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

Overview

Extracellular vesicles (EVs) are cell-derived particles in body fluids, which have excellent potential as next-generation biomarkers for the early diagnosis of common diseases, such 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 cell-specific 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. These will then be evaluated in an inter-laboratory comparison study across a range ($\geq$ 20) of standard flow cytometers (FCMs) in clinical labs.

Need

European healthcare costs are estimated to increase by 5 %- 6 % 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 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, FCM is one of the most appropriate techniques for single EV analysis in clinical samples, given the ready presence of FCM in clinical laboratories and their ability to identify cell-specific EVs at high throughput. An FCM measures light scattering and fluorescence intensity of single EVs in a flow. However, due to technical variations between different FCM models, measurements of EV concentrations are currently incomparable between most clinical laboratories. 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 EV 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 EV reference materials will also require testing and validation using biological test samples in clinical laboratories.

Objectives

The overall objective 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 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 FCMs 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 and expected results

The state-of-the-art for EV is defined by the preceding EMRP project HLT02 METVES. In the preceding project HLT02 METVES, procedures were developed for the collection and handling of EVs from biological fluids. The size distribution of EVs was also measured using 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 FCM has clinical potential, because FCMs 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 FCMs. The results were ground-breaking: although two out of three FCMs 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 FCMs 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 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, this project 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 FCMs. 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.

Impact

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 FCM 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) FCMs 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, project partner BD is very interested in the commercialisation and dissemination of the outputs of this project to industrial and clinical end users. In addition, project 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.

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. Due to the direct connections between this project's partners and stakeholders from industry and the healthcare sector, it is highly likely that academia and industry will consult NMIs and DIs to use this project's metrological infrastructure.

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 FCM measurements on EVs, the International Society for Advancement on Cytometry (ISAC), the International Society for Extracellular Vesicles (ISEV), the International Society on Thrombosis and Haemostasis (ISTH), and the National Institute of Health (NIH) started the EV Flow Cytometry Working Group. This project's partners are prominent members of these societies and the working group, and because the objectives of METVES II are aligned to the long-term mission of the EV Flow Cytometry Working Group, and therefore their support for the dissemination, uptake and exploitation of this project's developed infrastructure is ensured. To emphasise this, ISTH, has offered their "*international network of researchers for appropriate collaborations as well as our communication channels to inform about the project and its results*."

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, the Scientific and Standardisation Committees (SSC) on Vascular Biology of the ISTH, the Blood EV Working Group of ISEV, ISO/TC 229 JWG 2 and ISO/TC24/SC 4.

Longer-term economic, social and environmental impacts

In the long-term, we expect that the infrastructure and reference materials developed in this project will have major impact on 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

Project start date and duration: 01 June 2019	3 years	
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Internal Funded Partners: 1. VSL, Netherlands 2. BAM, Germany 3. LGC, United Kingdom 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