utilizing automated western blotting

Rachel Talis, Francisco Ramirez, Karolin Spitzer, Caroline Metzen, Nike Bahlmann, Chris Heger, Martin Schlumpberger, Markus Sprenger-Haussels
**Bio-Techne, Analytical Solutions Division, San Jose, CA; *QIAGEN GmbH, QIAGEN Strasse 1, Hilden, Germany,*

The study of extracellular vesicles (EVs) not only keeps producing new insights into fundamental biologic processes, but also continues to provide new opportunities in the diagnosis and treatment of a wide range of diseases. Despite that, the field is still lacking methods for isolation of EVs that provide not only the required efficiency and specificity, but also standardization, scalability, short processing time and potential for full automation, required for routine use. Here we present a novel, magnetic bead-based workflow, utilizing biochemical affinity, followed by characterization of the isolated EVs according to MISEV criteria using an automated capillary Immunoassay platform.