EV-HIT: Building an Extracellular Vesicle Index for Health

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STUDENTS AS CO-CREATORS

Background

The aim of the project was to develop an easy-to-use health index test based on extracellular vesicles (EVs) and key microRNA EV-markers derived from human plasma.

For this purpose, EVs were isolated from plasma from a range of 23 healthy volunteers. Profiles of EVs across this volunteer range were analysed using state of the art Nanoparticle Tracking Analysis (NTA) and NS300 'Malvern' Nanosight technologies to quantify EV numbers and assess modal EV size. These were correlated with age, BMI, fat mass, grip-strength and blood pressure. Additionally, 10 volunteers (5 males and 5 females respectively) were assessed for microRNA miR23b, a marker indicative for metabolism, associated with muscle hypertrophy and with functional roles in oncogenesis. This miR marker was shown to correlate with EV profile, demonstrating the potential for such EV profiling as indicative for health status.

A significant aspect of this research project involved the creation of a patient questionnaire as part of the study design, systematic recruitment of volunteers and review of the background literature in order to best select appropriate microRNAs based upon metabolic function. The subsequent part involved assessing and taking samples from the volunteers, with measurement of non-invasive parameters associated with their health and the isolation and analysis of EVs from their bloods.

Figure 1. The poster design used as the advertisement in the recruitment process of volunteers for the EV-HIT study.

Introduction

The discovery of extracellular vesicles (EVs) dates back to 1940, when Chargaff and West identified in the plasma the presence of subcellular factors capable of promoting blood coagulation. Initially, these vesicles were considered as inert membrane debris, devoid of biological significance, also termed cellular "dust".
The research on EVs has grown exponentially in the last decade with increasing recognition of EVs being key factors in cellular communication in physiological processes, as they carry a range of protein and genetic material, including microRNAs, between cells. Therefore, roles for EVs in a range of diseases, including cancer and chronic diseases, have also been studied widely. It has also been found that EVs can be reliable biomarkers when isolated from a range of body fluids, including plasma.

EVs are furthermore being engineered as drug-delivery vehicles and as therapeutic factors in regenerative medicine. In cell communication, EVs perform different functions that are connected to the cell type from which they derive.
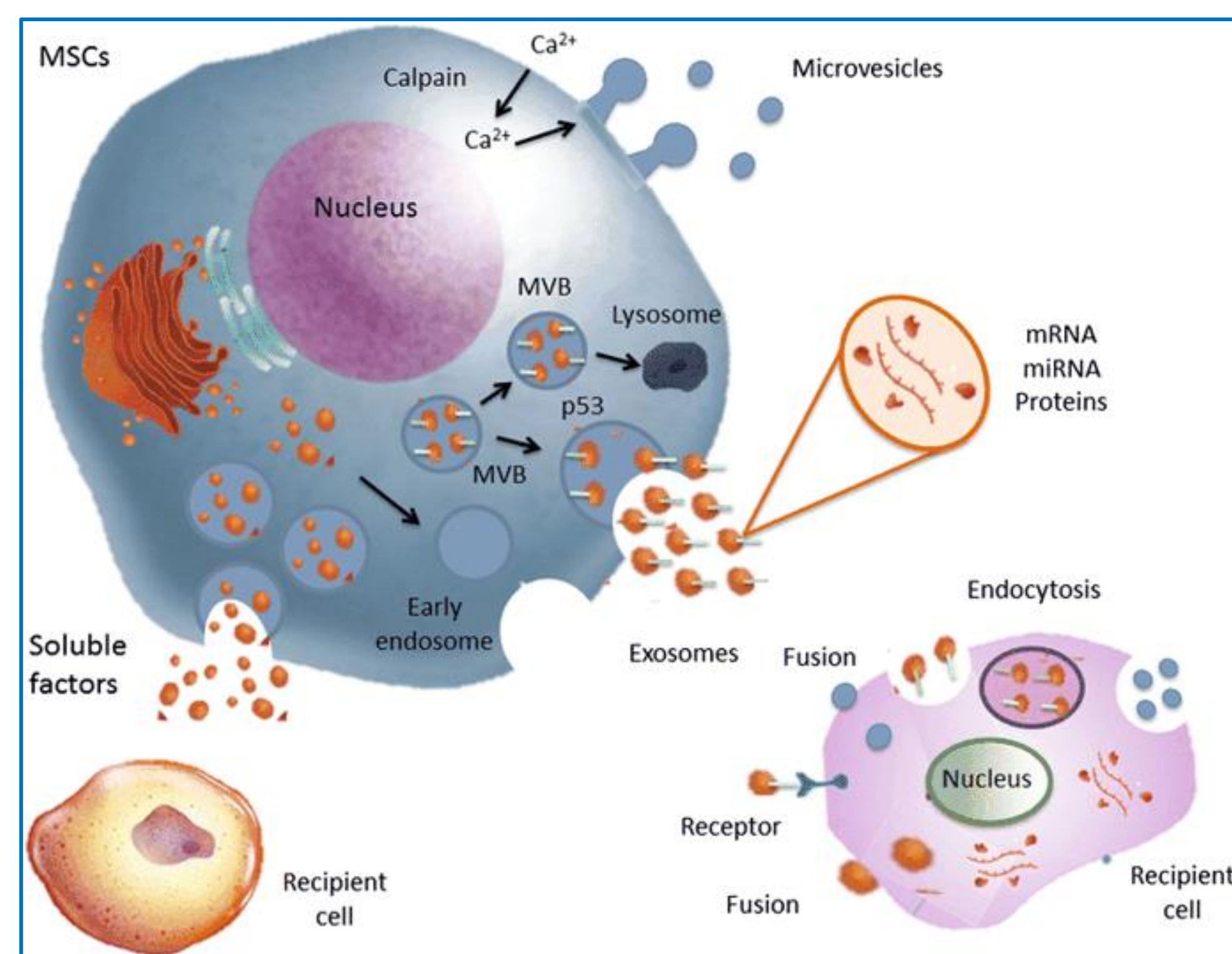

Figure 2. Cartoon representation of extracellular release from one cell in communication to a target recipient cell. Credit: BioMed Central ® (2019).
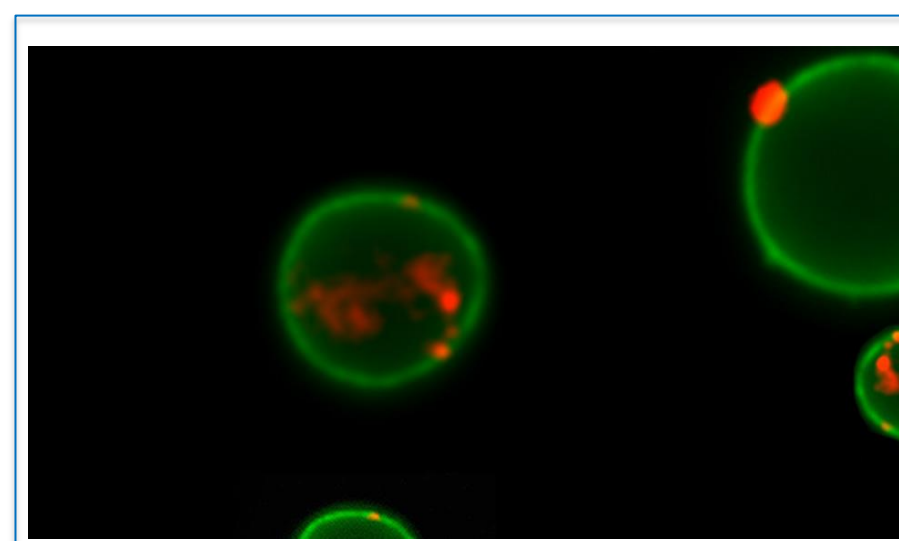

Figure 3: Flow cytometry imaging of EVs (red) harvested from cell-culture supernatants. Fluorescent dyes and antibodies are employed to visualise the cells and their EVs, which are represented by red. Credit: Görgens.A, Science (2016)

Results and Discussion

The samples were collected from healthy individuals which is ideal in order to reassure that the results will not be affected by any other factors such as any known diseases. The age range is also wide enough to cover the majority of the population. Our study revealed some significant correlation between EV profiles and age, fat mass and blood pressure.

Distinct individual differences were noted between EV profiles, with EVs ranging from sizes of 50- 300 nm. Some individual's EV profiles demonstrated wider distribution of sizes up to 600nm, and in others a monodispersed, narrower range focused around a 100 nm sized peak.
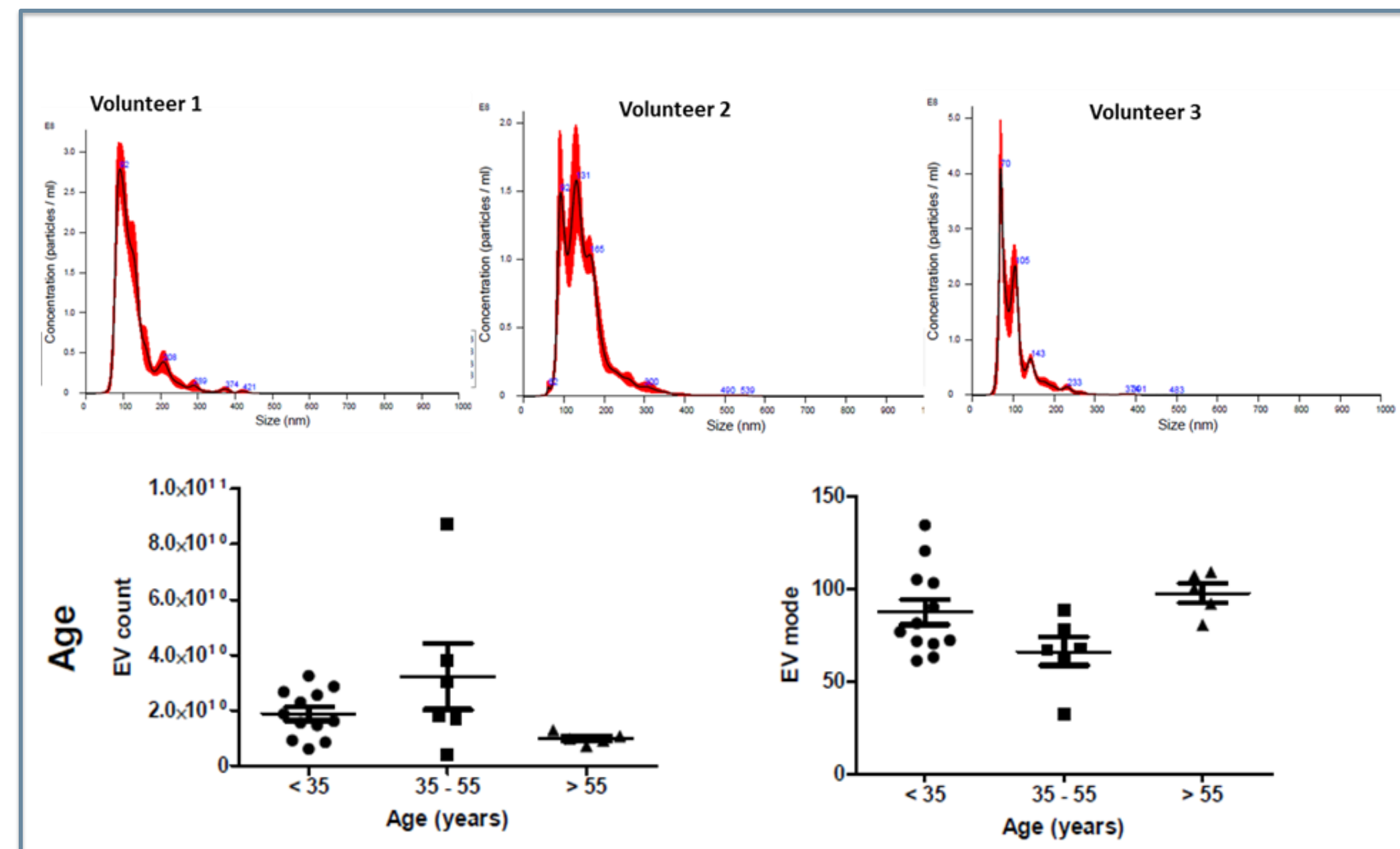

Figure 4. EV profiles from NTA analysis are shown for a few volunteers, highlighting differences in individual profiles. The correlation between age with EV number and modal EV size is shown.

EV numbers were seen to peak between the ages of 35 and 55, and drop after age of 55. EV modal size was higher below the age of 35 and above 55. It may be possible that EVs are responsible for helping cells to maintain a healthy state and for cell repair, both of which are pivotal in ageing. Our findings indicate that as cell age, their ability to produce EV lowers.

Gender did not seem to affect either EV numbers or EV size distribution significantly in plasma of this volunteer cohort (Fig. 5).
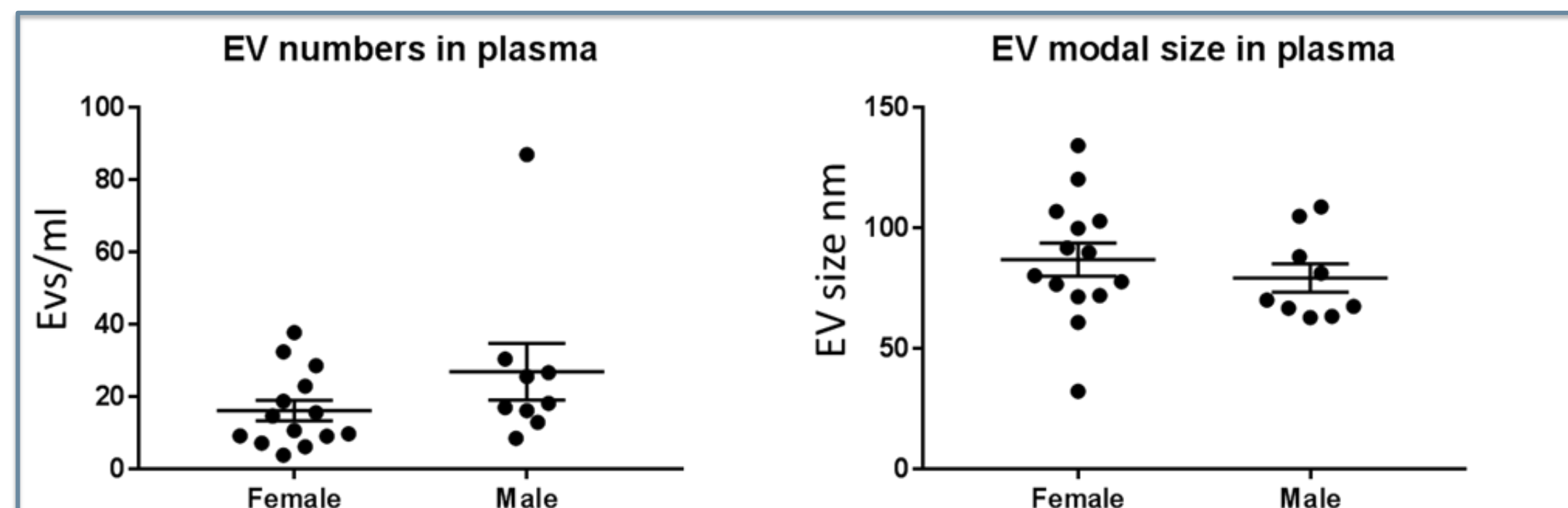

Figure 5. EV numbers and modal size in plasma compared between genders. EV count and modal size of EVs released was assessed by NTA analysis from plasma of 23 volunteers

No significant correlation was observed in this sample cohort between BMI, and EV number and size respectively. Likewise, no significant relationship was found between fat free mass (%) and modal size. Taking outliers into account, this could be partly due to the fact that profiles of biofluid-derived EVs may be affected by general lifestyle such as dietary habits and other factors which were not taken into consideration during the recruitment process. Some positive relationship was though noted between fat free mass and EV count as well as a correlation to diastolic blood pressure. (Fig. 6).
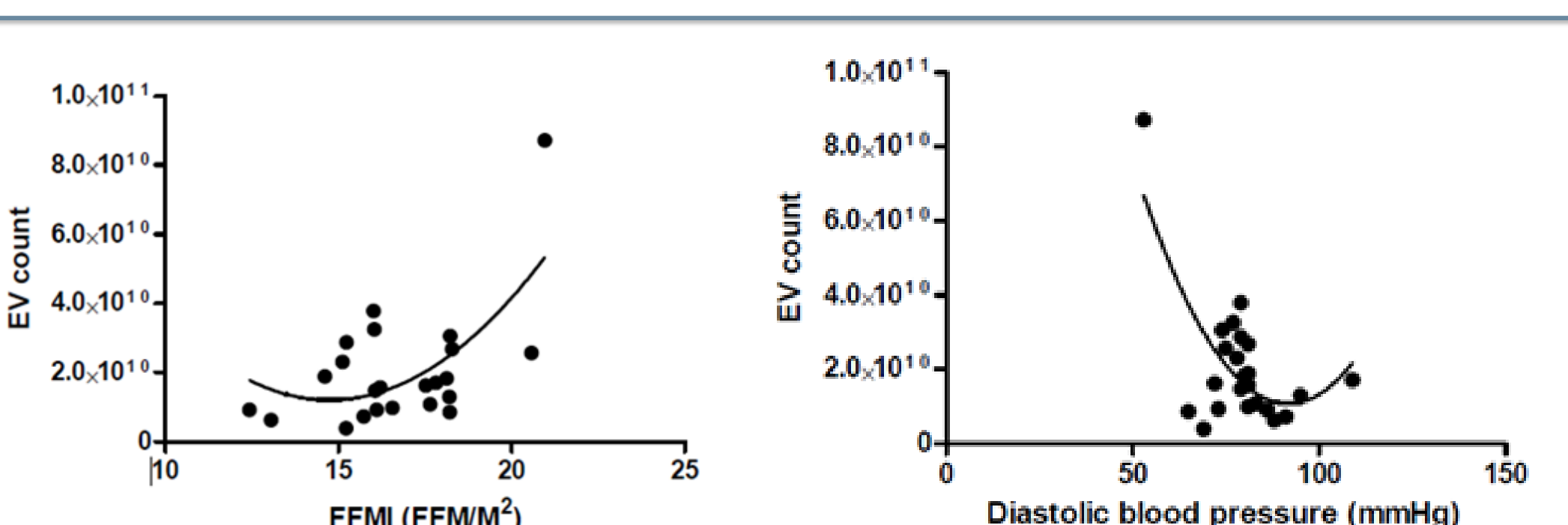

Figure 6. EV numbers in plasma correlated with free fat mass and diastolic blood pressure in the 23 volunteers tested.

MicroRNA-23 profiling revealed some gender-differences in EVs, although not statistically significant in this small cohort of 5 individuals per gender. Correlation with age was inconclusive in this small sub-cohort (Fig. 7 and Fig. 8).
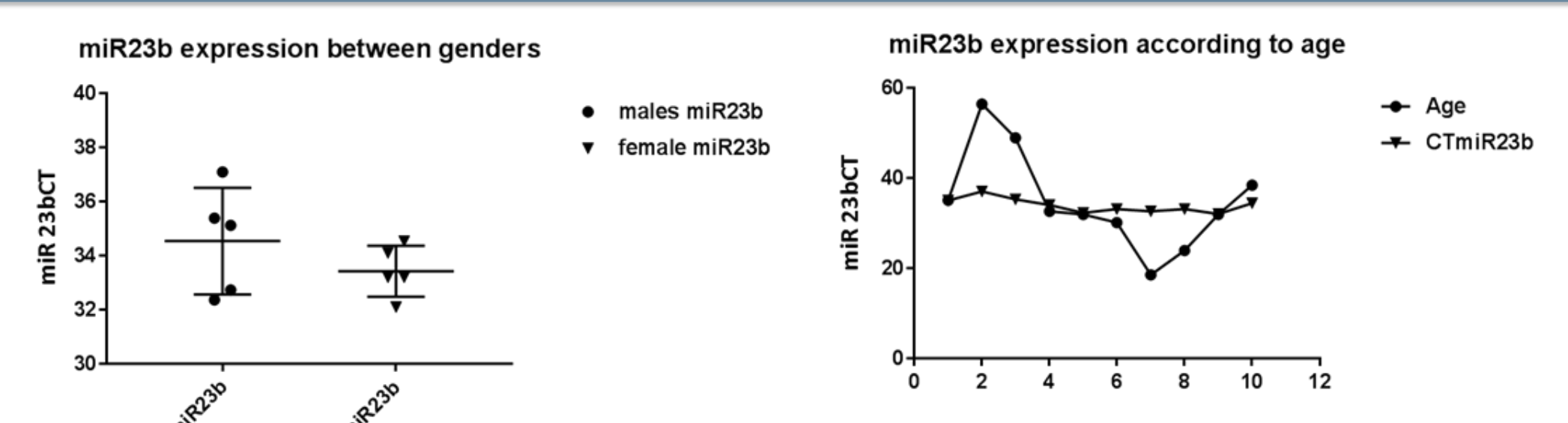

Figure 7. microRNA-23 expression in plasma-EVs of 5 female and 5 male volunteers.

Some, but not significant correlation was found between micro-RNA target marker miR23b expression and EV modal size and number, with age and BMI (Fig. 8).
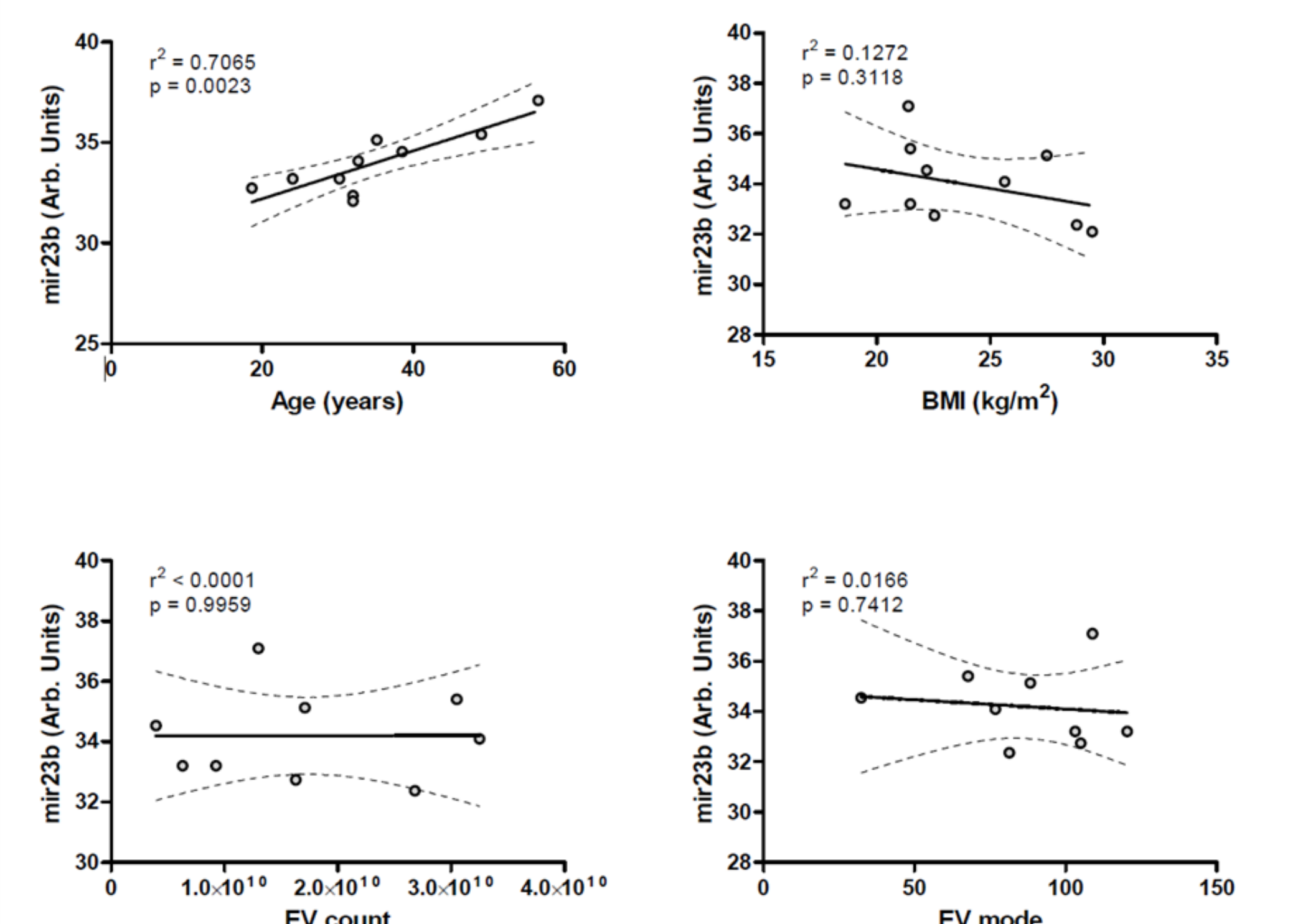

Figure 8. microRNA-23 expression in plasma-EVs of 5 female and 5 male volunteers in correlation with Age, BMI, count and EV mode.

AIMS

To develop an easy to use new health index test based on extracellular vesicle (EV) profile and key microRNA EV-markers from human plasma.

SUB-AIMS

- To isolate EVs from plasma from a range of volunteers and assess changes in numbers and size distribution profiles of EVs, relating them to easy to measure health markers (i.e. grip strength, BMI, age and blood pressure)

- To select a microRNA panel for key metabolic microRNAs that can be measured in the EV isolates and model a standard optimal health status EV-test from plasma, based on EV enumeration and EV microRNA biomarker profile.
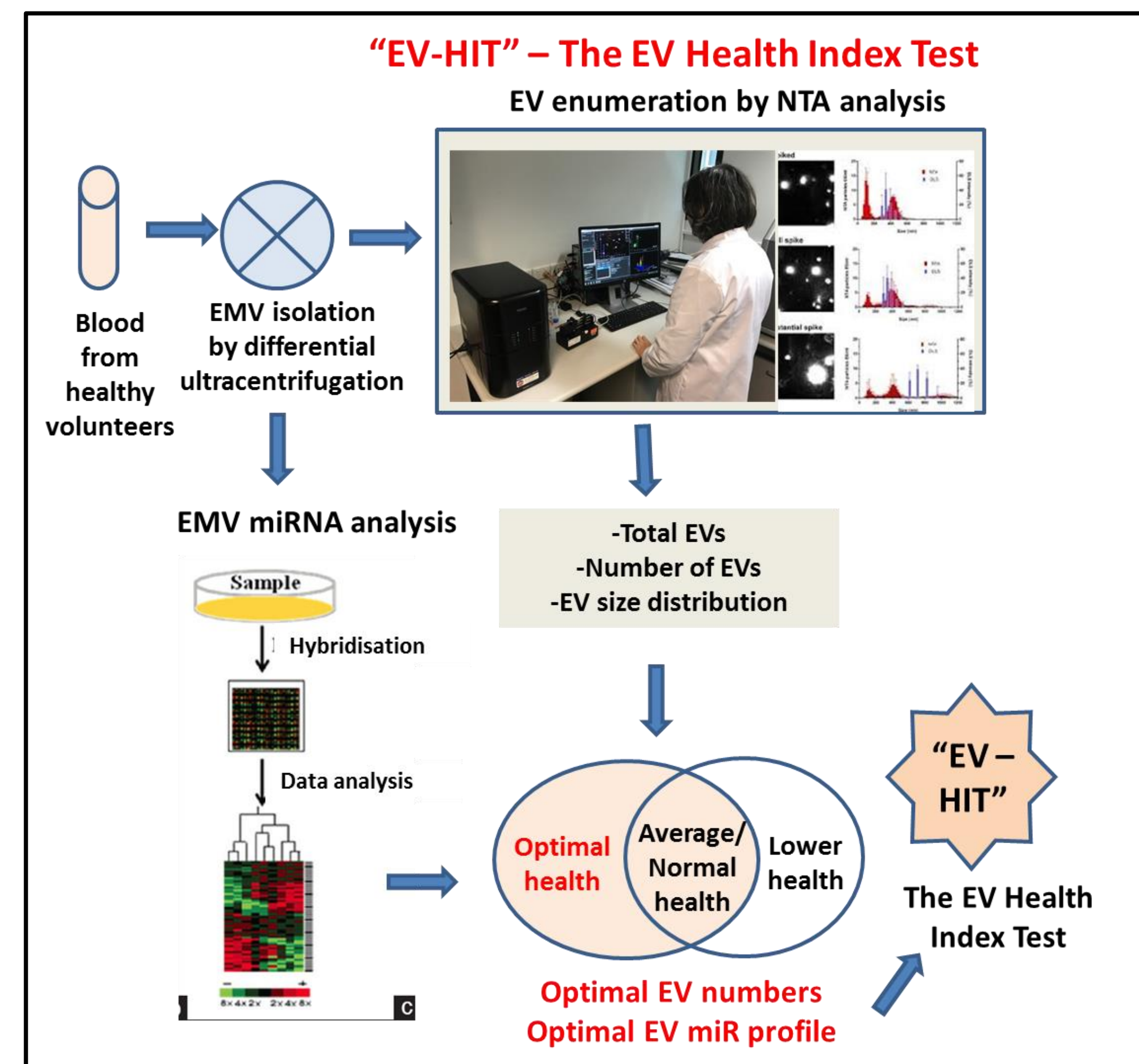

Figure 9. Flow-chart showing the process in the laboratory of extracting and analysing EVs for size profiling and microRNA cargo analysis.

Methods

Study design. The EV-HIT Student Team designed the recruitment poster and recruitment was advertised at UoW as well as in a range of gyms. EVs of "healthy" volunteers, from a range of volunteers with an age baseline of 18, were isolated from plasma to assess EV profiles based on EV enumeration and EV microRNA biomarker profile. An extensive literature search for key inflammatory and metabolic markers lead to the conclusion to focus on miR23b for assessing this marker in a selection of volunteers. All graphs were created in either GraphPad Prims (version 7) or Excel. Statistical significance was at $p < 0.05$ following ANOVA.

Subjects. 23 "healthy" volunteers, with an age range of 18-65, of mixed genders, with a BMI 18.6-29.7. Blood was collected from a vein in the arm into EDTA coated cuvettes.

1. EV isolation. Plasma was isolated from bloods and EVs isolated by step-wise ultracentrifugation according to established protocols and the recommendations of MISEV2018. The protocol was adjusted specifically in this pilot study for small scale samples of 100 µl plasma samples.

2. EV enumeration. Nanoparticle tracking analysis, based on Brownian motion of particles in suspension, was used to assess numbers and size distribution of EVs. The equipment used for this type of EV analysis is the NanoSight NS300 system, which is gold-standard for EV size profile analysis.

3. EV miRNA isolation and analysis. RNA was extracted from EV preparations from 5 male and 5 female volunteers, out of the total of 23 volunteers, ranging in age and BMI. The expression of miR23b, a key metabolic marker indicative of health, was assessed in these samples. The $2\Delta\Delta CT$ method was used for calculating relative miR expression levels.

Conclusion

Overall our findings show that EV profiles can be linked to some non-invasive health parameters and may therefore be developed as a novel tool to assess health status. Furthermore, refinement of the test, including more key microRNAs for metabolic health and inflammation status can be incorporated into such a health index test. The whole EV test then needs to be refined and validated in a much larger cohort of volunteers.

Our findings from this pilot study indeed do support the initial idea of development of a health index test based on EV profiling and associated EV cargo.

Recommendations for future research

For refinement of the test developed in this study, the cohort of volunteers will need to be increased and the current index developed here can then serve as a basis. Further data can be added for refined modelling of this new EV-HIT health index test.

In particular, in order to develop the project further, and to have a better understanding of the results and to reduce bias in terms of gender, early recruitment would be essential so that the likelihood of recruiting more participants is higher. Two recruitment processes could be adopted; first is to recruit a set number of males and the second would be to strictly recruit females.

Furthermore, the EV-HIT health index test can be refined by including more key microRNAs for metabolic health and inflammation status, as due to cost-effectiveness for this pilot project the focus was on one key metabolic miRNA only. In addition, participants with a wider range of ages could be recruited.

By targeting additional EV related microRNAs, it will be possible to create a panel to a range of health parameters including muscle health, athletic ability, cardiac function and frailty, with the potential for application in the optimisation of health assessment and prevention of disease.