Publishable Summary for 18HLT01 METVES II
Standardisation of concentration measurements of extracellular vesicles for medical diagnoses

Overview

Extracellular vesicles (EVs) are cell-derived particles present in body fluids, which have excellent potential as biomarkers for the diagnosis of diseases as cancer and thrombosis. This project aims to tap into the clinical potential of EVs by developing traceable measurements of number concentration, size distribution, refractive index (RI) and fluorescence intensity of EVs in human blood and urine. The project will develop synthetic reference materials with physical properties resembling EVs, ready-to-use biological test samples, and instrumentation and procedures to standardise EV measurements in clinical laboratories, which will be evaluated in an inter-laboratory comparison study across standard flow cytometers in clinical laboratories.

Need

European healthcare costs are estimated to increase by five to six percent annually for the next decade, and healthcare costs are projected to become unsustainable between 2040 and 2050. A dramatic reduction of treatment costs can be achieved by early diagnosis of disease, because the costs of early stage treatment are a fraction of late stage treatment. Moreover, early stage treatment improves the clinical outcome and the quality of life of patients, and hence a healthier society. However, early diagnosis requires real time diagnostic information from easily accessible samples. Body fluids are so well suited for this purpose that they are often called “liquid biopsies”. Current liquid biopsies are mainly based on the analyses of (macro)molecules, cell-free DNA or cells, however EVs are rapidly gaining interest as a new category of liquid biopsy biomarkers.

The exploitation of EVs as biomarkers requires reliable measurements, however this is currently very difficult as most EVs are smaller than 200 nm. At present, flow cytometry is one of the most appropriate techniques for EV analysis in clinical samples, because flow cytometers are present already in clinical laboratories and can measure EVs at high throughput.

A flow cytometer measures light scattering and fluorescence intensity of single EVs in a flow. However, due to technical variations between different flow cytometer models, measurements of EV concentrations are currently incomparable. Therefore, EV reference materials and methods are urgently needed to calibrate flow rate, light scatter intensity and fluorescence intensity in the sub-micrometre size range. The ideal reference material should contain particles with a traceable number concentration to calibrate flow rate, a traceable size and RI to calibrate scatter intensity, and a traceable fluorescence intensity. Applications of such dedicated reference materials will also require testing and validation using biological test samples in clinical laboratories.

Objectives

The overall goal of this project is to enable the standardisation of concentration measurements of cell-specific EVs in human body fluids by developing reference materials and related reference measurement methods. The specific objectives are:

1. To develop clinically relevant synthetic reference materials that contain stable spherical particles with (1) concentrations in the range of 10^9 - 10^{12} particles mL^{-1}, (2) discrete diameters between 50 nm and 1 000 nm, (3) an refractive index (RI) in the range of 1.37 - 1.42 and (4) a visible fluorescence intensity between 100 and 100 000 molecules of equivalent soluble fluorochromes (MESF).

2. To develop traceable measurement methods for the number concentration, size distribution, fluorescence intensity and RI of the reference materials from Objective 1. The uncertainty for each method will be determined.
3. To develop traceable methods to characterise the number concentration, size distribution, RI, and fluorescence intensity of biological test samples containing EVs from human body fluids. The uncertainty for each method will be determined.
4. To evaluate and validate the performance of the clinically relevant synthetic reference materials from Objective 1 via an inter-laboratory comparison with an adequate number of clinical end users. This should include an assessment of the reproducibility of measurements of the concentration of EV from the biological test samples from Objective 3, across a range ($\geq$ 20) of standard flow cytometers in clinical labs.
5. To facilitate the take up of the technology and measurement infrastructure developed in the project by the measurement supply chain (accredited laboratories, instrumentation manufacturers), standards developing organisations and end-users (medical practitioners, clinical and academic laboratories).

Progress beyond the state of the art

The state-of-the-art for EV is defined by the preceding EMRP project HLT02 METVES. In the preceding project, procedures were developed for collection and handling of biological fluids for EV research. The size distribution of EVs was measured using both metrological and clinical instruments. Because EVs are polydisperse and have a complex composition, and because suitable reference materials and methods were lacking, traceable size measurements proved unfeasible with both primary and clinical methods. However, HLT02 METVES revealed that flow cytometry has clinical potential, because flow cytometers can identify cell-specific EVs at a rate of thousands per second. Therefore, an inter-laboratory comparison study was initiated to measure cell-specific EVs within the same size range. In this study, commercial synthetic EV reference materials were characterised by metrological instruments and used to standardise EV size measurements by 46 flow cytometers. The results were ground-breaking: although two out of three flow cytometers were sufficiently sensitive to detect EVs, flow rates deviated two-fold from the set flow rate, and local preparation of EV samples lead to undesired inter-laboratory variability.

The HLT02 METVES project focused on size determination of EV reference materials, but not their number concentration, RI and fluorescence intensity. Consequently, neither flow rate, nor scattering and fluorescence intensity of flow cytometers could be calibrated. This means that currently, laboratories rely on polystyrene particles to calibrate these aspects, but these particles have four major shortcomings for EV research. Although some kits have a CE mark for *in vitro* diagnostics and hence are ready for clinical use, polystyrene particles designed for flow rate or fluorescence calibration firstly lack an uncertainty statement of number concentration or MESF, respectively, and, secondly, scatter 1 000-fold more light than EVs. Therefore, polystyrene particles often require different acquisition settings than EVs, which is impractical and can lead to errors. Thirdly, the MESF of the dimmest fluorescence calibration particles should be 10-fold dimmer for EV applications. Fourthly, polystyrene and silica particles have a higher RI than EVs and therefore are unsuitable to directly relate scatter intensity to EV size. Based on the results of the preceding project HLT02 METVES and Mie theory, the partner Exometry in this project has developed a kit to derive EV size from scattering intensity. However, this kit requires validation with particles having a size, RI, and therefore scattering intensity resembling EVs.

In summary, METVES II will build upon the outcomes of the preceding METVES project and will go beyond the state-of-the-art by:

- developing EV reference materials and methods to standardise flow rate, scattering intensity and fluorescence intensity of EV detection by flow cytometers. The concentration, size, RI, and fluorescence intensity of the reference materials will have uncertainty statements and resemble EV properties, so that calibrations are reliable and do not require a change of acquisition settings.
- developing stable, ready-to-use, and well-characterised biological test samples containing pre-labelled and pre-diluted EVs to eliminate variation of the EV concentration due to sample preparation in different laboratories.

- conducting an inter-laboratory comparison study to demonstrate reproducible flow cytometry measurements of the EV concentration with a coefficient of variation (CV) < 20 % using the developed EV reference materials, reference methods, and biological test samples produced in this project.

Results

To develop clinically relevant synthetic reference materials that contain stable spherical particles with (1) concentrations in the range of 10^9 to 10^{12} particles mL^{-1}, (2) discrete diameters between 50 nm and 1 000 nm, (3) an RI in the range of 1.37 to 1.42 and (4) a visible fluorescence intensity between 100 and 100 000 MESF

An online survey was sent to and completed by members of the project's Stakeholder Committee and the EV Flow Cytometry Working Group (www.flowcytometry.org) to gain insight into the desired properties for the development of reference materials to standardise EV measurements by flow cytometry. The EV Flow Cytometry Working Group is a collaboration between flow cytometry experts from the International Society on Extracellular Vesicles (ISEV), the International Society for the Advancement of Cytometry (ISAC), and the International Society for Thrombosis and Hemostasis (ISTH), and National Institute of Health (NIH). The outcomes from the questionnaire on the desired properties aligned with the objectives of this project, and thus have formed the basis of the development of clinically relevant synthetic EV reference materials, which can be subsequently used in the inter-laboratory comparison study in Objective 4.

Candidate EV reference materials of 3 types were produced, as described below: (1) hollow organosilica beads (HOBs), (2) liposomes and (3) polymer-based solid low-RI particles. After optimisation of synthesis procedures, candidate EV reference materials were distributed to METVES II partners for development of measurement techniques to determine the number concentration and RI.

1. To prepare HOBs (i.e. candidate EV reference materials), monodisperse seed particles in the size range between 100 nm and 500 nm were prepared in house in large volumes (100 mL), and from a commercial source. Quality control methods for synthesis procedures such as transmission electron microscopy, resisitive pulse sensing and flow cytometry, were established by collaborations between the consortium (MTA TTK, AMC) and external collaborators (University of Pannonia, Hungary). The quality control methods enabled us to identify the reproducibility issues of the synthesis methods. Candidate HOBs were distributed from Nov 2019 (M6) to May 2020 (M12) to project partners. To prepare fluorescent candidate HOB reference materials, amino-propyl-modified and biotinylated HOBs were prepared and characterised by dynamic light scattering (DLS) and Zeta-potential measurements. Amino-propyl-modified HOBs were labelled with fluorescein isothiocyanate (FITC) and biotinylated HOBs were labelled with avidin-conjugated PE and will be distributed March (M22) or April 2021 (M23).
2. FITC-labelled liposomes (i.e. candidate EV reference materials), were prepared with proprietary centrifugation technology. To improve the homogeneity of the liposomes, they were sorted by flow cytometry and characterised with dynamic light scattering and flow cytometry to determine scatter intensity, polydispersity, and fluorescence intensity. Five lots of FITC-labelled liposomes of different sizes were prepared and delivered to project partners for method development. In parallel, liposomes with a variety of lipid compositions were prepared and measured by flow cytometry. The measurements revealed that the side scatter of liposomes depends upon their lipid composition, which can then be used to enable the fine tuning of the RI of liposomal candidate EV reference materials.
3. To produce polymer-based solid low-RI particles (i.e. candidate EV reference materials), a variety of materials were produced from different polymer cores, i.e. commercial polystyrene particle cores of a variety of sizes (100, 200 and 500 nm), poly(methyl methacrylate) particles, and self-synthesised poly(methyl methacrylate)/ polyvinylpyrrolidone (PMMA) particle cores (800 nm) using different reaction conditions. All polymer cores and candidate materials have now been characterised by DLS, electron microscopy and small-angle X-ray scattering. Due to problems with uniformity and reproducibility of the mesoporous silica shells, it was decided to proceed with fluorescently-labelled or stained PMMA particles without mesoporous silica shells, which will have a higher RI than EVs, but fulfil the projects' requirements regarding particle size distribution and number concentration, and fluorescence intensity.

To develop traceable measurement methods for the number concentration, size distribution, fluorescence intensity and RI of the reference materials from Objective 1. The uncertainty for each method will be determined.

The developed candidate EV reference materials from objective 1 have been characterised using a variety of methods.

The development of reference methods for mean diameter and size distribution width determination are in progress. Using atomic-force microscopy measurements (AFM), challenges specific to hollow shells have been identified, such as the fragility of the particles and presence of pores on the surface and the sensitivity of the measured particle size on the parameters of the AFM measurement have been determined. The very narrow size distribution and hollow shell structure of the HOBs candidate EV reference materials was confirmed by small-angle X-ray scattering (SAXS), and the particles could be successfully modelled with a shell thickness of less than 5 % of the diameter.

For candidate EV reference material liposomes, the size distribution and concentration were identified as a critical parameter for SAXS measurements. Additionally, a metrological flow cytometer was commissioned based on a computational microscope and tailored to the specific requirements of flow cytometry. One of the core components, the liquid cell for the sheath fluid, was installed and tested. In addition, different laser beam shapes for the best suitability to measure the size distribution were evaluated.

The development of reference methods for number concentration determination are in progress. Contrast-variation SAXS was applied to HOBs and liposomes candidate EV reference materials. In the case of the HOBs, the addition of surfactant was evaluated to overcome diffusion of contrast media into the HOBs. A method to determine the dry content of candidate HOBs was developed using freeze-drying, which has the advantage of consuming less sample volume than traditional thermogravimetric analysis. The sensitivity of single-particle Inductively Coupled Plasma Mass Spectrometry (spICPMS) for silicon, essential to measure the silica-based HOBs, was also further improved by replacing quartz glass parts with sapphire, and by the application of nonstandard ultrafast microsecond detection, which allows the determination of preliminary concentration values for some particles.

The development of reference methods for fluorescence intensity determination is in progress. The fluorescence intensity of FITC-labelled liposomes and Zn porphyrine-stained particles was determined using calibrated fluorescence spectrometers and a commercial integrating sphere setup. The expected sensitivity of spICPMS for different elements in the elemental composition analysis was also determined. The metrological flow cytometer was upgraded by integrating a micro-Raman spectroscopic detector to measure the fluorescence yield of fluorescent particles. The spectroscope remains to be fully tested and characterised by the project.

The development of reference methods for RI determination are in progress. A custom-made integrating sphere setup, a possible measurement method for the RI, was recalibrated and characterised for wavelength accuracy, detector linearity, and spectral responsivity. The calibration was validated using BAM's certified spectral fluorescence standards dye kit. Further to this, a goniometric setup for the analysis of the RI, employing precise spectrometric goniometry and a prismatic liquid cell were used for measurements of the RI of liquids and the RI of PBS buffer and H_2O was determined with better than 0.1 % accuracy. Further measurements of fluids relevant to flow cytometry will take place after improving the uncertainty. Finally, simulations of collimated extinction spectroscopy (SEMPA) for the HOB particles were performed which will allow the project to determine the RI from measured spectra.

To develop traceable methods to characterise the number concentration, size distribution, RI, and fluorescence intensity of biological test samples containing EVs from human body fluids. The uncertainty for each method will be determined.

In order for the project to best meet end-user's requirements, the online survey from Objective 1 also included questions regarding the desired properties of the biological test samples containing EVs from human body fluids. The outcome of the completed surveys by stakeholders and members of the EV Flow Cytometry Working Group (www.evflowcytometry.org) aligned with the project's objectives and the development of (stable) biological test samples ((S)BTS) containing EVs from human blood (partner AMC) and urine (partner UH) is in progress.

Plasma EVs have been fluorescently labelled with antibodies against a platelet protein (CD61-APC), an erythrocyte protein (CD235a-PE), and lactadherin-FITC, and EVs in urine with lactadherin-FITC. Experiments testing the stability of the BTS were performed for plasma, serum, and urine, and have shown that stable storage of pre-labeled BTS is possible for at least 2 weeks at -80°C in the presence of cryopreservation agents e.g. dimethyl sulfoxide (DMSO), glycerol, or trehalose, when measured by flow cytometry. Furthermore, partner AMC improved the plasma BTS by developing a procedure to remove residual platelets without affecting the EV concentration. The long-term stability study is ongoing at AMC (4 months) and has just started at partner UH.

To test if the developed plasma BTS is stable during shipment and handling, AMC has shipped plasma BTS to a US collaborator (Cytek Biosciences) and a Swiss (industrial) partner (BD). The concentration of pre-labelled EVs measured in the BTS differed 20-fold between the measurements made by partner AMC and the US collaborator without instrument calibration. Both AMC and the US collaborator used flow cytometers with different optical configurations. However, the difference in measurements was less than 20 % after instrument calibration. Measurement results from partner BD are expected soon.

To evaluate and validate the performance of the clinically relevant synthetic reference materials from Objective 1 via an inter-laboratory comparison with an adequate number of clinical end users. This should include an assessment of the reproducibility of measurements of the concentration of EV from the biological test samples from Objective 3, across a range (≥ 20) of standard flow cytometers in clinical labs.

The interlaboratory comparison study is scheduled for M34 of the project, therefore no preparations have been made yet.

Impact

A stakeholder committee of 10 members has been setup, including two members from industry, one clinician, one member from the National Institute of Standards and Technology (USA), four well respected EV researchers from the EU and two from the USA.

A website (www.metves.eu) for the project has also been setup, as well as a “SharePoint” that is used by the consortium to share documents and presentations, etc.

So far, 25 conference presentations and posters were presented, and 3 peer-reviewed open access scientific publications have been published in journals the American Journal of Reproductive Immunology, the Journal of Extracellular Vesicles and Materials. Further to this a press release was given on ‘finding solutions to the diagnostics and treatment of severe diseases through research on extracellular vesicles’ by the University of Helsinki (UH).

Impact on industrial and other user communities

EVs in liquid biopsies behold the promise of becoming new biomarkers for common diseases. In 2022, the estimated liquid biopsy market size is expected to exceed $ 2.1 billion with a compound annual growth rate of > 23 %. Consequently, there is a growing demand for biomarker research from industry. One of the industrial partners in this project is BD, which is one of the largest players in the global flow cytometry market and due to the connections of the other project partners, it is expected that the metrological basis developed in this project will become a prerequisite for clinical acceptance and routine application of EV-based diagnostics. This will enable the direct uptake, exploitation and use of the developed EV reference materials, reference methods and metrological services by academia and industry active in the development of (1) reference materials for EV, virus or bacteria measurements, (2) flow cytometers dedicated to nanoparticle detection, (3) diagnostic kits, and (4) drug-loaded therapeutic EVs or liposomes.

As one of the world leaders in measurement procedures, reagents and instruments for research & clinical cell analysis, partner BD is very interested in the commercialisation and dissemination of the outputs of this project to industrial and clinical end users. In addition, partner AMC has recently set up three clinical studies to exploit EVs as a biomarker to recognise the underlying cause of stroke, predict cardiac arrest, and diagnose prostate cancer. These linked clinical studies at AMC will directly benefit from metrology developed in this project, thereby supporting the use of the outputs of this project by clinical end users.

In the process of acquiring a new flow cytometer, an EV test sample developed by AMC was shipped to Cytek Biosciences (https://cytekbio.com/), a US flow cytometer manufacturer. As part of the work of objective 3, the EV test sample allowed the comparison of the performance of the Aurora flow cytometer from Cytek Biosciences and the performance of the flow cytometer at AMC. The senior scientist at Cytek Biosciences was enthusiastic about the usability of the EV test sample and how close the EV test sample resembles freshly immunostained EV samples.

Impact on the metrology and scientific communities.

The growing clinical and industrial interest in biomarkers offers metrology a unique and timely opportunity to create impact on a new and rapidly expanding clinical research field and on future medical applications of EVs. This project will create a novel metrology infrastructure to characterise EV reference materials developed and to standardise (biological) nanoparticle measurements. These new procedures will involve the traceable determination of the RI and scattering intensity of nanoparticles in suspension, and this new infrastructure will increase the measurement capabilities of the European NMIs and DIs.

From a scientific perspective, there is a growing concern about reproducibility in medical sciences, which particularly affects the growing EV-field due to an absence of nomenclature, suitable reference materials, appropriate quality control, appropriate calibrations, and the appearance of new instruments which lack reference procedures. To improve standardisation of flow cytometer measurements on EVs, the ISAC, ISEV, ISTH, and NIH started the EV Flow Cytometry Working Group. This project's partners are prominent members of these societies and the EV Flow Cytometry Working Group was consulted as part of the online survey in Objectives 1 & 3. Thereby linking the project's work with key end user needs.

The influence and impact of metrology in the research field of EVs is gaining momentum. The output from a recent ISEV workshop on reference materials for EV research (Belgium, December 2019) was a manuscript entitled "*Towards defining reference materials for extracellular vesicle size, concentration, refractive index and epitope abundance*". This manuscript contains input from the METVES II consortium and a link/text on this project, as well as an entire section explaining the term "metrology".

As part of this project, partners AMC and VSL, (a university hospital and a metrology institute respectively), are sharing a PhD student, in order to further knowledge exchange on optics and fluidics of flow cytometry, and in development of a metrological flow cytometer and goniometer. Further to this, three foreign students (from China, Italy and American) and partner UH have been trained by AMC in calibration of a flow cytometer for EV measurements

Finally, the project has produced 2 interactive online training tools for end users. One online training tool is on 'Metrology for measurement of nanoparticle size by electron microscopy & atomic force microscopy - In-situ nano particle metrology using traceable flow cytometry'. The other online training tool is on 'Metrology for extracellular vesicles as a prerequisite in the pursuit of an early stage minimally invasive new medical diagnostic tool' and it is available for end users at https://www.youtube.com/watch?v=esfDXlSyXqQ.

Impact on relevant standards

At present, no directives of the EU and no appropriate measurement standards exist with regard to EVs. Therefore, this project aims to standardise EV concentration, size, RI and fluorescence measurements by developing specific reference materials and methods. Because reference materials aimed for comparisons of clinical measurement equipment and new traceable measurement procedures will be developed, the relevant standardisation working groups of the BIPM's CCQM (Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology) i.e. Working Group on Cell Analysis (CAWG), Working Group on Surface Analysis (SAWG) and Working Group on Inorganic Analysis (IAWG) will be informed of the project results for the future establishment of entries for calibration and measurement capabilities and performing metrological comparisons. Project partners are also actively involved in the EV Flow Cytometry Working Group of ISAC, ISEV and ISTH and the Blood EV Working Group of ISEV.

So far, the project has been presented to ISO/TC 229 Nanotechnologies JWG 2JWG 2 Measurement and characterization and ISO/TC 24 Particle characterisation including sieving/ SC 4 Particle characterization, as well as the the Scientific and Standardisation Committees (SSC) on Vascular Biology of the ISTH at the meeting in (Melbourne, July 2019). The ISTH's SSC on Vascular Biology is particularly important because

earlier interlaboratory comparison studies on measuring the concentration of EVs have been organised by this body, including the inter-comparison in the preceding HLT02 METVES project.

Longer-term economic, social and environmental impacts

In the long-term, the infrastructure and reference materials developed in this project is expected to impact healthcare by enabling (1) the establishment of normal values for concentrations of EVs in healthy subjects, (2) the comparison of EV concentrations between healthy and non-healthy subjects for biomarker research, and (3) the comparison of potential EV biomarkers to available biomarkers and prediction models.

The high potential of liquid biopsies and hence EV is reflected by the projected global market size, which is expected to grow from at least $ 2.1 billion in 2022 to $ 6.0 billion in 2030 with reported compound annual growth rates up to 37 %. Cell-specific EVs can be used to facilitate early diagnosis, as there is increasing evidence that changes in their concentration and function directly reflects health and disease. Due to the aging of the EU population and increasing costs of healthcare this project will support a reduction in European healthcare costs, as the costs of early stage treatment are a fraction of late stage treatment. The inter-laboratory comparison studies in this project will also help to establish EVs as biomarkers, and the standardisation of EV measurement results between instruments. This in turn, will pave the way towards new biomarker development, thereby facilitating earlier diagnosis and improving patient survival.

List of publications

[1]. Kuiper M, van de Nes A, Nieuwland R, Varga Z, van der Pol E, Reliable measurements of extracellular vesicles by clinical flow cytometry, American Journal of Reproductive Immunology, 2020, e13350

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[2]. Welsh JA, van der Pol E, Bettin, BA, Carter DRF, Hendrix A, Lenassi M, Langlois, MA, Llorente A, van de Nes AS, Nieuwland, R, Tang V, Wang L, Witwer KW, Jones JC. Towards defining reference materials for measuring extracellular vesicle refractive index, epitope abundance, size and concentration. Journal of Extracellular Vesicles 2020, 9: 1816641,

https://doi.org/10.1080/20013078.2020.1816641

[3]. Al-Khafaji MA, Gaál A, Wacha A, Bóta A, Varga Z. Particle size distribution of bimodal silica nanoparticles: a comparison of different measurement techniques. Materials 2020, 13(14), 3101

https://doi.org/10.3390/ma13143101

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Coordinator: Rienk Nieuwland, VSL Tel: +31205664851 E-mail: r.nieuwland@amsterdamumc.nl Project website address: www.metves.eu		
Internal Funded Partners: 1. VSL, Netherlands 2. BAM, Germany 3. LGC, UK 4. LNE, France 5. PTB, Germany 6. VTT, Finland	External Funded Partners: 7. AMC, Netherlands 8. Exometry, Netherlands 9. MTA TTK, Hungary 10. UH, Finland	Unfunded Partners: 11. BD, Switzerland 12. PolyAn, Germany
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