Final Publishable JRP Summary for HLT02 MetVes

Metrological characterisation of microvesicles from body fluids as non-invasive diagnostic biomarkers

Overview

Diagnosis requires reliable information, which in part comes from analysis of body fluids. Body fluids contain cell fragments (microvesicles; MV) that differ in common diseases such as cancer and cardiovascular disease, and thus provide novel biomarkers. Unfortunately, prior to the MetVes project the results of MV measurements were incomparable between instruments and hospital laboratories. Therefore the MetVes project developed (i) a procedure that is now being used worldwide to isolate MV, (ii) reference materials to improve standardisation of MV measurements, (iii) discovered a method to measure a hitherto unknown but important physical property of MV required for standardisation, and (iv) improved the comparability of MV concentration measurements in 30 hospital laboratories worldwide; supported by the International Society on Thrombosis and Haemostasis.

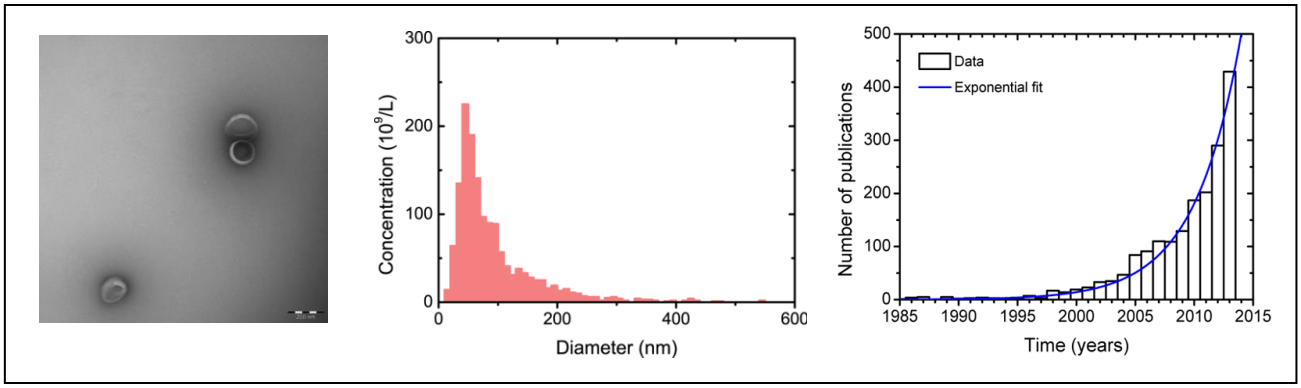

Figure 1. Left: Microvesicles (MV) in the urine of a normal healthy subject visualised by electron microscopy (scale bar: 200 nm). Centre: Estimated size distribution based on analysis of 5,000 MV by electron microscopy in human urine. Right: Number of publications on MV per year.

Need for the project

The costs of health care are increasing rapidly due to ageing of the population throughout Europe. One of ways of reducing health care costs is the early diagnosis of disease, which improves the efficacy of medical treatment. However, making a medical decision requires reliable information. Predominantly such information comes from the routine analysis of body fluids such as blood and urine, as these fluids contain cells and biomarkers that reflect health or disease. During the last decade, human body fluids were shown to contain not only cells and soluble biomarkers, but also numerous and extremely small cell-derived MV. MV are membrane-surrounded cell fragments, which can trigger blood clotting, thrombosis and inflammation, promote tumour growth and metastasis and may cause diseases such as preeclampsia, etc.

Although there is consensus that the concentration, cellular origin, composition and function of MV differ in most if not all diseases the measurement results for MV were incomparable between methods and laboratories, prior to the start of the MetVes project. For example, even recently (2010-2013) reported concentrations of MV in plasma of healthy humans differed 10^8-fold between methods and laboratories. Furthermore, most of the available methods used to detect MV lacked sensitivity, and there was a consensus that only “the tip of the MV iceberg” could be detected. Therefore, in order to exploit the full potential of MV as novel clinical biomarkers, improved understanding of MV measurements and the standardisation of measurement results were required.

EMRP
European Metrology Research Programme
Programme of EURAMET

The EMRP is jointly funded by the EMRP participating countries within EURAMET and the European Union

In hospital laboratories two methods were commonly used to measure single MV; both methods measure the amount of light scattered by a single particle or MV in suspension. However, the problem is that the amount of scattered light depends on the size (diameter) and composition of the particle or MV. For example, a plastic (polystyrene) particle scatters more light than a MV having the same diameter. This property is called the refractive index (RI), and in order to derive the diameter of a MV from the amount of scattered light, it is essential to know the RI. Unfortunately, a method to determine the RI of nanometre-sized particles did not exist prior to the MetVes project. But once the RI can be determined, the diameter of MV in suspension can be derived, which then allows estimation of the detection limit of applied methods and should improve the comparison of measurement results between instruments and institutes.

Methods measuring light scattering also lack specificity as they do not provide information on the type of particle that is scattering light. This lack of specificity caused problems especially when studying MV in plasma, because (human) plasma contains not only MV but also lipoprotein particles which overlap in size (diameter) with MV. Therefore, to solve this problem, information on the (bio)chemical composition or other properties, such as morphology were needed to distinguish MV from other biomaterials in body fluids.

Previous studies showed that MV in body fluids are dependent on pre-analytical variables, such as blood collection conditions. Therefore the comparison of MV measurements results also requires standardisation of pre-analytical variables. However such standard operating procedures for sample procedures for the collection, handling, and storage of body fluids for MV did not exist prior to the MetVes project. In addition, MV research commonly requires isolation/purification of MV for analysis and although laboratories apply centrifugation-based protocols, numerous erroneous artefacts have been published in MV analysis. Therefore, there was a need for an isolation and purification method for MV from body fluids.

Scientific and technical objectives

The scientific and technical objectives for the MetVes project were:

1. To develop methodologies for the dimensional characterisation (measuring size and size distribution) of MV.
2. To develop MV reference standards. Standards should be stable, provide repeatable measurements, and should have properties similar to MV (size, morphology, refractive index (RI)).
3. To develop reliable MV sample procedures, including controlled and standardised collection, handling, and storage of body fluids for MV and for the isolation and purification of MV from these body fluids.
4. To develop methodologies for measuring the concentration, morphology and (bio)chemical composition of MV, and to distinguish MV from other biomaterials in body fluids (lipoproteins, viruses).

Key results and conclusions

1. To develop methodologies for the dimensional characterisation of MV

The MetVes project developed a light scattering-based method that is capable of measuring the RI of small particles, including MV, thus making it possible to derive the true diameter of MV in suspension for the first time (*Nano Letters, 2014*). By measuring the RI of MV and the RI of the most widely used MV reference materials, it has become possible for end-users (such as biomedical research laboratories and companies developing flow cytometers), to correct for differences in scattered light between reference materials and MV, which means that they now can determine the real detection limit for MV in instruments.

Using this knowledge, the project studied the ability of flow cytometers to detect MV in 33 clinical laboratories worldwide (see Figure 2 for participating laboratories). The results of the study showed that 1 in 3 of the flow cytometers was unable to detect MV in the largest MV size range 1,200 to 3,000 nm. Because all the clinical laboratories included in the study have a track record for MV analysis by flow cytometry, the obtained results provide important information about the (lack of) sensitivity of commercially available flow cytometers, and should be taken into account by end-users when referring to previously published scientific work in which insensitive flow cytometers have been used. This work was supported by Exometry B.V, who provided the synthetic monodisperse polystyrene reference materials (as selected in objective 2), to the clinical

laboratories and the International Society on Thrombosis and Haemostasis (ISTH) who supported the shipping/transport of the samples.

Figure 2. Location of the clinical laboratories participating in the MetVes flow cytometer comparison study

2. *To develop MV reference standards. Standards should be stable, provide repeatable measurements, and should have properties similar to MV*

To improve the standardisation of MV measurements between instruments and laboratories, reliable and well characterised reference materials must be available. Based on the results of a questionnaire distributed by the project to 40 stakeholders and end-users, the MetVes project selected 14 commercially available synthetic reference materials made out of silica and polystyrene with a particle diameter between 30 nm to 315 nm. After measuring the size distribution of the particles in the reference materials by Atomic Force Microscopy (AFM), 5 synthetic monodisperse particle samples were selected for their high quality monodispersity (i.e. their particle uniformity). In addition, 2 synthetic bimodal reference particle standards were also chosen to mimick polydispersity as in most clinical samples MV have a polydisperse size distribution. The size distributions and stability of the 5 monodisperse and 2 bimodal synthetic reference materials were measured over 18 months by a variety of clinically used methods including Nanoparticle Tracking Analysis (NTA), Tunable Resistive Pulse Sensing (TRPS), Transmission Electron Microscopy (TEM) and AFM and the results of the methods compared. The results showed that (i) TRPS and NTA overestimate the mean size of silica particles (of 48nm) by up to 88% as these beads are below the detection limits for the methods and therefore the method often measures aggregates of the particles instead, and (ii) NTA is incapable of measuring the size distribution of a bimodal MV reference materials.

3. *To develop reliable MV sample procedures, including controlled and standardised collection, handling, and storage of body fluids for MV and for the isolation and purification of MV from these body fluids*

Prior to the start of the MetVes project, published recommendations for the standardisation of pre-analytical variables for blood collection, handling and storage for MV analysis (e.g. Thrombosis Research 2011; 127(4): 370-377) relied on flow cytometry for MV detection, and were therefore based on measuring only 1-2% of the MV present (the lack of sensitivity of flow cytometry is described in objective 1). In addition, no MV sample procedures existed for urine and saliva. Therefore the project developed standard operation procedures (SOPs) for the collection, handling and storage of blood, salvia and urine for MV analysis. To do this the project determined the effects of centrifugation, single freeze-thaw cycle, time between collection and handling, storage temperature and storage duration on blood, urine and saliva, using not only flow cytometry but also TRPS, NTA and TEM. One of the most important findings was the fact that centrifugation of samples, even at relatively low g forces, results in losses of MV sub populations. The SOPs are available for end users to download from the project website, and so far 226 unique end-users have downloaded the SOPs (Dec 2014 – Aug 2015 website statistics).

In terms of the isolation and purification of MV from body fluids; the MetVes project has pioneered the use of an old, but unused isolation method for extracellular vesicles, for use with MV isolation. The method, size exclusion chromatography (SEC) allows fast isolation of MV from complex body fluids such as plasma in a single step, and is sufficiently reliable to be used in medical clinics. Once MV have been isolated by SEC, ‘downstream’ MV analysis becomes possible e.g. by proteomics, lipidomics and mRNA analysis, which allows end-users to study the biochemical composition of MV in detail; this was hitherto impossible. The biochemical composition of MV is important as this can be used for the identification of unique proteins, lipids and mRNAs, which can be used as biomarkers for the diagnosis of disease. The SEC method developed by

the project has been commercialised by partner AMC in collaboration with iZon and within the space of a year the use of the method has increased to 170 laboratories worldwide (see Figure 3).

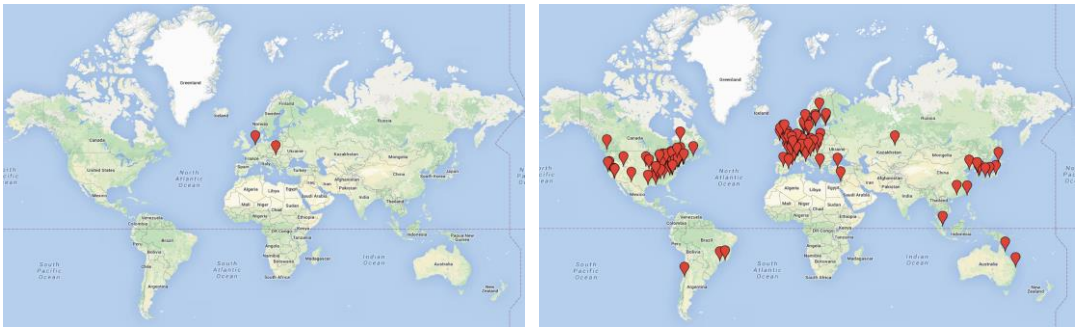

Figure 3. Left: Use of SEC in 2014. Right: Use of the MetVes introduced MV isolation procedure SEC in May 2015 (over 170 laboratories)

4. *To develop methodologies for measuring the concentration, morphology and (bio)chemical composition of MV, and to distinguish MV from other biomaterials in body fluids*

The results of the previous objectives 1, 2 and 3 have made it possible for end users, for the first time, to be able to compare MV detection results, from body fluids, between different instruments, methods and laboratories [5]. The project has shown that differences in MV size distribution and concentration measurements (of the same MV sample) are primarily due to differences in the minimum detectable MV size of different methods, i.e. 70–90 nm for NTA, 70–100 nm for TRPS, 150–190 nm for ultrasensitive flow cytometry, and 270–600 nm for conventional flow cytometry. This knowledge now enables end-users to compare MV concentration measurements from different methods, once the minimum detectable MV size is known.

For the (bio)chemical composition of MV, the project developed a method using AFM with “funcionalised tips” to study the cellular origin of single MV. The functionalised tips are coated with antibodies against a protein present on only a single type of MV. This method allows end-users to establish the cellular origin of MV by AFM, and can also be used to distinguish MV from other biomaterials in body fluids.

Actual and potential impact

Since the start of the MetVes project in 2011, it has had an important impact in the biomedical sciences community as it was the first project involved in the standardisation of MV measurement. The project has introduced the term “metrology” and corresponding terminology to a large and global (bio)medical audience and provided a sound metrology structure for MV measurement that will be further developed and explored within a newly initiated working group on the standardisation of vesicle measurements by flow cytometry. This new working group is a collaboration between the International Society for Extracellular Vesicles (ISEV), ISTH and the International Society for Advancement of Cytometry (ISAC) (www.evflowcytometry.org). The MetVes project coordinator (who is the Chair of the Scientific and Standardisation Committee on Vascular Biology of the ISTH) has been asked to participate in this international working group and so far has given 2 invited presentations to this working group.

The project has received ongoing interest from a large group of renowned (medical) institutes and universities such as Harvard Medical School and the Mayo Clinic in the US and Oxford University in the UK, as well as support from relevant international societies such as the ISAC, ISEV and ISTH. For example, the ISTH invited the MetVes project to present a State of the Art Lecture on “Innovation in detection of microparticles and exosomes” at the XXIV ISTH Congress meeting in Amsterdam in July 2013 and to contribute with an invited review in the ISTH’s associated state of the art book which was published as an additional volume of the Journal on Thrombosis and Haemostasis.

In addition to this, to ensure the uptake and dissemination of the MetVes project’s results to stakeholders and end-users a wide range of activities were undertaken. These activities included (i) the production of the MetVes website (www.metves.eu), (ii) publication of peer-reviewed papers in health journals (8 published, 4 in preparation), (iii) presentations (46) at international scientific and medical conferences, (iv) presentations

at seminars/workshops in the Europe and USA (8), (v) training of graduate and PhD students (total 19 students) of 7 different European countries, (vi) production of 4 instructional movies, (vii) two articles in national newspapers and 4 other press releases, and (viii) a METVES workshop attended by over 50 metrologists and health care professionals.

The MetVes project also developed (i) MV reference standards and SOPs for the collection, handling and storage of blood, urine and saliva for MV analysis, (ii) a method for the isolation of MV by SEC that is being used in 170 (mainly clinical) laboratories worldwide, and (iii) reliable procedures for the dimensional characterisation of MV and for measuring the concentration, morphology and (bio)chemical composition of MV.

The SOPs developed by the project, to standardise the pre-analytical phase and the development of a method for isolation and purification of MV from body fluids are being widely used by investigators in the MV field, including researchers in hospital laboratories, not only in Europe but also worldwide. The method to isolate MV from body fluids using SEC has also been widely accepted, and is being used by many research organisations and hospital laboratories, worldwide. For example, investigators of the National Institute of Health (Washington DC, USA) are using SEC to isolate MV for downstream analysis. Although SEC is not a novel method as such, the application of SEC in within the field of MV is novel, and therefore it was commercialised by the project, in order to facilitate standardisation.

Finally, the project developed a novel and label-free method for distinguishing MV from other biomaterials in body fluids using the RI of MV that offers clinical laboratories the opportunity to analyse body fluids containing MV more efficiently and economically.

All these results have enabled the comparison of MV measurement results between different instruments and different medical institutions, which means that for the very first time it will become possible to perform multi-centre MV trials. The results have also provided a sound basis for the standardisation of MV measurements and contributed to the further establishment of MV as biomarkers of disease, an important step towards earlier diagnosis of many common and rare diseases and thus more efficient medical treatment.

List of publications

[1]. Coumans FA, van der Pol E, Böing AN, Hajji N, Sturk G, van Leeuwen TG, Nieuwland R. Reproducible extracellular vesicle size and concentration determination with tunable resistive pulse sensing. J Extracell Vesicles 2014; 3: 25922 (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4263901/)

[2]. Böing AN, van der Pol E, Grootemaat AE, Coumans FA, Sturk A, Nieuwland R. Single-step isolation of extracellular vesicles by size-exclusion chromatography. J Extracell Vesicles 2014; 3 (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4159761/)

[3]. van der Pol E, Coumans FA, Sturk A, Nieuwland R, van Leeuwen TG. Refractive index determination of nanoparticles in suspension using nanoparticle tracking analysis. Nano Lett 2014; 14: 6195-201 (http://pubs.acs.org/doi/abs/10.1021/nl503371p)

[4]. Yuana Y, Levels J, Grootemaat A, Sturk A, Nieuwland R. Co-isolation of extracellular vesicles and high-density lipoproteins using density gradient ultracentrifugation. J Extracell Vesicles 2014; 3 (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4090368/)

[5]. van der Pol E, Coumans FA, Grootemaat AE, Gardiner C, Sargent IL, Harrison P, Sturk A, van Leeuwen TG, Nieuwland R. Particle size distribution of exosomes and microvesicles determined by transmission electron microscopy, flow cytometry, nanoparticle tracking analysis, and resistive pulse sensing. J Thromb Haemost 2014; 12: 1182-92 (http://onlinelibrary.wiley.com/doi/10.1111/jth.12602/abstract;jsessionid=3060E081BBB1F6D211458AD75C4D1D8E.f03t01)

[6]. Varga Z, Yuana Y, Grootemaat AE, van der Pol E, Gollwitzer C, Krumrey M, Nieuwland R. Towards traceable size determination of extracellular vesicles. J Extracell Vesicles 2014; 3 (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3916677/)

[7]. van der Pol E, Coumans F, Varga Z, Krumrey M, Nieuwland R. Innovation in detection of microparticles and exosomes. J Thromb Haemost 2013; 11 Suppl 1: 36-45 (http://onlinelibrary.wiley.com/doi/10.1111/jth.12254/abstract)

[8]. Yuana Y, Sturk A, Nieuwland R. Extracellular vesicles in physiological and pathological conditions. Blood Rev 2013; 27: 31-9 (http://www.bloodreviews.com/article/S0268-960X(12)00076-8/abstract)

JRP start date and duration:	1st June 2012, 36 months
JRP-Coordinator: Rienk Nieuwland, Dr, VSL, Tel: +31 20 566 4851 E-mail: rnieuwland@vsl.nl JRP website address: www.metves.eu	
JRP-Partners: JRP-Partner 1 VSL, Netherlands JRP-Partner 2 EJPD, Switzerland	JRP-Partner 3 PTB, Germany JRP-Partner 4 SMD, Belgium JRP-Partner 5 AMC, Netherlands
REG-Researcher (associated Home Organisation):	Yuana Yuana AMR, Netherlands
REG-Researcher (associated Home Organisation):	Zoltán VARGA RCNS HAS, Hungary
REG-Researcher (associated Home Organisation):	Edwin van der Pol AMC, Netherlands
RMG-Researcher (associated Home Organisation):	Sami Valkonen UH, Helsinki

The EMRP is jointly funded by the EMRP participating countries within EURAMET and the European Union