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 METVES II project has developed 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 were 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 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 to 10^{12} particles mL^{-1}, (2) discrete diameters between 50 nm and 1 000 nm, (3) a 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 (≥ 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 was 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 interlaboratory 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 has built upon the outcomes of the preceding METVES project, and its work has gone beyond the state-of-the-art by:

- the development of 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 have uncertainty statements and resemble EV properties, so that calibrations are reliable and do not require a change of acquisition settings.
- the development of 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.
- the completion of 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

Objective 1 was to develop reference materials to calibrate flow cytometry measurements of single EVs. The quantities for calibration included the flow rate, the scattering intensity and fluorescence intensity to enable the standardisation of size, concentration and fluorescence measurements of EVs by flow cytometry.

An online survey was conducted at the beginning of the project to determine the desired properties of these reference materials. The survey was sent to the members of the Stakeholder Committee of METVES II and to the members of the EV Flow Cytometry Working Group, which was established by flow cytometry experts from the International Society for Extracellular Vesicles (ISEV), International Society for the Advancement of Cytometry (ISAC), and the International Society on Thrombosis and Haemostasis (ISTH). The survey findings confirmed the targeted ranges for the physical properties of the reference materials in Objective 1.

To ensure that all the necessary criteria were met, three types of reference materials were considered: (1) hollow organosilica beads (HOBs), (2) liposomes, and (3) low-RI solid particles. Small-angle (SAXS) and wide-angle X-ray scattering (WAXS) and flow cytometry measurements were performed for the initial characterisation of the 3 types of candidate reference materials.

HOBs have similar light-scattering properties to EVs due to their high refractive index shell and low refractive lumen, and they can be produced in small and reproducible quantities. Producing larger amounts of HOBs was difficult, however, due to the complex chemical processes that are involved. It was possible to label HOBs with small-molecular fluorophores (such as fluorescein isothiocyanate, FITC), a commonly used fluorochrome to label EVs, by using traditional methods, as confirmed by bulk fluorescence measurements. However, it was challenging to produce HOBs with MESF (molecules of equivalent soluble fluorophores) values reaching the detection limit of a flow cytometer. As a result, HOBs were traceably characterised for both size and concentration and are considered EV reference materials for these properties.

Liposome preparation is straightforward by using established methods, as well as fluorescent labelling of liposomes with fluorophores commonly used to label EVs. For example, by using lipids conjugated with fluorophores (such as carboxyfluorescein-conjugated phosphatidylethanolamine, PE), or by using functionalised lipids that can bind protein-based fluorophores (such as allophycocyanin, APC). However, using liposomes as reference materials for EVs posed two challenges, i.e. determining their (i) size and (ii) concentration accurately, as the size distribution of liposomes produced by extrusion methods becomes wider with increasing pore size. As a result, liposomes were characterised for fluorescence properties and were considered as an EV reference material for this property. The possibility of traceable size and concentration characterisation of gel phase liposomes extends beyond the scope of the METVES II project.

The polymer core-based low-RI particles were found to be unsuitable as candidate EV reference materials for the scattering and fluorescence detection of EVs by flow cytometry. This was due to the difficulties encountered with the preparation of these particles including non-homogeneous layer formation, melting of the polymer core during synthesis, and stickiness of the particles to the tubing of a flow cytometer. In contrast, solid PMMA particles were useful reference materials for traceable measurements of the RI and were used further in Objective 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.

For each of the relevant properties from Objective 1, the reference values could be determined with sufficient accuracy. For the size characterisation of silica-based particles, both solid silica particles and HOBs, measurement results from multiple methods were found in agreement. While mostly standard methodologies could be applied, a new numerical model for the SAXS data analysis of the HOBs was devised comprising independent size distributions for the core size and the shell thickness.

The number concentration of the solid silica particles was determined by SAXS and single particle inductively coupled plasma mass spectrometry (spICPMS), and the number concentrations of HOBs was derived from

spICPMS. The reference values of both results were in agreement with the (indirectly) traceable method particle tracking analysis (PTA), which for the first time allowed the determination of the number concentration of silica-based particle suspensions. The number concentration was also determined using flow cytometry, but no uncertainty budget has been derived so far for flow cytometry. Nevertheless, the results were in general agreement with the traceably determined reference values.

For each of the measurements of the RI and the fluorescence intensity, only one method for each measurand was available. The results obtained from these methods agree with the expected values, and therefore were accepted as reference values. The RI reference values of the PMMA particles in a size range from 2 µm down to 600 nm were determined using spectrally resolved collimated transmission measurements with a custom-built setup. This device allowed the exclusion of unwanted contributions to the directed transmittance when analysing the measurements, which is not possible in standard spectrophotometers. The RI was then determined by an analysis of the measured ensemble averaged extinction cross section. The fluorescence intensity of the fluorescent liposomes was determined using an integrating sphere setup. This was the only possible method in the scattering media due to the strong scattering background which resulted in the failure of typical relative quantum yield determinations.

Additionally, the RI of commonly used liquids in EV flow cytometry was successfully determined with an expanded (k = 2) uncertainty below 2×10^{-6}. This was achieved with a custom-built setup of the minimum deviation angle method on top of a precision goniometer.

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.

A procedure was developed to produce a stable biological test sample derived from human plasma. This procedure included a new strategy to remove blood platelets (small cells) without affecting the concentration of EVs. The project's developed plasma-derived EV test sample was called PEVTES. Plasma and derived PEVTES both contain EVs originating from different cell types, and high concentrations of non-EV particles (mostly lipoproteins). Since EVs from two cell types (platelets, erythrocytes) were pre-labelled in PEVTES, not all EVs were labelled, and the non-EV particles remained unlabelled. The size range of EVs and non-EV particles in PEVTES ranged from about 70 nm to 1 µm. Consequently, the composition of particles present in PEVTES is very complex and heterogeneous when compared to synthetic EV reference materials, and this heterogeneity hampered the traceable determination of the physical properties of labelled EVs in PEVTES, i.e. those EVs that were detected in the interlaboratory comparison study to standardise concentration measurements of EVs (Objective 4).

The concentration, size distribution and fluorescence of the EVs in the PETVES test sample was stable at - 80 °C for at least 12 months as determined by flow cytometry.

The size distribution of the particles in PEVTES was determined by PTA and AF4-UV-MALS. Since both methods detect all particles and not exclusively labelled EVs, the obtained data should be interpreted with care as labelled EVs are only a small fraction of all particles present in PEVTES. For example, the mean total particle concentration determined by flow cytometry was $2.19E^{+08}$ particles/mL, whereas the concentration of labelled EVs was $2.33E^{+05}$ /mL for erythrocyte-derived EVs and $2.09E^{+06}$ /mL for platelet-derived EVs. Similarly, the number concentration determined with PTA should be carefully interpreted.

The number concentration of labelled EVs in PEVTES was determined by flow cytometry. However commercial flow cytometers lack measurement uncertainty, because essential information such as sample stream width and exact optical alignment of the instrument have not been traceably measured. Thus, traceable number concentration measurements using commercial flow cytometry were not possible. Instead, the coefficient of variation (CV) of the concentration of labelled EVs was determined, which was 11 % when including all measurements of labelled platelet-derived and erythrocyte-derived EVs, which was considered a repeatability score sufficient to use PEVTES as test sample in the interlaboratory comparison study (Objective 4).

The fluorescence intensity of EVs could not be traceably determined because the instruments were either too insensitive or required a sample volume larger than was practically possible to prepare in a single batch (fluorescence spectrometers and integrating sphere spectroscopy).

The RI of the diluted PEVTES was measured using the METVES II project's newly developed precision goniometer with an expanded uncertainty of $1.4E^{-6}$. A metrological flow cytometer was also developed and is nearly operational. This metrological flow cytometer is expected to be the first instrument in the world that will be capable of concurrently measuring the RI, size, shape and fluorescence of single particles directly in suspension. Once operational, this instrument can be used to traceably characterise single submicrometer particles in suspension.

Despite the above successes, not all goals could be achieved regarding the development of traceable methods to characterise the developed PEVTES. We faced challenges regarding uncertainty evaluation, instrument sensitivity, and required volumes. As explained, the complexity and composition of PEVTES makes it less suitable for characterisation with high-end methods that are used for traceable particle characterisation. But use of PETVES with commercial flow cytometers, lacked traceability as essential information is unavailable for such flow cytometers. Therefore, the further development of the project's metrological flow cytometer will be indispensable to overcome these hurdles.

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.

A global interlaboratory comparison study was performed amongst participating laboratories who were members of (i) the EV Flow Cytometry working group, (ii) participating members of the HLT02 METVES-organised interlaboratory comparison study, (iii) stakeholders of this project METVES II, and/or (iv) members of the Reference materials task force of ISEV. Additional criteria for the participating laboratories were a track record of EV detection by flow cytometry, and that they should have at least one flow cytometer that was capable of (1) differentiating 100 nm polystyrene beads from background noise by light scattering, (2) detecting APC, FITC, and PE fluorescence, and (3) measuring EVs directly in diluted blood plasma at high throughput (>1,000 events/s). 49 laboratories were invited to participate, 24 laboratories from 12 different countries fulfilled all the inclusion criteria, and a total of 39 different flow cytometers were registered for participation in the interlaboratory comparison study.

Distributed reference materials included the project's characterised solid silica particles for flow rate calibration, and HOBs as potential light scatter calibrators (Objectives 1 & 2). The METVES II-developed PEVES (Objective 3) and liposomes (Objectives 1 & 2) were selected as test samples. A webinar was organised to inform and instruct participants about the measurement procedures for the interlaboratory comparison study. Complete data sets from 21 flow cytometers were received and analysed. Due to the unforeseen COVID-19 pandemic, the study was delayed but preliminary data analysis has clearly demonstrated that calibration significantly reduced the variation in concentration measurements of labelled EVs on flow cytometers with different optical configurations.

Impact

A stakeholder committee of 10 members was set up, 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 was also set up (www.metves.eu) for stakeholders to find out important information and events for the project. Further to this, 41 conference presentations and posters have been presented on the project's results 13 peer-reviewed open access scientific publications have been published, and a patent application has been filed for the production of liposomes.

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 was expected to exceed $ 2.1 billion, with a compound annual growth rate of > 23 %. Consequently, there is a growing demand for biomarker research from the industry. One of the industrial partners in this project is BD, which is one of the leaders in the global flow cytometry market and due to the connections of the other project partners (AMC, Exometry, PTB, TTK, VSL, UH), 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 acceptance will support the uptake and use of the project's developed EV reference materials (Objectives 1 & 3) and reference methods (Objective 2) in the future development of (i) reference materials for EV, virus or bacteria measurements, (ii) flow cytometers dedicated to nanoparticle detection, (iii) diagnostic kits, and (iv) 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 project's outputs to industrial and clinical end users. Software developed by partner Exometry will be used by BD for flow cytometry calibration in conjunction with BD's FlowJo Plug-in that was developed to support this (Objective 3). In addition, BD has filed a patent application on the production of liposomes from Objective 1 will produce a report on potential interest in the commercialisation, licensing, and/or distribution of the project's reference materials (Objectives 1 & 3) and reference procedures (Objective 2).

Indeed, one of the reference particles (Objective 1) has already been commercialised by Exometry together with partner TTK as "verity shells" (https://www.exometry.com/products/verity-shells).

For industry, it is important that reliable and comparable data is generated with commercial equipment. Commercial flow cytometers were used by 24 participants in the project's interlaboratory comparison study, including 3 industrial laboratories.

Furthermore, comparing the preliminary results from this project METVES II interlaboratory comparison study (Objective 4) to the results of the previous METVES projects' comparison study, demonstrates that commercial flow cytometers have already become more sensitive for small particle detection and hence industry is investing in this area.

Impact on the metrology and scientific communities.

The METVES II project has developed new methods for traceable number concentration and diameter measurements of submicron particles. VSL together with AMC developed the first ever precision goniometer with an expanded uncertainty of $1.4E^{-6}$ (Objective 3). In METVES II the goniometer was used to determine the RI of fluids commonly used in clinical flow cytometry such as sheath fluid and physiological buffers such as phosphate-buffered saline. This information is critical because EVs have a low RI, and the RI contrast affects the size determination of EVs, and hence affects the measured concentrations of EVs.

VSL together with AMC also constructed a metrological flow cytometer (Objective 3). This metrological flow cytometer is nearly operational and has already gained considerable interest at international scientific meetings because this instrument, will (in principle) be capable of simultaneously measuring the RI, size and fluorescence of single particles in suspension. When operational, this metrological flow cytometer, will be the first of its kind and can be used as a metrological instrument to characterise single reference materials, biological particles as EVs, etc.

The PEVTES sample developed by this project (Objective 3) is unique as it expresses the complexity and heterogeneity of a real human plasma sample. The procedure to develop stable labelled EV-containing test samples is also completely new, although future research is needed to explore its use in standardisation studies.

METVES II partners and stakeholders are prominent members or chairs of the EV Flow Cytometry Working Group (a collaboration between ISAC, ISEV and ISTH) and standardisation bodies of ISEV and ISTH. In these international organisations, results have been presented and disseminated. The developed infrastructure of METVES II is new to the EV field, and this knowledge has been shared with the scientific and medical communities e.g.

- at the "EV Club seminars" (ISEV initiative) https://www.youtube.com/watch?v=ghub6emZDAA)
- at an industry-sponsored Nature webinar (https://www.nature.com/webcasts)
- and at a METVES II organised workshop (https://www.metves.eu/downloads/workshop/Workshop_METVES_II.pdf).

A major element of generating impact is training and education. Two PhD students, one with an engineering background and one with a biomedical background, were employed and trained during METVES II to strengthen collaboration between NMI's (VSL) and hospitals (AMC, UH). Further to this, (bio) medical students

were trained by AMC to calibrate flow cytometers, an annual summer course (starting 2023) is scheduled by AMC to teach (bio) medical students to calibrate flow cytometers using reference materials, test samples and software developed in the METVES II project. Furthermore, ISEV is setting up an online training course on EV detection and isolation methods, that will explain the relevance of metrology and calibration to an audience of clinicians and (bio) medical researchers using the knowledge and information developed in the METVES II project.

Impact on relevant standards

At present, no EU directives or appropriate measurement standards exist with regard to EVs. Partners in METVES II have provided input to both international ISO TC24 Particle characterisation including sieving and TC229 Nanotechnologies and national METSTA SR229 Nanotechnology standards development organisations that are related to methods used for characterising reference particles. Input was also provided by the consortium to existing technical reports, specifications and the revisions of existing technical specifications, including ISO 19430 “Determination of the particle size distribution and number concentration by the Particle Tracking Analysis (PTA) Method”, and ISO/TS 19590 “Nanotechnologies — Characterisation of nano-objects using single particle inductively coupled plasma mass spectrometry“. Furthermore, the project’s results were used in new standards and technical reports, including ISO 23484 “Determination of particle concentration by small-angle X-ray scattering (SAXS)”, and ISO/ TS 24672 “Nanotechnologies - Guidance on the measurement of nanoparticle number concentration'. Finally, the project has presented its results to the Scientific Standardisation Committee (SSC) of the ISTH Subcommittee on Vascular Biology and at the EV Flow Cytometry working group (Objective 4).

Longer-term economic, social and environmental impacts

In the long-term, the infrastructure and reference materials developed in this project to calibrate flow cytometers is expected to have a considerable impact on biomarker research. Firstly, the concentration of EVs in a body fluid depends on collection, handling and storage procedures. Until now, there was no method available to quantify and monitor the concentration of EVs in a reproducible and standardised manner. But thanks to the METVES II project, calibration of flow cytometers to standardise concentration measurements of EVs (and other submicron particles) has become possible, which will be used in future experiments to optimise pre-analytical procedures.

One of the hallmarks of clinically relevant biomarkers is the establishment of a reference range. A reference range is the normal concentration of a biomarker in a particular body fluid of healthy human. When the biomarker concentration (e.g. EV) is outside the reference range, this may be relevant for diagnosis, prognosis, and monitoring of disease. But to establish biomarker reference ranges, all instruments and laboratories must generate comparable data. This goal of establishing reference ranges for EVs in body fluids can become possible in future using the project’s outputs; (i) synthetic EV reference materials, (ii) traceable measurement methods for them, (iii) biological test samples (PETVES) and (iv) an interlaboratory comparison study with clinical end users, of the EV reference samples and methods.

Comparable concentration measurements of EVs, should pave the way towards clinically relevant multicentre studies of the concentration of cell-type specific EVs and their biomarker potential. Since all body fluids contain EVs from multiple cell types, the developed infrastructure will be broadly applicable, and is expected to find new and non-invasive disease biomarkers that may facilitate earlier diagnosis, reduce healthcare costs, and improve patient survival rates. Importantly, the reference materials and methods developed in METVES II could also be used in the future to standardise concentrations of non-EV particles, e.g. lipoproteins, viruses, bacteria and non-biological particles.

List of publications

[1]. Al-Khafaji, M. A.; Gaál, A.; Jezsó, B.; Mihály, J.; Varga, Z. Amino Surface Modification and Fluorescent Labelling of Porous Hollow Organosilica Particles: Optimization and Characterization. *Materials* **2022**, *15* (7), 2696. https://doi.org/10.3390/ma15072696

[2]. Geißler, D.; Nirmalananthan-Budau, N.; Scholtz, L.; Tavernaro, I.; Resch-Genger, U. Analyzing the Surface of Functional Nanomaterials—How to Quantify the Total and Derivatizable Number of Functional Groups and Ligands. *Microchim Acta* **2021**, *188* (10), 321. https://doi.org/10.1007/s00604-021-04960-5

[3]. Gankema, A. A. F.; Li, B.; Nieuwland, R.; van der Pol, E. Automated Fluorescence Gating and Size Determination Reduce Variation in Measured Concentration of Extracellular Vesicles by Flow Cytometry. *Cytometry Part A* **2022**, *101* (12), 1049–1056. https://doi.org/10.1002/cyto.a.24665

[4]. Kuiper, M.; Koops, R.; Nieuwland, R.; van der Pol, E. Method to Traceably Determine the Refractive Index by Measuring the Angle of Minimum Deviation. *Metrologia* **2022**, *59* (5), 055006. https://doi.org/10.1088/1681-7575/ac8991

[5]. van der Pol, E.; van Leeuwen, T. G.; Yan, X. Misinterpretation of Solid Sphere Equivalent Refractive Index Measurements and Smallest Detectable Diameters of Extracellular Vesicles by Flow Cytometry. *Sci Rep* **2021**, *11* (1), 24151. https://doi.org/10.1038/s41598-021-03015-2

[6]. Al-Khafaji, M. A.; 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

[7]. 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* **2021**, *85* (2), e13350. https://doi.org/10.1111/aji.13350

[8]. Bettin, B.; Gasecka, A.; Li, B.; Dhondt, B.; Hendrix, A.; Nieuwland, R.; van der Pol, E. Removal of Platelets from Blood Plasma to Improve the Quality of Extracellular Vesicle Research. *Journal of Thrombosis and Haemostasis* **2022**, *20* (11), 2679–2685. https://doi.org/10.1111/jth.15867

[9]. Al-Khafaji, M. A.; Gaál, A.; Jezsó, B.; Mihály, J.; Bartczak, D.; Goenaga-Infante, H.; Varga, Z. Synthesis of Porous Hollow Organosilica Particles with Tunable Shell Thickness. *Nanomaterials* **2022**, *12* (7), 1172. https://doi.org/10.3390/nano12071172

[10]. Welsh, J. A.; van der Pol, E.; Bettin, B. A.; Carter, D. R. F.; Hendrix, A.; Lenassi, M.; Langlois, M.-A.; Llorente, A.; van de Nes, A. S.; Nieuwland, R.; Tang, V.; Wang, L.; Witwer, K. W.; Jones, J. C. Towards Defining Reference Materials for Measuring Extracellular Vesicle Refractive Index, Epitope Abundance, Size and Concentration. *Journal of Extracellular Vesicles* **2020**, *9* (1), 1816641. https://doi.org/10.1080/20013078.2020.1816641

[11]. Pol, E. van der; Welsh, J. A.; Nieuwland, R. Minimum Information to Report about a Flow Cytometry Experiment on Extracellular Vesicles: Communication from the ISTH SSC Subcommittee on Vascular Biology. *Journal of Thrombosis and Haemostasis* **2022**, *20* (1), 245–251. https://doi.org/10.5281/zenodo.7634583

[12]. Welsh, JA; Arkesteijn, GJA; Bremer, M; Cimorelli, M; Dignat-George, F; Giebel, B; Görgens, A; Hendrix, A; Kuiper, M; Lacroix, R; Lannigan, J; van Leeuwen, TG; Lozano-Andrés, E; Rao, S; Robert, S; de Rond, L; Tang, VA; Tertel, T; Yan, X; Wauben, MWH; Nolan, JP; Jones, JC; Nieuwland, R; van der Pol, E. A compendium of single extracellular vesicle flow cytometry. *Journal of Extracellular Vesicles* **2023**, *12*: 12299. https://doi.org/10.1002/jev2.12299

[13]. Nieuwland, R.; Siljander, P. R.-M.; Falcón-Pérez, J. M.; Witwer, K. W. Reproducibility of Extracellular Vesicle Research. *European Journal of Cell Biology* **2022**, *101* (3), 151226. https://doi.org/10.5281/zenodo.7635515

This list is also available here: https://www.euramet.org/repository/research-publications-repository-link/

Project start date and duration:	1 June 2019, 42 months	
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. TTK, Hungary 10. UH, Finland	Unfunded Partners: 11. BD, Switzerland 12. PolyAn, Germany
RMG: –		