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ORIGINAL ARTICLE

Preanalytical, analytical, and biological variation of blood plasma submicron particle levels measured with nanoparticle tracking analysis and tunable resistive pulse sensing

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ABSTRACT

Background: Nanoparticle tracking analysis (NTA) and tunable resistive pulse sensing (TRPS) enable measurement of extracellular vesicles (EVs) in blood plasma but also measure other particles present in plasma. Complete isolation of EVs from similarly sized particles with full EV recovery is currently not possible due to limitations in existing isolation techniques.
Aim: This study aimed to evaluate preanalytical, analytical, and biological variation of particle measurements with NTA and TRPS on blood plasma.
Methods: Blood from 20 healthy subjects was sampled in the fasting and postprandial state. Platelet free plasma (PFP) was analyzed immediately and after a freeze-thaw cycle. Additionally, the effect of prandial state and a freeze-thaw cycle on EV-enriched particle fractions obtained via size-exclusion chromatography (SEC) was examined.
Results: We observed analytical linearity in the range of $1.0–10.0 \times 10^8$ particles/mL for NTA and $1.0 \times 10^8–1.8 \times 10^9$ particles/mL for TRPS. The analytical variation was generally below 10%. A considerable intra- and inter-individual variation was demonstrated with estimated reference intervals of $1.4 \times 10^{11}–1.2 \times 10^{12}$ particles/mL for NTA and $1.8 \times 10^8–1.6 \times 10^9$ particles/mL for TRPS. Food intake and to a lesser extent a freeze-thaw cycle affected particle populations in PFP and, similarly, in EV-enriched fractions.
Conclusion: In this study NTA and TRPS enabled acceptably precise concentration and size measurement of submicron particles in PFP. An appreciable intra- and inter-individual biological variation was observed. In studies on particle populations in PFP or EV-enriched fractions, we recommend analysis of fresh, fasting samples.

Introduction

The amount of knowledge on extracellular vesicles (EVs), i.e. cell derived vesicles with a lipid bilayer membrane, has increased considerably in recent years, resulting in potential new diagnostic, prognostic, and therapeutic applications [1,2]. EVs can be divided into exosomes and microvesicles (MVs) based on size and release mechanisms [3], but several aspects of their common and diverse features are still unknown [4]. Preanalytical and analytical standardization in EV measurement is lacking [5] and at this point no single method is able to provide comprehensive information on both the total concentration, size distribution, and phenotypical features of the EVs in a plasma sample [6]. A widely applied method for EV detection and phenotyping is flow cytometry (FC) [7,8]. Despite recent sensitivity improvements [9,10], FC lacks the ability to detect the smallest EVs that are abundantly present in human plasma [11] and may possess pivotal physiological and pathological functions.

In recent years, methods have been developed for single particle detection, allowing for the measurement of particles smaller than those detectable by conventional FC. Nanoparticle tracking analysis (NTA) and tunable resistive pulse sensing (TRPS) represent two such methods with the ability to deliver data on particle number and size distribution in body fluid samples [12,13]. NTA, a light scatter based method, visualizes particles by focusing the laser light scattered by the particles in a solution using a microscope. The Brownian movements of the particles in the field of view are recorded with a camera for determination of size distribution besides particle concentration [11]. The particle size range that can be detected by NTA is approximately 30–1000 nm, depending on the refractive index of the analyzed particles [14]. TRPS is based on the Coulter principle in which transient reductions of the electric current flow in an electric field in a conductive liquid are measured [15]. In TRPS the rate and magnitude of the changes of current flow through a stretchable nanopore caused by particles present in the sample (that represents the conductive liquid) passing through the pore are measured. These measurements provide information on particle concentration and size distribution [16]. The particle size range that can be measured by TRPS depends on the specific type of nanopore and the stretch that is applied to it. In this study, we applied an NP200 nanopore which is optimized for the detection of particles with a size between 100 and 400 nm [13].

In blood plasma, particles within the size range measured by NTA and TRPS are of diverse nature. At their current stage of development, NTA and TRPS do not enable particle phenotyping and thus do not facilitate distinction between EVs and other similarly sized particles, which include protein aggregates, cell debris components, and substantial numbers of phospholid monolayer-bounded lipoproteins [11,17,18]. Therefore, we use the word 'particles' for the measured analytes. At present, no isolation method can fully isolate EVs from other particles, and the most widely applied isolation method, differential centrifugation, has some notable limitations as it may damage EVs, the pellet from a high-speed spin contains high density lipoproteins (HDLs), and the centrifugation may trigger the formation of extravesicular protein aggregates [5,19,20]. Moreover, it may not be possible to completely sediment EVs in plasma by ultracentrifugation [21]. Another approach to EV isolation, size-exclusion chromatography (SEC), allows for extraction of particle fractions that are enriched in EVs compared to HDLs and proteins [22] but expectedly contain other types of lipoproteins, such as very low density lipoproteins (VLDLs) and chylomicrons [20]. Analyzing EV-enriched fractions, extracted by SEC as suggested by Böing et al. [20], we have previously found that while these fractions were almost completely purified of low density lipoproteins (LDLs) as well as HDLs, they did indeed contain some triglyceride (TG) [23], which is the main component of VLDLs and chylomicrons. Furthermore, the fractions with the highest degree of EV enrichment have been depleted of the smaller EVs, which are expected to represent a considerable fraction of the total EV population [20]. Until improved methods for EV isolation and purification have been developed, researchers applying NTA and TRPS for studies on EVs in plasma have to endure EV isolation methods that allow for a certain degree of 'simplification of the complexity of vesicle preparations' [24] at the expense of the integrity of the original EV population. In this context, we focused on mapping the overall particle content in the physiological medium of EVs in the blood, which is plasma [5], in order to establish a basis for the interpretation of measurements of uncharacterized particles in less complex but more radically manipulated media in which EVs have to a greater or lesser extent been isolated and purified.

The aim of this study was to evaluate the analytical performance of NTA and TRPS measuring the general population of particles in platelet free plasma (PFP), the impact of applying a preanalytical freeze-thaw cycle, and the inter- and intra-individual biological variation, including the impact of prandial state, on the particle population. Since lipoproteins represent some of the particles in the same size range as EVs in plasma [11], the impact of prandial state may be important. In order to compare these results on raw PFP with results on EV-enriched fractions of PFP, the effects of food intake and a freeze-thaw cycle on particle concentration and size distribution were also measured in such fractions obtained by SEC.

Materials and methods

Preanalytical and biological variation

Study population

Inclusion of participants was approved by The North Denmark Region Committee on Health Research Ethics. 22 adult persons, 13 females and nine males, were recruited. Inclusion criteria were age above 18 years and the persons' own statement of absence of acute or chronic disease at the time of enrollment. The participants completed a medical questionnaire on age, height, weight, smoking and health status on the day of blood sampling and had blood drawn for additional blood tests to screen for ongoing disease. Exclusion criteria were indication of an ongoing medical condition in the questionnaire or signs of disease in the additional blood samples. Two participants, both males, were excluded because of illness as one had a seasonal influenza and the other an ongoing hepatic disease. The remaining 20 healthy individuals were enrolled in the study.

Specimen collection

Fasting samples were drawn at 08.15 h after an overnight fast. At 08.30 h the participants consumed a non-standardized breakfast with a selection of breadrolls, butter, cheese, jam, marmalade, cake, coffee, tea, milk, and sugar to simulate an everyday life postprandial state. Postprandial samples were drawn at 09.45 h. All blood samples were collected after application of a light tourniquet from the median cubital vein through a Vacuette Safety Blood Collection Set (Greiner Bio-One, Kremsmünster, Austria) with a 21 gauge needle and a 19 cm tube into a 9 mL Vacuette 3.2% sodium citrate plastic tube (Greiner Bio-One) after discarding the initial 3.5 mL of blood. The test tubes were gently inverted 10 times and then placed vertically in a rack until centrifugation. Blood for the additional blood tests, which included glucose, hematology tests, liver and kidney function tests, and lipid levels was drawn into standard tubes and analyzed at the Department of Clinical Biochemistry, Aalborg University Hospital, Denmark.

Sample handling

All samples for NTA and TRPS measurements were centrifuged twice at 2500 g for 15 min at room temperature in order to obtain PFP as recommended by Lacroix et al. [25]. The first centrifugation was initiated within 30 min after the sample was drawn. Samples were divided into separate aliquots in 2 mL capped microtubes (Sarstedt, Nümbrecht, Germany). One aliquot was kept at room temperature until particle analysis by NTA and TRPS within the day of the blood sampling. The impact of prandial state on the particle concentration and size distribution was investigated by comparison of the results from the fasting and postprandial samples. An aliquot from the fasting and postprandial sample from each participant was stored at −80 °C for 2–5 weeks and then thawed in a water bath at 37 °C and analyzed at room temperature. For homogenization, samples were gently inverted several times by hand immediately prior to each NTA and TRPS measurement. To study stability of refrigerated PFP, an aliquot of fresh fasting PFP was kept at 4 °C and analyzed with NTA and TRPS after equilibration to room temperature for 10 subsequent days.

Eight of the participants in the reference interval study, five females and three males, were included in the intra-individual variation study. From these persons a second fasting

blood sample was drawn between 1 and 15 weeks after the first fasting sample at 08.15 h and the fresh sample was analyzed.

Additional blood tests including lipoprotein measurements were performed with a Cobas 8000 Modular Analyzer (Roche Applied Science, Penzberg, Germany) using dedicated reagents from Roche and an Advia 2120 Hematology System (Bayer Healthcare, Leverkusen, Germany). LDL cholesterol concentration was estimated using the Friedewald equation [26].

Analytical linearity and variation

For the linearity study NTA and TRPS measurements were performed on an independent dilution series of a PFP sample from a person also included in the inter-individual variation study using Dulbecco's Phosphate Buffered Saline (DPBS) (Lonza, Basel, Switzerland) that had been filtered using a sterile Q-Max 0.2 μm syringe filter (Frisenette, Knebel, Denmark). Analytical linearity was evaluated by plotting the measured concentration against the expected concentration (see Figure 1) for each dilution.

Investigation of between-day variation was performed on separate aliquots of the same PFP sample. The aliquots were kept at −80 °C and then thawed and analyzed with NTA and TRPS on 7 and 10 different days, respectively. Due to a relatively high between-day variation of TRPS when analyzing the PFP aliquots, we performed additional experiments to closely examine the analytical variation of TRPS. This was achieved by conducting experiments on a microbead solution prepared by diluting a qNano SKP200B polystyrene microsphere concentrate (Izon, Oxford, UK) in filtered DPBS and analyzing it on 8 different days as well as a study on a

fasting PFP sample which was thawed and then kept at room temperature and analyzed with TRPS 11 times during the same day with half-hourly intervals. In the following sections we refer to these three different types of variability studies as the Between day$_{PFP}$, Between day$_{beads}$, and Within day$_{PFP}$ study, respectively. Furthermore, within run variation (Within run$_{PFP}$) was calculated from duplicate measurements of all dilutions within the linear range in the analytical linearity study.

A minimum of 10 μL of plasma or stock solution of artificial microspheres was transferred using calibrated pipettes throughout the study.

EV-enriched plasma fractions by SEC

PFP from four healthy subjects (two females and two males), collected as described for the preanalytical and biological variation study, was used for particle isolation by SEC using a 10 mL qEV column (Izon). The qEV column was washed with 10 mL DPBS (pH 7.4, 0.22 μm filtered). 0.5 mL PFP was loaded followed by elution with DPBS, and 0.5 mL fractions were collected. Separation by qEV was performed in duplicates at room temperature on the same column (with a washing in-between). Based on results from Böing et al. [20] and experience in the laboratory [23,27], fraction 9 and 10 were expected to be EV-enriched, i.e. the HDL and protein content was expected to be markedly more reduced than particle concentration. Fraction 9 and 10 as well as the corresponding PFP were analyzed by NTA using the same settings as for the other samples. In order to reduce potential inter-column variation the same column was used for all samples from each subject (fasting, postprandial, fresh,
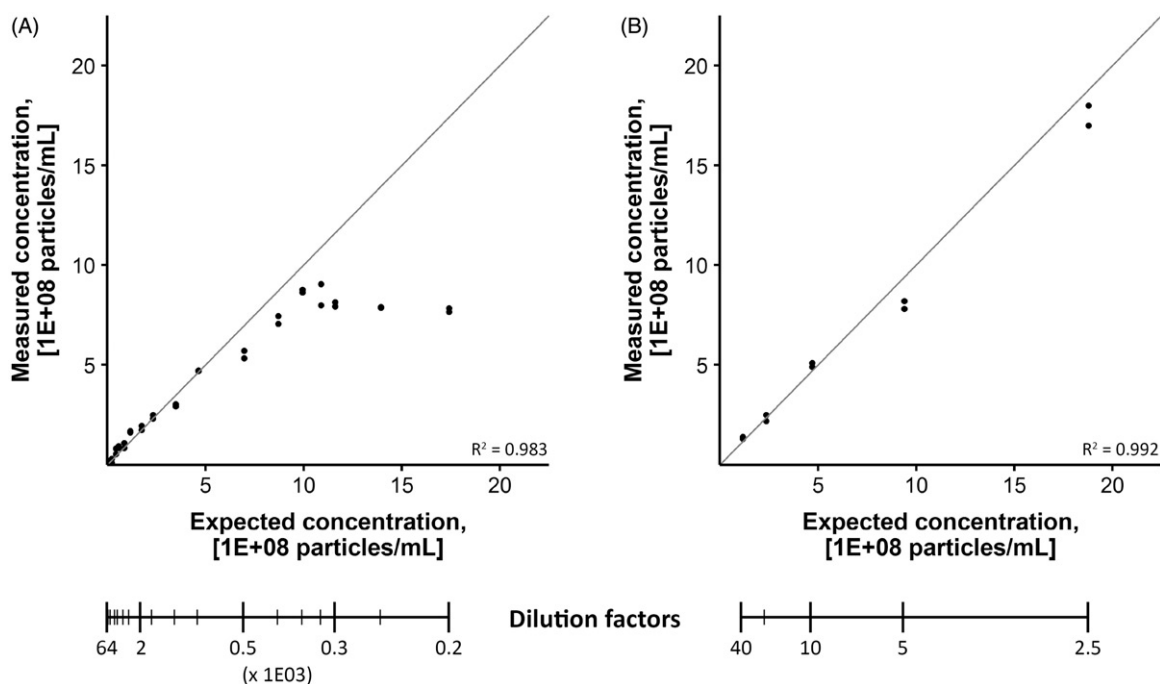
Figure 1. Analytical linearity of NTA (A) and TRPS (B). Linear regression performed on measured concentrations plotted against expected concentrations for NTA (A, $R^2 = 0.983$) and TRPS (B, $R^2 = 0.992$). Line of equality depicted. Expected concentrations were calculated as the mean of all raw concentrations of measurements within the ranges considered linear by visual assessment, divided by the dilution factor. The left and right dilution factor scales indicate the dilution factor used for each measurement represented in (A) and (B), respectively.

frozen). To verify reproducibility of SEC, protein absorbance (optical density at 280 nm) was measured in fraction 9 and 10 as well as in the subsequent fractions for comparison. Between the separations using the same column it was washed with DPBS until protein absorbance was zero in the eluate and kept in refrigerator between the runs.

Instruments and analytical settings

NTA

NTA was performed using a NanoSight LM10-HS (Malvern Instruments Ltd, Malvern, UK) with a 405 nm laser and a Luca DL-658M-OEM EMCCD camera (Andor Technology, Belfast, UK) and NanoSight NTA 2.3 software.

For all plasma samples, the camera shutter was set to 500, camera gain to 300, and detection threshold to 3. Dilution factors for the samples ranged between 500 and 2000 to ensure that all NTA measurements were performed within the linear range. Therefore, the sample viscosity is linked to the corresponding viscosity for water at the given temperature. Temperature was measured with the temperature probe and the value was entered manually in the software before each measurement. For minimum expected particle size, blur, and minimum track length, the automatic settings were selected.

Samples were loaded manually with a syringe into the sample chamber and the microscopic field of view was positioned as close as possible to the so-called 'thumbprint', which is a visual reference point in the instrument [11], in the area where the particles were most clearly visualized. To obtain duplicate measurements, two videos of 60 sec each were recorded for each sample immediately following two separate injections of the diluted plasma into the chamber. For each recording the background signal, which refers to pixels in the recorded video that are generated by background contamination [11], was subtracted before processing. In case of the software indicating 'high noise' or 'too high particle concentration', samples were further diluted.

To monitor any drift in the daily results, a control measurement was performed on a diluted 100 nm polystyrene microsphere standard (Malvern Instruments Ltd) with identical instrumental settings.

TRPS

All samples were analyzed using a qNano (Izon) with an NP200 nanopore (Izon) at a constant 47 mm stretch and with a constant pressure of 7 cm H_2O applied on the system. Measurements were performed at a baseline current between 70 and 150 nA and were continued for at least 180 sec or until at least 500 particles were counted. The system was calibrated with a qNano CPC200B microsphere concentrate (mean diameter 212 nm, mode diameter 203 nm) which had been diluted 1000-fold in filtered DPBS.

The impact of minimum detected particle size on concentration measurements has been emphasized in recent publications [16,28]. Using the NP200 nanopore with a fixed 47 mm stretch, TRPS has a lower size limit of particle detection at about 150 nm and the predominant number of particles detected by TRPS in PFP samples are within a size range of 150–400 nm. In the preliminary phase of the study, we also used a smaller nanopore (NP100), designed for detection of smaller particles than those detectable with the NP200. However, applying this nanopore on plasma samples resulted in almost immediate pore clogging, i.e. obstruction of movement of ions and other particles through the nanopore caused by substances being trapped in the pore, thus partially or totally blocking passage through it, practically rendering analysis with the NP100 on PFP samples unfeasible.

Immediately prior to each PFP sample measurement, the qNano CPC200B microsphere solution was run as a control sample to check if the particle flow rate was comparable to the particle flow rate observed in the calibration run. In case of a noticeable shift in the particle flow rate, the pore was processed by applying additional washing steps, tapping the shielding cap, temporarily changing the pore stretch, or increasing the applied pressure using the variable pressure module or the plunger. If a particle flow rate corresponding to the starting point could not be re-established by implementing these steps, recalibration was performed before PFP sample measurement.

Statistics

All PFP samples and bead dilutions were analyzed in duplicates and the mean value given except in the analytical linearity study in which the two distinct values from the duplicate measurements were presented.

The 95% reference intervals for particle concentrations were established using parametric analysis of log-transformed and untransformed data for particle concentration and particle size data, respectively. For normality testing, the Anderson-Darling test was applied.

Results on analytical variation were reported as SD (standard deviation) and CV (coefficient of variation).

In the intra-individual variation study and the Within run$_{PFP}$ study, a pooled CV was calculated as the square root of the mean of the squared CVs.

The effect of prandial state and a freeze-thaw cycle on particle numbers and mean size, and the effect of prandial state on lipid levels were evaluated with Wilcoxon matched pairs signed rank test.

To examine the degree of correlation between plasma particle levels and plasma lipid levels as indicators for lipoprotein content in the samples, the measured particle concentrations in the PFP samples from the inter-individual variation study were plotted against the corresponding concentrations of total cholesterol, LDL cholesterol, HDL cholesterol, and triglyceride, respectively, and Spearman's rank correlation was applied.

All p-values are given as two-sided values and p-values below 0.05 considered significant.

Statistical analysis was performed with R version 3.1.1 for Windows (R Foundation for Statistical Computing, Vienna, Austria). Reference intervals were established using Analyse-it version 2.24 (Analyse-it Software Ltd, Leeds, UK).

Results

Analytical linearity

Figure 1 shows the results of the analytical linearity study. For NTA (Figure 1(A)) we observed linearity with an acceptable level of agreement between expected and measured particle concentrations within the range of $1.0–10.0 \times 10^8$ particles/mL. This implied that 80% of the fresh fasting PFP samples analyzed in the inter-individual biological variation study could be measured within the linear range after being diluted by a factor of 1000, the remaining after dilution by a factor of either 500 or 2000. For the solutions with a particle concentration above the observed linear range for NTA measurements, an indication of high noise or too high particle concentration in the field of view was conferred by the software.

For TRPS (Figure 1(B)) we observed linearity with an acceptable level of agreement between expected and measured particle concentrations within the range of $1.0 \times 10^8–1.8 \times 10^9$ particles/mL. Thus all fresh fasting PFP samples analyzed in the inter-individual biological variation study were within the linear range without any dilution. However, most samples were diluted by a factor of 2.5 or 5, which in our experience reduces the risk of nanopore clogging.

Analytical variation

Figure 2 summarizes the results of the analytical variation studies. TRPS exhibited a considerably higher variation in particle concentration (CV 24.1%) than NTA (CV 6.4%) in the Between day$_{PFP}$ study. To further examine the analytical variation of TRPS we performed the additional Between day$_{beads}$ study and the Within day$_{PFP}$ study (see *Materials*

and methods) which both presented markedly lower CVs of the concentration measurements (5.9 and 5.0%, respectively) than the Between day$_{PFP}$ study.

Biological variation

Characteristics of the study population in the biological variation studies are listed in Table 1. No postprandial change in plasma cholesterol levels was observed whereas the TG concentration increased significantly ($p < 0.001$) postprandially, the mean increase being 22.3%.

Fasting versus postprandial samples

Figure 3 summarizes the results of the study of the impact of prandial state on particle content in the samples. Using NTA we found a higher particle concentration for all included persons in the postprandial as compared to the fasting state. For the fresh samples the median increase in particle concentration after food intake was 61% (IQR 34–110%). This general

Table 1. Study population characteristics ($n = 20$).

	Fasting	Postprandial
Gender		
Females, n	13	
Males, n	7	
Age (years)	45 (13)	
BMI (kg/m²)	23.4 (4.6)	
Smoking status		
Current, n	2	
Former, n	3	
Never, n	15	
Plasma lipids (mmol/L)	*Fasting*	*Postprandial*
Total cholesterol	5.1 (1.1)	5.1 (1.0)
LDL cholesterol	2.9 (1.0)	2.8 (0.9)
HDL cholesterol	1.8 (0.4)	1.8 (0.5)
Triglyceride	1.0 (0.6)	1.2 (0.6)

Continuous data are given as mean (SD).

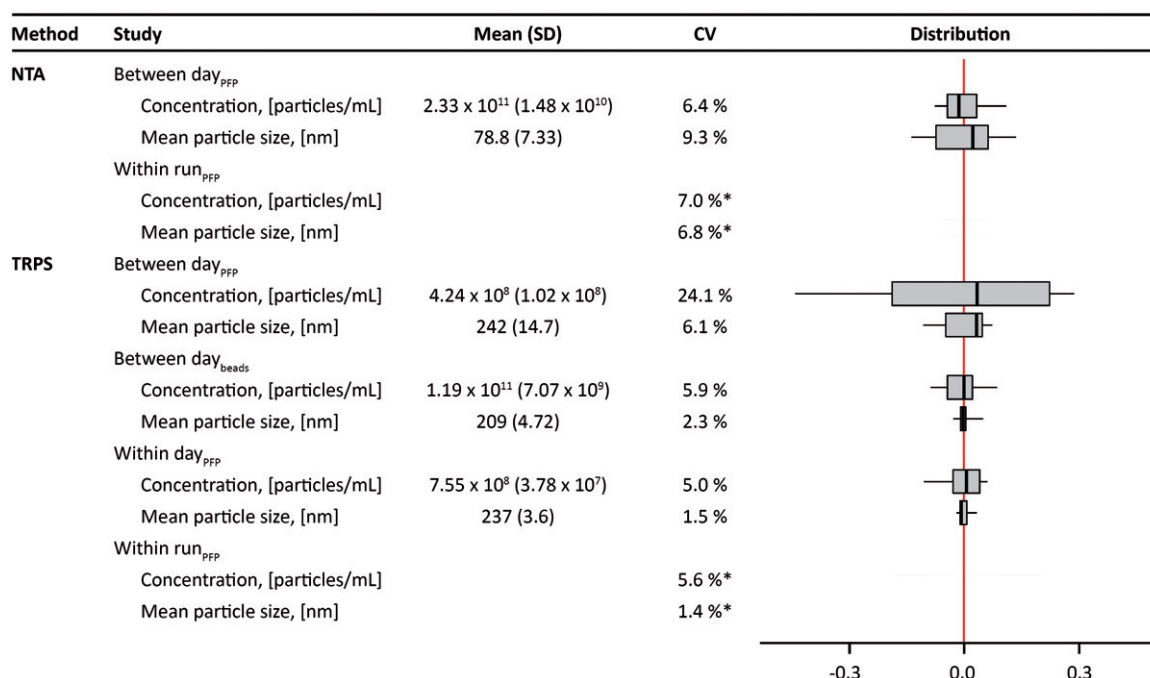| Method	Study	Mean (SD)	CV	Distribution
NTA	Between day$_{PFP}$			
	Concentration, [particles/mL]	2.33×10^{11} (1.48×10^{10})	6.4 %	
	Mean particle size, [nm]	78.8 (7.33)	9.3 %	
	Within run$_{PFP}$			
	Concentration, [particles/mL]		7.0 %*	
	Mean particle size, [nm]		6.8 %*	
TRPS	Between day$_{PFP}$			
	Concentration, [particles/mL]	4.24×10^8 (1.02×10^8)	24.1 %	
	Mean particle size, [nm]	242 (14.7)	6.1 %	
	Between day$_{beads}$			
	Concentration, [particles/mL]	1.19×10^{11} (7.07×10^9)	5.9 %	
	Mean particle size, [nm]	209 (4.72)	2.3 %	
	Within day$_{PFP}$			
	Concentration, [particles/mL]	7.55×10^8 (3.78×10^7)	5.0 %	
	Mean particle size, [nm]	237 (3.6)	1.5 %	
	Within run$_{PFP}$			
	Concentration, [particles/mL]		5.6 %*	
	Mean particle size, [nm]		1.4 %*	

Figure 2. Analytical variation of NTA and TRPS. Standardized data, generated by subtracting the mean of all measurements from each measurement and finally dividing by the mean of all measurements within each respective experiment, are shown in box plots indicating the first, second, and third quartile and the range. '0,0' and the through-going line indicate the standardized mean. The given concentrations are adjusted for dilution. *Pooled CV for duplicate measurements.

and statistically significant increase in particle concentration was accompanied by a significant increase in mean particle size. In the fasting samples, the median of the mean particle sizes in all participants was 62 nm, whereas in the postprandial samples it was 93 nm.

Using TRPS, a more pronounced increase in measured particle concentration after food intake in all participants was observed. The median effect in the fresh samples was a 14-fold increase (IQR 5- to 23-fold increase), whereas no change in mean particle size was observed.
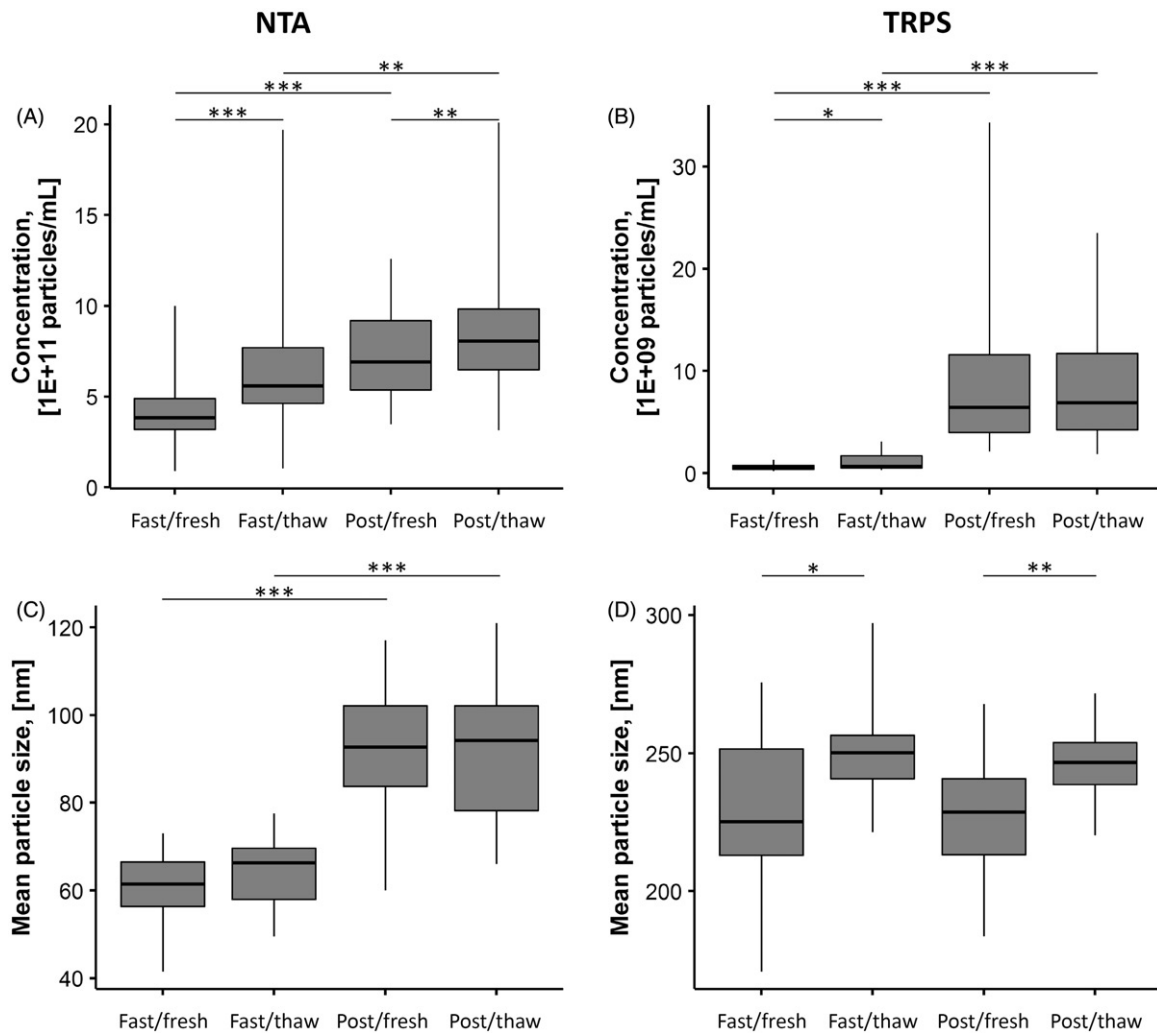
Inter-individual variation

Between-subject variation in particle concentrations in the fresh fasting samples is shown in Table 2. Since the applied normality test did not reject a Gaussian distribution (concentration data were logarithmically transformed, size data were not transformed) the 95% central interval was calculated parametrically. However, the estimated reference intervals exceeded the total range of the actual measured concentrations for both methods.

Intra-individual variation

Figure 4 presents the differences between the measured concentrations in the two different samples from the eight participants in the intra-individual variation study. The pooled within-subject CV for particle concentrations measured by NTA was 33.6% and the corresponding CV for TRPS was 20.9%, whereas the pooled within subject CV for mean
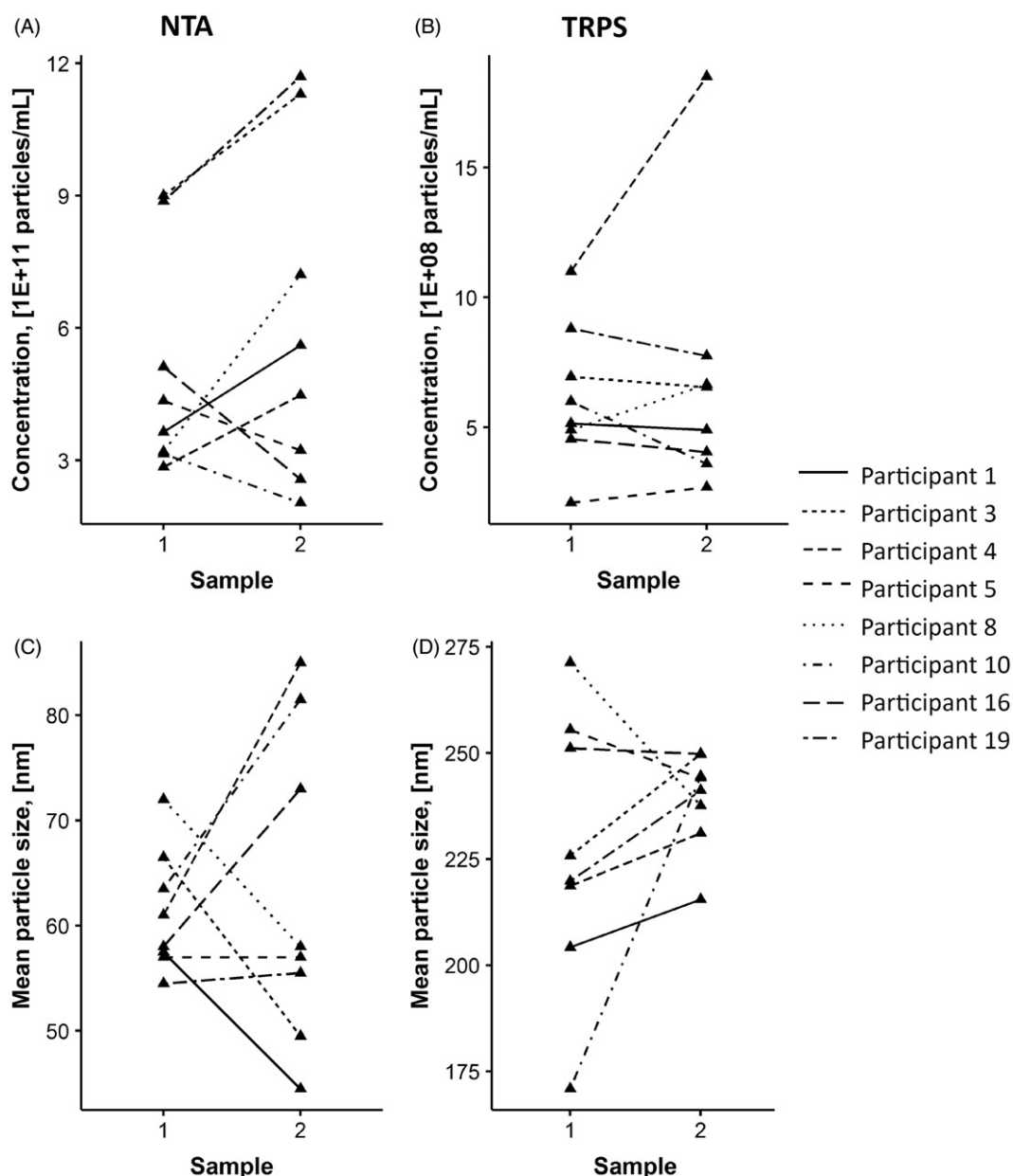

Figure 3. Preanalytical and inter-individual variation. Results on particle concentration and mean particle size in fasting and postprandial samples from 20 healthy individuals ($n = 20$) analyzed in their fresh form and after one freeze-thaw cycle by NTA (A, C) and TRPS (B, D), respectively. Box plots indicate the first, second, and third quartile and the range. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. Fast/fresh, Fasting fresh; Fast/thaw, Fasting thawed; Post/fresh, Postprandial fresh; Post/thaw, Postprandial thawed.

Table 2. Inter-individual variation in particle concentration in fresh fasting PFP ($n = 20$).

	Indication of variation	NTA	TRPS
Particle concentration, (particles/mL)	Observed range	8.9×10^{10}–1.0×10^{12}	2.1×10^{8}–1.3×10^{9}
	95% reference interval	1.4×10^{11}–1.2×10^{12}	1.8×10^{8}–1.6×10^{9}
Mean particle size, (nm)	Observed range	41.5–73.0	170.9–275.7
	95% reference interval	45.5–76.0	178.5–283.2

NTA: Nanoparticle tracking analysis; (TRPS) tunable resistive pulse sensing.

Figure 4. Intra-individual variation. Results on particle concentration and mean particle size in the two separate blood samples from 8 healthy individuals ($n = 8$) shown for NTA (A, C) and TRPS (B, D).

particle size was 16.2% and 10.3% for NTA and TRPS, respectively.

Preanalytical variation

For PFP stored at 4 °C, daily measurements with NTA showed that particle concentration and mean size are retained, i.e. within the expected analytical variation, within a 10 days storage period, however, TRPS measurements resulted in a reduced particle concentration after 8 days of storage.

The effect of applying a freeze-thaw cycle is shown in Figure 3. For NTA one freeze-thaw cycle resulted in a moderate increase of particle concentration with a median increase in particle concentration by 29% (interquartile range (IQR) 20–41%) in the fasting samples and 38% (IQR 3–59%) in the postprandial samples but no change in mean particle size.

For TRPS, the effect was more variable with a median increase in particle concentration in the fasting samples after one freeze-thaw cycle by 32% (IQR −10–96%). In four cases the concentration more than doubled, whereas no change in concentration was observed after freezing and thawing the postprandial samples. The particle size increased after freezing with a median increase of mean particle size by 11% (IQR −2–22%) in the fasting samples and 10% (IQR −3–17%) in the postprandial samples.

Correlations between particle concentrations and blood lipid levels

Our data suggested a modest positive correlation ($r_s = 0.50$; $p = 0.026$) between fresh fasting TG levels and particle concentrations measured by NTA (Figure 5(A)), but not TRPS
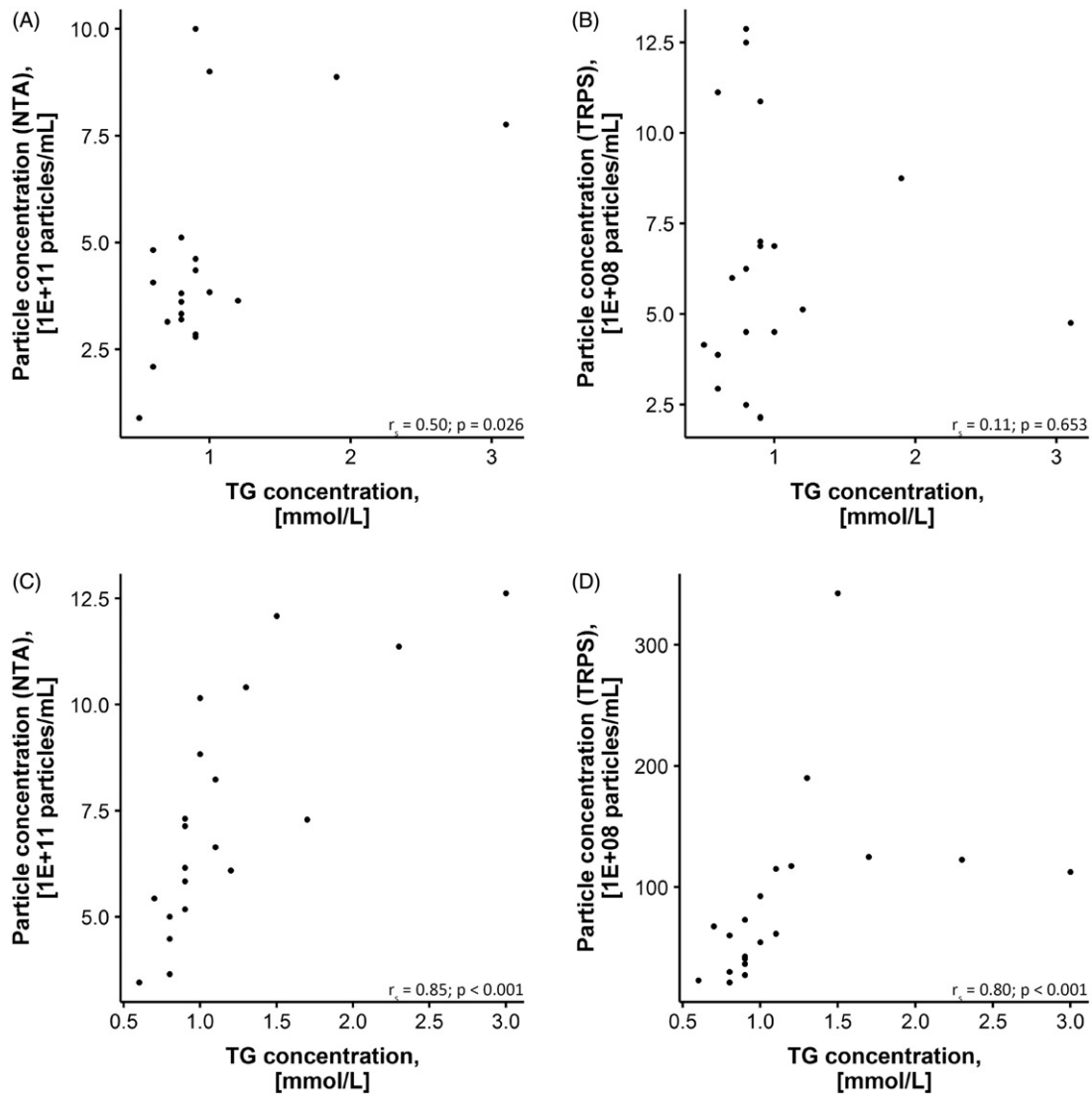
Figure 5. Correlation between TG concentrations and particle concentrations measured by NTA (A, C) and TRPS (B, D), respectively, on fresh fasting (A, B) and fresh postprandial (C, D) PFP samples from 20 healthy individuals ($n = 20$). The degree of correlation is indicated with Spearman's r_s.

(Figure 5(B)), whereas no association between any of the other lipid parameters and particle concentrations measured with neither NTA nor TRPS was observed.

Plotting particle concentrations against TG levels in the fresh postprandial samples we observed stronger correlations between plasma TG levels and particle concentrations measured by both NTA ($r_s = 0.85$; $p < 0.001$) and TRPS ($r_s = 0.80$; $p < 0.001$) as shown in Figure 5(C) and 5(D), respectively.

### EV-enriched plasma fractions by SEC

In the four subjects included in this study, NTA results on fasting vs. postprandial and fresh vs. thawed PFP samples, reflected the tendencies observed in the main study on the 20 subjects, see Figure 6(A). The separations for each sample were run in duplicate (same column) and CV calculated from the duplicates was 10–15% when counting particles in the various kinds of samples and fractions. In fresh fasting PFP the mean concentration of particles in the four subjects was 3.0×10^{11} (range 1.8–3.9×10^{11}) particles/mL and in fraction 9 and 10 it was 4.1×10^{10} (range 2.3–5.4×10^{10})

particles/mL and 3.5×10^{10} (range 2.3–4.9×10^{10}) particles/mL, respectively. Results on each study participant are provided in Figure 6. In the fresh postprandial samples a mean increase in particle concentration of 65% was seen in PFP. In fraction 9 and 10 a mean postprandial increase in particle concentration of 113% and 62%, respectively, was observed.

Applying a freeze-thaw cycle on the fasting PFP resulted in a mean increase in particle concentration of 35% in PFP, while the corresponding increases in fraction 9 and 10 were 43% and 24%, respectively.

In general, a higher particle concentration in PFP predicted a higher particle concentration in the EV-enriched fractions by SEC.

The mean value of mean particle sizes in the fresh fasting samples was 74 (range 66–80) nm in PFP. In fraction 9 and 10 it was 81 (range 70–94) and 80 (range 65–100) nm, respectively. In fresh postprandial samples the mean particle size increased to 91 (range 83–101) nm in PFP and to 104 (range 95–108) nm in fraction 9 and 83 (range 78–95) nm in fraction 10. No systematic effect of a freeze-thaw cycle on mean particle size was observed.
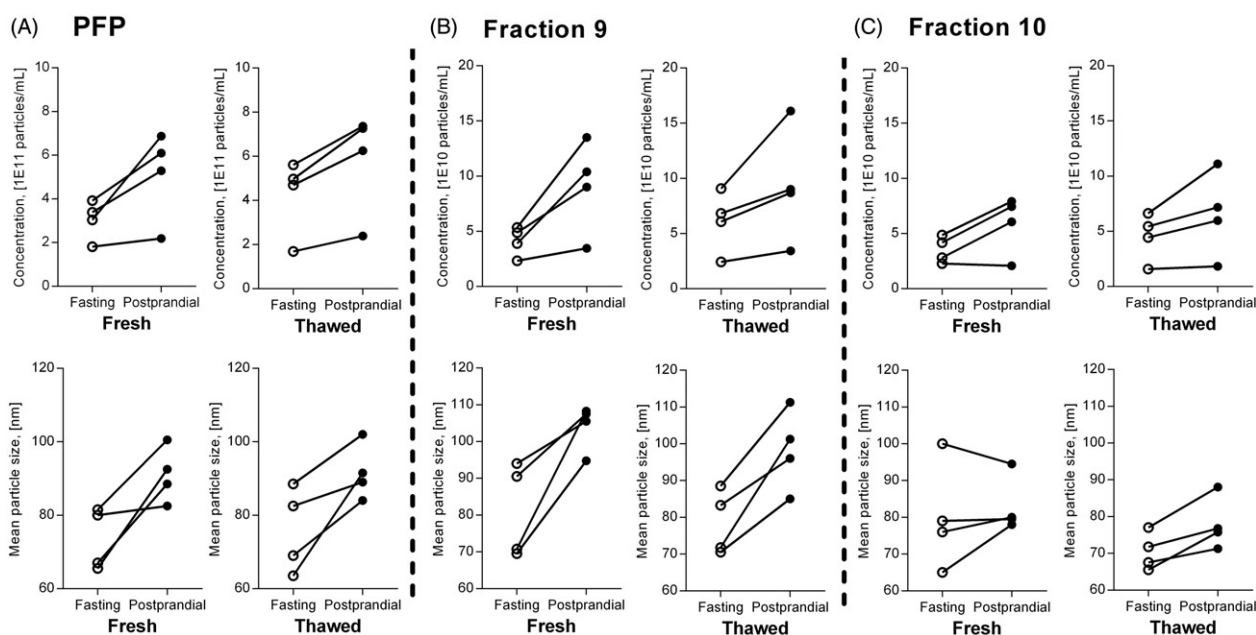
Figure 6. Fasting (open circles) and postprandial (closed circles) particle concentrations in PFP (A), SEC fraction 9 (B), and SEC fraction 10 (C) ($n = 4$).

As expected, optical density measurements revealed minor protein content in fraction 9 and 10 compared with later fractions.

Discussion

In this study we have examined the analytical linearity and variation of particle measurements on PFP by NTA and TRPS as well as the inter- and intra-individual biological variation and the impact of storing PFP at $-80\,°C$ and $4\,°C$. The prandial state demonstrated a substantial impact on the particle measurements on PFP, whereas freezing had a less pronounced effect on the particle population and PFP stored in a refrigerator appeared to be stable for a week. In addition, the prandial state and a freeze-thaw cycle had an effect on the particle population measured in EV-enriched fractions obtained by SEC that was comparatively similar to the effect observed when analyzing PFP directly.

Analytical linearity and variation

We demonstrated analytical linearity for plasma particle measurements with NTA and TRPS within the shown concentration ranges. Our data on PFP samples were in close proximity to earlier findings on NTA and TRPS linearity in measurements of artificially manufactured polystyrene particles [29,30]. As TRPS is based on detection of single particles passing through a pore the possibility of coincidence events, also referred to as 'swarm detection' [31], i.e. the phenomenon whereby a number of smaller particles simultaneously passing a flow cytometer's laser beam are registered as one larger particle [32], could be considered for this method. However, the observed direct proportionality between the expected and the measured particle concentrations suggests that swarm detection is not a substantial issue in TRPS.

We consider the observed analytical variation acceptable with regard to the potential of the methods for future research on particle content in PFP samples. The high CV found for TRPS in the Between day$_{PFP}$ study on thawed PFP samples may indicate a preanalytical issue related to the freezing or thawing process rather than an analytical one since the method produced considerably more consistent results when tested in the Between day$_{beads}$ and the Within day$_{PFP}$ study. In general, it should be noted that a limitation of this study is that the given indications of analytical variation inevitably contain an element of preanalytical variation and vice versa just as the given indications of biological variation contain an element of both aforementioned.

Biological variation

The intra-individual variation study demonstrated a considerable within-subject CV for particle concentration measurements by both NTA and TRPS, but not unlike certain other biomarkers. To provide a frame of reference it can be mentioned that the within subject CVs are approximately the same size as for plasma bilirubin [33].

To our knowledge, reference intervals for particle concentrations in plasma as measured by NTA and TRPS have not previously been given, although Gardiner et al. reported a typical PFP particle concentration of $1–5 \times 10^{12}$ particles/mL using NTA (prandial state not specified) [11], and De Vrij et al. reported particle concentrations in non-purified plasma of approximately 2×10^8 particles/mL measured with TRPS using an NP200 nanopore [13]. These levels are more or less comparable to the levels observed in the current study, but the differences that exist may reflect differences in both preanalytical handling of the samples, prandial state of the study participants, and instrumental settings. Our results clearly indicated changes in the particle population in PFP following food intake, and for purposes such as group comparisons, we recommend collection of blood from fasting participants.

With our methodological set-up NTA and TRPS obviously detect distinctively different particle populations as

NTA detects a manifold higher amount of particles of which the predominant part has a diameter of less than 100 nm. The two different studied particle populations measured by the methods respond differently to food intake. The numerous amounts of smaller particles detected in fasting PFP by NTA are not to the same extent outnumbered by particles appearing in the postprandial state as are the fewer particles measured by TRPS. The main part of these postprandially appearing particles may plausibly be accounted for by lipoproteins, since food intake is followed by the release of TG rich lipoproteins in the form of chylomicrons and VLDLs into the circulation [34]. In the fasting state the lipoprotein particles in plasma should primarily be a mixture of VLDLs, intermediate density lipoproteins (IDLs), LDLs, and HDLs which, except for a less abundant subgroup of so-called large VLDLs, have a diameter of less than 100 nm [35]. Chylomicrons have diameters between 75 and 1200 nm [36] and will thus appear in the size range of particles detectable by TRPS. The concentration of chylomicrons rises significantly during the postprandial phase [36] which is in agreement with our TRPS findings. The observed postprandial increase in both particle concentration and mean particle size measured by NTA is likewise expected to reflect increased levels of chylomicrons but also VLDLs which are at their largest when they are secreted and become progressively smaller during transfer of lipids away from them as they circulate in the blood [37]. The stronger correlation between particle concentrations and TG levels found in the postprandial than in the fasting state is in accordance with this. Thus, our data suggest that particle levels in postprandial PFP samples are influenced by lipoprotein concentrations, although they do not clarify how large a fraction of particles in the fasting state and their postprandial increase is accounted for by lipoproteins. Gardiner et al. noted that over 98% of particles measured in PFP by NTA appear to be lipoproteins [11]. However, the lack of association between fasting cholesterol and particle levels measured by NTA and TRPS and only a modest association between fasting TG levels and particle concentrations measured by NTA indicates that the fraction, at least in the fasting state, may be lower than 98%, although lipid levels do not directly reflect the number of lipoprotein particles [38].

The effect of food intake on EVs is not well known [5], but FC studies have demonstrated an increase of total EV numbers [39] as well as erythrocyte-derived [40] and endothelial cell-derived [41] EV numbers in samples collected in the postprandial state.

NTA results on the EV-enriched fractions obtained by SEC indicated that food intake induces an increase in particle concentrations and mean particle size in these fractions comparable to the effect observed in the original PFP samples. Moreover, the data suggested that higher particle concentration in PFP predicted higher particle concentration in the EV-enriched fractions. Our comparison of fasting and postprandial particle concentrations in fraction 9 and 10 clearly substantiate the advice by Böing et al. [20], recommending collection of fasting blood samples for SEC-based isolation of EVs to minimize the potential contamination by chylomicrons and VLDLs. However, previous results using SEC on

PFP indicated that TG is present in these particular fractions also when blood samples were obtained from fasting subjects although reduced considerably more than the number of particles [23], suggesting that even in EV-enriched fractions from fasting samples VLDLs may account for a non-negligible part of the uncharacterized particles detected by, e.g. NTA. A limitation of this part of the study is the low number of subjects included but the results clearly indicate the same systematic differences between fasting and postprandial samples.

Since plasma is the physiological medium of EVs it would be desirable to perform EV measurements directly on unpurified PFP, avoiding harsh isolation protocols that may affect the particle composition in the sample [5,42] and for which reproducibility among laboratories may be a challenge. However, NTA and TRPS do not enable distinction between EVs and similarly sized particles present in unpurified plasma such as lipoproteins, protein aggregates and cell debris particles but development of the techniques detecting fluorescently labeled EVs is in progress. Preliminary data indicate that raw particle measurements on plasma may have a potential for demonstrating clinically relevant differences between healthy and diseased subjects and at least be a supplement to other investigations [43,44].

Preanalytical variation

Thawed fasting samples can be analyzed by NTA and TRPS, but in our study the particle concentrations and their variation increased as an effect of the freeze-thaw cycle, and a similar increase was found in the EV-enriched fractions obtained by SEC. The mean size of the particles in the population measured by TRPS increased, whereas mean size was unchanged in the particle population measured by NTA (in EV-enriched fractions as well as in raw PFP) of which the majority was within the size range of exosomes. In accordance, Sokolova et al. [45] reported that storage at $-20\,^{\circ}$C did not affect the size of exosomes that had been isolated and purified from cell culture supernatants by applying a combination of multiple centrifugations, filtering, and SEC, whereas exosome concentration was not measured. Jayachandran et al. [46] reported that freezing of plasma samples had no effect on flow cytometric counts of MVs (subtype of EVs) isolated from plasma by a combination of multiple centrifugation steps and filtering. However, our data indicate that analysis of PFP with NTA and TRPS should preferably be done on fresh samples or alternatively samples stored at $4\,^{\circ}$C for up to 1 week.

Technical considerations

Our study has some methodological limitations associated with the technical platform contained within NTA and TRPS. Gardiner et al. stated that imprecision of concentration measurements by NTA due to adherence to the sample chamber and tubing can be minimized by making several short measurements [11]. However, we did not experience pronounced adherence when applying two measurements of 60 sec. The pooled CV calculated from the duplicates was

7.0% (Figure 2), indicating an acceptable precision in our analytical setup. Furthermore, it has been suggested to use different camera settings [11] which can give a more varied picture of the particles, but our simplified approach requires fewer subjective decisions and is thus more manageable measuring many samples. Some further technical considerations regarding challenges to instrument calibration in NTA, maintenance of nanopore stability in TRPS, and implementation of advanced particle analysis by applying additional sample runs with different settings can be found as a supplementary note.

Conclusion

NTA and TRPS both exhibited reasonable analytical variation, while an appreciable intra- and inter-individual biological variation in particle concentration and mean particle size in plasma was observed. For analysis of platelet free plasma in its raw form or after EV enrichment by size exclusion chromatography, we recommend analysis of fresh fasting samples or samples stored at $4\,^{\circ}C$ for up to 1 week, since a freeze-thaw cycle and, more strongly, food intake affect the results.

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Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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