CHAPTER SEVEN

# Role of Blood Sampling in Assessment of Concentration of Extracellular Nanovesicles in Isolates from Peripheral Blood

**Roman Štukelj[*], Ines Hribar Ignaščenko[†], Sonja Peternelj[*,‡], Magda Peruško[*,‡], Tatjana Blažič[*,‡], Manca Pajnič[*], Špela Bračun Vnuk[‡,§], Vid Šuštar[§], Apolonija Bedina Zavec[*,¶], Karin Schara[‡], Rado Janša[†], Veronika Kralj-Iglič[*,1]**

[*]Laboratory of Clinical Biophysics, Faculty of Health Sciences, University of Ljubljana, Ljubljana, Slovenia
[†]Clinical Department of Gastroenterology, Ljubljana University Medical Centre, Ljubljana, Slovenia
[‡]Department of Orthopaedic Surgery, Ljubljana University Medical Centre, Ljubljana, Slovenia
[§]Laboratory of Clinical Biophysics, Chair of Orthopedics, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia
[¶]National Institute of Chemistry, Ljubljana, Slovenia
[1]Corresponding author: e-mail address: veronika.kralj-iglic@fe.uni-lj.si

## Contents



## Abstract

*Background*: Cell-derived nanovesicles (NVs) are membrane-enclosed fragments of cell interior. They are formed in the last stage of membrane budding or in cell fragmentation. NVs are more or less free to move in body fluids and thereby constitute a cell–cell communication system. They can be harvested from body fluids, so they are potential novel biomarkers of health and disease. Advantages of NVs as biomarkers of disease are that they can be readily isolated from peripheral blood which can be obtained by phlebotomy. Blood sampling is minimally invasive while harvesting of NVs by centrifugation

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and washing of blood is a simple, low cost, and widely available method. Great promises of the method have, however, not yet been fulfilled, one of the reasons being poor repeatability and accuracy of the harvesting procedure.

*Aim*: To study the effect of the blood sampling needles' dimensions on the concentration of NVs in isolates from peripheral blood.

*Methods*: Milliliters of blood were taken from an author with no record of disease into tubes containing trisodium citrate by a free flow. Sampling was performed 37 times by using 4 types of needles differing with respect to dimensions. The time of blood flow and the volume of the acquired blood were measured at the sampling. A mathematical model was applied assuming that the blood is a Newtonian fluid. The Navier–Stokes equation for flow through a narrow cylindrical tube was considered and the shear velocity at the needle inner wall was calculated. NVs were isolated from fresh blood by repetitive centrifugation and washing and counted by flow cytometry. Shear velocity at the tube wall was correlated with concentration of NVs in the isolates by the Pearson correlation coefficient ($r$) and the corresponding probability ($p$).

*Results*: Results of mathematical models have shown that the shear stress depends on the length of the needle and its radius, or on the radius, the volume of the acquired blood, and on the time of the blood flow through the needle. We found a statistically significant correlation between the concentration of NVs in isolates and the calculated shear velocity of blood at the needle wall ($r = 0.56$, $p = 0.004$).

*Conclusions*: It is indicated that the concentration of NVs in the isolates from blood is proportional to the shear velocity of blood during the flow through the needle. In order to minimize artefact NV production at sampling, the shear velocity should be minimized. This can be achieved by using long needle with small inner radius which, however, enables continuous blood flow.

## 1. INTRODUCTION

Extracellular nanovesicles (NVs) are membrane-enclosed fragments of cell interior which are released from cells by different mechanisms such as budding and apoptosis [1]. Formerly considered as inert, NVs are now appreciated as an important impact factor in basic mechanisms [2] including genetic [3] and epigenetic [4] processes, spreading of infections [5] and inflammation [6], and progression of cancer [7] and of neurodegenerative diseases [8]. NVs are recognized as cell–cell communication system within the organism [9]. They carry the constituents of the mother cell, convey these constituents to distant cells, transfer material to other cells, and influence the function of the host cells. Specific proteins [10,11], surface-bound ligands and receptors [12], and different forms of RNA molecules (e.g., messenger RNAs and microRNAs) [13] and DNA molecules [14] were found to be transferred between cells by NVs.

NVs are present in body fluids [15–17] and can be harvested from them (Fig. 7.1). Commonly applied method for harvesting of NVs from blood is centrifugation and washing of samples whereas different protocols give populations of different sizes. Other methods include filtration, flow cytometry, chromatography, immunoadsorption/bead capture, microfluidics, and polymer-based extraction. Assessment of NVs is performed by a combination of protein content/surface proteins by Western blotting, transmission and scanning electron microscopy, atomic force microscopy, dynamic light scattering, nanoparticle tracking analysis based on Brownian motion, atomic force microscopy, mass spectrometry, and analysis of nucleic acid content.

The mechanisms underlying the NV formation have not yet been fully understood. A mechanism was suggested where invaginations of plasma membrane form intracellular vesicles within which a similar process yields exosomes [18]. Exosomes may be released from the cell by exocytosis if

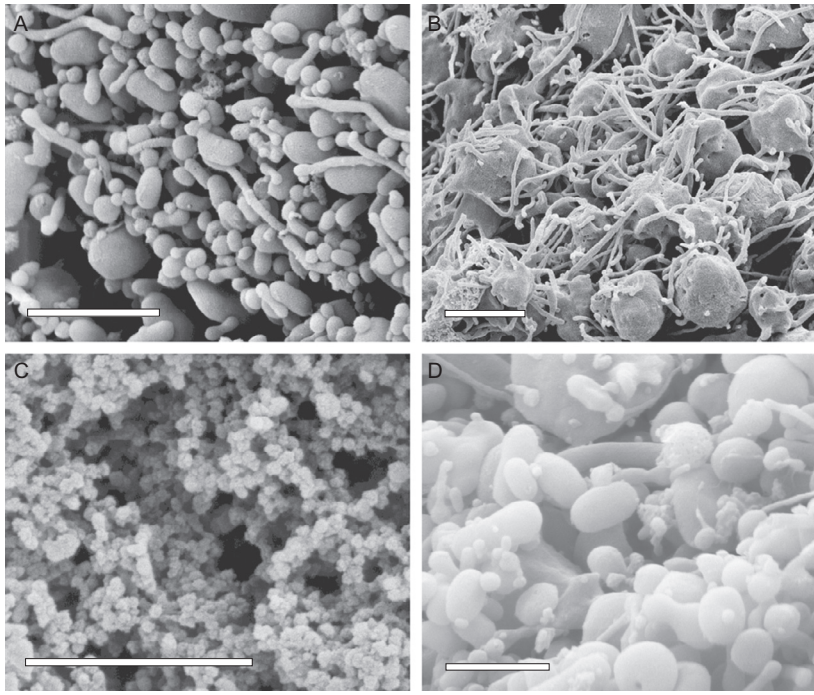


**Figure 7.1** Scanning electron micrographs of extracellular nanovesicles (NVs) and related mechanisms. (A) NVs harvested from cerebrospinal liquor, (B) activated platelets from harvesting pellet, (C) NVs harvested from pleural effusion, and (D) NVs isolated from peripheral blood. Bars = 1 μm.

the endosome merges with the plasma membrane; another mechanism is fragmentation of cells that undergo apoptosis [1,14]. Further, membrane exovesiculation takes place in the last phase of membrane budding when the bud is pinched off from the membrane to become a free vesicle. Packing and distribution of membrane constituents creates local membrane curvature which is consistent with lateral sorting of membrane constituents and drives the formation of buds and vesicles [19]. NVs are enriched in proteins and lipids associated with membrane rafts [20]. Nanovesiculation is affected by pathophysiological processes [21] such as cell activation, hypoxia, irradiation, oxidative injury, exposure to complement proteins, and exposure to shear stress.

However, despite evident perspectives for minimally invasive diagnostic procedures, NV-based methods have not yet been introduced into clinical practice. Methods and protocols for harvesting, keeping, and determining NVs have not yet overcome problems such as poor repeatability and accuracy, complexity in the interpretation of assessment methods, as well as insufficient understanding of mechanisms of NV formation in the organism and during the harvesting [22]. Further, it is unclear how a subject should be prepared for sampling with respect to food intake and activity. All these reasons provide an explanation of the fact that NV-based diagnostic and therapeutic methods are not yet commercially available.

Harvesting of NVs is the first and crucial step of any NV-based procedure and represents a bottleneck to a clinically relevant quantitative method of assessment of NVs. The assessment depends on external parameters [22] and NVs are lost in the procedure due to sticking to laboratory material with which they come in contact (tubes, pipette tips). Freezing and thawing seems to be deleterious for NVs and there is presently no other acknowledged method to keep and transport NV samples. Due to technological shortcomings, the number of samples treated under the same conditions is limited to relatively small numbers; while validation of the methods is hindered as at present state of the art of the harvesting methods, multicentric studies have not been practiced.

It was found [22] that most of the NVs harvested from blood derive from fragmentation of blood cells during the isolation process due to thermal and mechanical stress and that native particles that are already present in samples compose a minority of the population; the concentration of NVs in blood isolates is smaller at higher temperatures due to activation of platelets at room temperature or lower temperature and due to increased viscosity of plasma,

which influences sedimentation and fragmentation of blood cells (mostly platelets) during centrifugation of samples. Despite recent indications that most of the NVs harvested from fluid samples are fragmented cells which are formed during the isolation process, the analysis of the isolates is clinically relevant. Namely, the content of the isolates reflects structural properties of blood cells and plasma which are affected by the clinical status.

It is suggested that in blood sampling, a needle with larger radius should be used as to avoid excessive shear stress; however, to our best knowledge, quantitative results on the effect of the needle dimensions on concentration of NVs in isolates have not been reported. Here we present a theoretical analysis of the dimensions of the needle on shear velocity in free flow sampling. Within a series of measurements on a single subject, we tested the hypothesis that the concentration of NVs in blood isolates reflects the shear velocity at the inner wall of the needle. Thereby, this work aims at a contribution to a better understanding of nanovesiculation during isolation of NVs from peripheral blood.

## 2. MATERIAL AND METHODS

### 2.1. Blood sampling

After at least 12 h fasting, blood was taken from one of the authors (female, 53 years) with no record of a disease into 2.7-mL tubes containing 270 μL of 0.109 mol/L trisodium citrate (BD Vacutainers; Becton Dickinson, CA). For free flow sampling, the covers of the tubes were previously removed so that the pressure in the tubes was equal to the atmospheric pressure. Blood was taken 37 times from metacarpal dorsal and medial cubital veins. In each sampling, processing of the sample(s) started within 15 minutes from the sampling. Different needles (Microlance; Becton Dickinson, NJ) were used. Prior to sampling, vacutubes were prewarmed to 37 °C to minimize activation of platelets due to thermal stress [22]. The time needed to obtain a required volume of blood was measured. Three samples were discarded (two obtained by using the 23-gauge needle and one by using the 21-gauge needle) due to intermittent flow, and another three samples were discarded (one obtained by using the 18-gauge needle and two by using the 21-gauge needle) as the phlebotomy was not immediately successful. The final analysis included 31 samples. In three samples, we were unable to assess the concentration of NVs due to technical problems with the flow cytometer; however, we retained the data on the blood flow of these samples.

### 2.2. Isolation of NVs

Samples were centrifuged at 1550 × *g* and 37 °C for 20 min in the centrifuge Centric 400/R (Domel, Železniki, Slovenia) within 15 min after blood sampling to separate cells from plasma. The upper 250 μL of plasma was slowly removed using a tip with wide opening and placed into the 1.8-mL Eppendorf tube. Samples were centrifuged at 17,570 × g and 37 °C for 30 min in the centrifuge Centric 200/R (Domel, Železniki, Slovenia). Supernatant (210 μL) was discarded while pelleted NVs (40 μL) were resuspended by 210 μL citrated phosphate-buffered saline (PBS). Samples were centrifuged again at 17,570 × *g* and 37 °C for 30 min. Supernatant (210 μL) was discarded while pelleted NVs (40 μL) were resuspended by the addition of 75 μL of citrated PBS.

### 2.3. Flow cytometric analysis

Flow cytometric analysis was performed by using the Altra Flow Cytometer (Beckman Coulter Inc., Fullerton, CA) with a 488-nm water-cooled laser. The Coulter software EXPO32 was used for data acquisition and analysis of results. The presence of particles was determined by forward and side scatter parameters. Flow-Count Fluorospheres (diameter 10 μm; Beckmann-Coulter, Fullerton, CA) of a known concentration ($1 \times 10^6$/mL) were used for calibration. At least 10,000 events were recorded for each sample analysis.

## 3. THEORY

### 3.1. Blood flow in the needle

The blood flow through the horizontally positioned needle with the inner radius $r_0$ is described by the law of motion proposed by Navier and Stokes. In the cylindrical coordinate system (with $r$ the radius, $\phi$ the angle, and $z$ the symmetry axis), the three components of the Navier–Stokes equation are

$$\frac{\partial v_r}{\partial t} + (\text{v.grad})v_r - \frac{v_\phi^2}{r} = -\frac{1}{\rho}\frac{\partial p}{\partial r} + \frac{\eta}{\rho}\left(\Delta v_r - \frac{2}{r^2}\frac{\partial v_\phi}{\partial \phi} - \frac{v_r}{r^2}\right), \tag{7.1}$$

$$\frac{\partial v_\phi}{\partial t} + (\text{v.grad})v_\phi + \frac{v_r v_\phi}{r} = -\frac{1}{\rho r}\frac{\partial p}{\partial \phi} + \frac{\eta}{\rho}\left(\Delta v_\phi + \frac{2}{r^2}\frac{\partial v_r}{\partial \phi} - \frac{v_\phi}{r^2}\right), \tag{7.2}$$

$$\frac{\partial v_z}{\partial t} + (\text{v.grad})v_z = -\frac{1}{\rho}\frac{\partial p}{\partial z} + \frac{\eta}{\rho}\Delta v_z, \tag{7.3}$$

where

$$(\text{v.grad}) = v_r \frac{\partial}{\partial r} + \frac{v_\phi}{r}\frac{\partial}{\partial \phi} + v_z \frac{\partial}{\partial z}, \tag{7.4}$$

$$\text{grad} = \left(\frac{\partial}{\partial r}, \frac{1}{r}\frac{\partial}{\partial \phi}, \frac{\partial p}{\partial z}\right), \tag{7.5}$$

$$\Delta = \frac{1}{r}\frac{\partial}{\partial r}\left(r\frac{\partial}{\partial r}\right) + \frac{1}{r^2}\frac{\partial^2}{\partial \phi^2} + \frac{\partial^2}{\partial z^2}, \tag{7.6}$$

$\rho$ is the density of the blood, $\eta$ is its viscosity, and $p$ is the pressure driving the flow. It is assumed that the flow in the needle is in the $z$ direction only, uniform and laminar and it is taken that it is completely developed. For simplicity, blood is described as an incompressible Newtonian liquid. These assumptions yield a single differential equation deriving from Eq. (7.4),

$$\frac{\partial p}{\partial z} = \eta\left(\frac{1}{r}\frac{\partial}{\partial r}\left(r\frac{\partial v_z}{\partial r}\right)\right). \tag{7.7}$$

It is taken that the pressure gradient along the needle is uniform,

$$\frac{\partial p}{\partial z} = \frac{\Delta p}{l}, \tag{7.8}$$

where $\Delta p$ is the difference across the needle and $l$ is the length of the needle. The solution of differential equation defined by (7.7) and (7.8) is

$$v_z = v_{z,0}\left(1 - \left(\frac{r}{r_0}\right)^2\right), \tag{7.9}$$

where

$$v_{z,0} = \frac{\Delta p r_0^2}{4\eta l} \tag{7.10}$$

is the velocity of blood in the middle of the needle while the volumetric flow rate is given by the Poiseuille–Hagen law

$$\frac{\mathrm{d}V}{\mathrm{d}t} = \frac{\pi r_0^4 \Delta p}{8\eta l}, \tag{7.11}$$

where $V$ is the volume of blood. The only nonzero component of the stress tensor in this flow is

$$\sigma_{zr} = \eta\frac{\partial v_z}{\partial r}. \tag{7.12}$$

### 3.2. Sampling with free flow

In the sampling, the needle is inserted into a vein and blood is allowed to flow into the tube as the pressure in the vein is higher than the atmospheric pressure,

$$\Delta p = p_a - p_v, \tag{7.13}$$

where $p_v$ and $p_a$ are the pressure in the vein and the atmospheric pressure, respectively. If we assume that this difference is constant throughout the blood sampling, it follows from (7.11) and (7.13) that

$$\frac{V}{t} = \frac{\pi r_0^4 (p_a - p_v)}{8\eta l}, \tag{7.14}$$

where $V$ is the volume of the harvested blood and $t$ is the time required to harvest this volume of blood.

The shear velocity at the inner wall of the needle is calculated by using Eq. (7.9),

$$\frac{dv}{dr}(r_0) = -\frac{2v_0}{r_0}. \tag{7.15}$$

With Eq. (7.10), we obtain

$$\frac{dv}{dr}(r_0) = -\frac{(p_a - p_v) r_0}{2\eta l}. \tag{7.16}$$

Expressing $(p_a - p_v)/\eta l$ from Eq. (7.14) finally gives

$$\frac{dv}{dr}(r_0) = \frac{4V}{\pi r_0^3 t}. \tag{7.17}$$

## 4. EXPERIMENTAL RESULTS ON THE FREE FLOW

Table 7.1 shows measured parameters and data regarding the needle, calculated shear velocity, and measured concentration of NVs in isolates for 31 blood samplings performed on the same subject. Needles with four different radii and three different lengths were used.

Table 7.2 shows Pearson coefficients for the correlations between the shear velocity and the time of blood flow, the inner thickness of the needle, the length of the needle, and the concentration of NVs in the isolate. One-tailed probability was calculated where the dependencies were predicted by

**Table 7.1** Measured parameters of the blood flow through the needle: time $t$ and volume $V$, data on the needle: length $l$ and inner radius $r_0$, calculated shear velocity at the inner wall of the needle $dv/dr_{r_0}$, and concentration of NVs for a subject with no record of disease

| $t$ (s) | $V$ (mL) | $r_0$ (mm) | $r_0$ (gauge) | $l$ (mm) | $\frac{dv}{dr}(r_0)$(1/mm) | $c_{NV}/c_{beads}$ |
|---|---|---|---|---|---|---|
| 20 | 2.80 | 0.6 | 18 | 38 | 0.65 | 1.96 |
| 24 | | 0.6 | 18 | 38 | | |
| 39 | 2.30 | 0.6 | 18 | 38 | 0.27 | |
| 27 | 2.75 | 0.6 | 18 | 38 | 0.47 | 0.68 |
| 75 | 3.20 | 0.6 | 18 | 38 | 0.20 | 0.53 |
| 27 | 2.95 | 0.6 | 18 | 38 | 0.51 | 0.7 |
| 3 | 3.50 | 0.6 | 18 | 38 | 5.40 | 5.58 |
| 3 | 2.95 | 0.6 | 18 | 38 | 4.55 | 0.7 |
| 17 | 2.70 | 0.6 | 18 | 38 | 0.74 | 0.39 |
| 5 | 2.80 | 0.6 | 18 | 38 | 2.59 | 0.83 |
| 4 | 2.95 | 0.6 | 18 | 38 | 3.41 | 0.4 |
| 10 | 2.70 | 0.6 | 18 | 38 | 1.25 | |
| 74 | 2.65 | 0.4 | 21 | 38 | 0.56 | 0.69 |
| 54 | 2.70 | 0.4 | 21 | 38 | 0.78 | 0.79 |
| 64 | 2.68 | 0.4 | 21 | 38 | 0.65 | 0.74 |
| 28 | 2.80 | 0.4 | 21 | 38 | 1.56 | 0.86 |
| 22 | 2.80 | 0.4 | 21 | 38 | 1.99 | 0.41 |
| 39 | 2.95 | 0.4 | 21 | 38 | 1.18 | 0.32 |
| 63 | 2.80 | 0.4 | 21 | 38 | 0.69 | |
| 43 | 2.80 | 0.35 | 22 | 30 | 1.52 | 0.57 |
| 114 | 2.80 | 0.35 | 22 | 30 | 0.57 | 0.51 |
| 93 | 2.80 | 0.35 | 22 | 30 | 0.70 | 0.73 |
| 99 | 3.00 | 0.35 | 22 | 30 | 0.71 | 0.32 |
| 30 | 2.80 | 0.35 | 22 | 30 | 2.18 | |
| 158 | 2.70 | 0.3 | 23 | 25 | 0.63 | 1.25 |

*Continued*

**Table 7.1** Measured parameters of the blood flow through the needle: time $t$ and volume $V$, data on the needle: length $l$ and inner radius $r_0$, calculated shear velocity at the inner wall of the needle $dv/dr_{r_0}$, and concentration of NVs for a subject with no record of disease—cont'd

| $t$ (s) | $V$ (mL) | $r_0$ (mm) | $r_0$ (gauge) | $l$ (mm) | $\frac{dv}{dr}(r_0)$(1/mm) | $c_{NV}/c_{beads}$ |
|---|---|---|---|---|---|---|
| 33 | | 0.3 | 23 | 25 | | |
| 141 | 2.65 | 0.3 | 23 | 25 | 0.70 | |
| 92 | 2.70 | 0.3 | 23 | 25 | 1.09 | 0.75 |
| 74 | 2.90 | 0.3 | 23 | 25 | 1.45 | 0.39 |
| 58 | 3.00 | 0.3 | 23 | 25 | 1.92 | 0.73 |
| 120 | 1.60 | 0.3 | 23 | 25 | 0.49 | |

**Table 7.2** Pearson coefficients ($r$) and the corresponding probabilities ($p$) of the correlations between the shear velocity and the time of blood flow, the radius of the needle, the length of the needle, and the concentration of NVs in the isolate

| Parameter | $r$ | $p$ | Tails |
|---|---|---|---|
| $1/t$ (s) | 0.47 | 0.003 | 1 |
| $1/r_0^3$(mm) | −0.21 | 0.128 | 1 |
| $l$ (mm) | 0.19 | 0.306 | 2 |
| $c_{NVs}/c_{beads}$ | 0.51 | 0.004 | 2 |

the theory, and two-tailed probability was calculated where such dependence did not exist. Statistically significant correlations were found between the shear velocity and the inverse time of the blood flow and between the shear velocity and the concentration of NVs in the isolate but no statistically significant correlations were found between the shear velocity and needle dimensions.

Figure 7.2 shows the time of blood flow as a function of the radius (A) and the length of the needle (B), respectively. The average time decreases with increasing radius and increasing length of the needle; however, large scattering of the measured values can be observed.

## 5. DISCUSSION

Recently, NVs are being explored as possible biomarkers of various diseases. It was found that their concentration is increased in isolates from

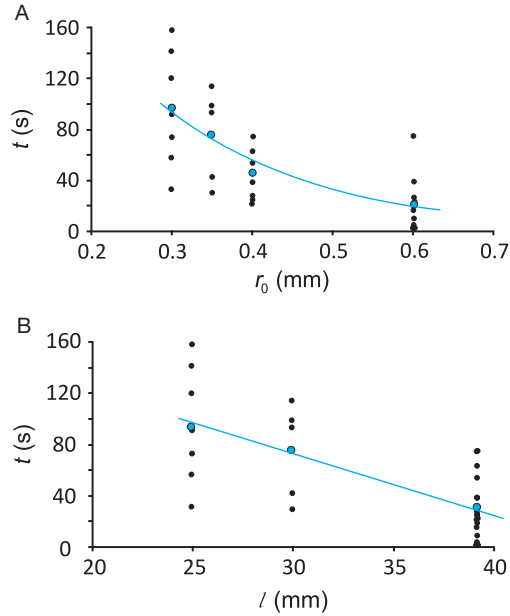


**Figure 7.2** The time of blood flow as a function of the inner radius (A) and of the length (B) of the needle.

blood of patients with various diseases, for example, autoimmune diseases [23,24], cardiovascular diseases [23], different types of cancer [25,26], diabetes mellitus of type 2 [27], and multiple sclerosis [23]. Studies of the origin of NVs isolated from peripheral blood have shown that the largest pool comes from platelets (around 80%), followed by erythrocytes (around 10%) and other cells (T-helper cells, T-suppressor cells, monocytes, B-lymphocytes, granulocytes, and endothelial cells) [27]. Cancer cells are prone to shed NVs; however, a significant increase of the number of NVs found in peripheral blood of patients with certain types of cancer originates from platelets which can be activated by NVs from cancer cells [26,28].

It was found that NVs deriving from platelets contain compounds which catalyze formation of blood clots [29,30]; moreover, the area-to-volume ratio of platelet-derived NVs is much larger than that of intact platelets, so that nanovesiculation significantly increases the catalytic surface for blood clot formation and is considered as a procoagulant mechanism. The interplay between these processes takes part especially through NV-mediated interaction between platelets, endothelial cells, and tumor cells, and is reflected in secondary thromboembolic events (e.g., in cancer [31–33] and autoimmune

diseases [34]) and in tumor progression [28,35]. Platelet-derived NVs were found to be involved in the formation of blood clots in blood vessels [29]. Further, manipulation of NVs could be used in treatment and prevention of various diseases. Using NVs as biomarkers for the disease would enable early diagnosis (with better prognosis) and continuous following of the treatment. It is believed that NV-based methods will in the future complement methods based on genetics in search of the "magic bullet" for common and widespread diseases such as cancer and neurodegenerative disorders.

Blood sampling is a basic part of any method involving NVs and is therefore key to clinical relevance of the respective method. Since blood is a dynamic system and its state depends on external parameters as well as on intrinsic properties of the components, sampling itself changes its biophysical as well as functional properties. In this work, we have studied the effect of the needle used for blood sampling on the concentration of NVs in the isolates. We have found that the concentration of NVs in isolates correlates with shear velocity at the needle wall as estimated by using the Poiseuille–Hagen law for Newtonian fluid, indicating the importance of blood flow through the needle. It follows from Eq. (7.16) that the shear velocity is higher if the radius of the needle is larger and if the needle is shorter which was supported also by the statistically significant correlation between the measured concentration of NVs and the calculated shear velocity (Table 7.2). These results disagree with the advice that thicker needle should be used to avoid activation of platelets [36]. Instead, our results indicate that a needle with a larger radius is likely to cause activation of platelets as the shear velocity near the needle wall is high in the thick needle due to high average velocity of blood in the needle.

Blood flow through the needle is driven by the difference between the pressure in the vein and the atmospheric air pressure (in the case of free flow) or pressure in the tube (in the case of sampling with vacutubes) which in turn depends on the donor's physiological and psychological state. In free flow sampling, the donor should retain equal average pressure in arteries and veins throughout the sampling which is however more difficult to achieve with thin and long needles which require more time to gather the required volume of blood. It can be seen in Table 7.1 that sampling can take more than 2 min.

We found that the shear velocity correlated statistically significantly with the time required to gather certain volume of blood and with the concentration of NVs in the isolates while no statistically significant correlation was found between the shear velocity and the needle thickness and between the

shear velocity and the needle length (Table 7.2) alone. This implies the importance of the model instead of considering each parameter separately.

Use of the Poiseuille–Hagen law is an approximation as blood is not a Newtonian liquid and the pressure difference changes periodically according to the heartbeat. In thin needles with low volume flux, the flow in the needle may cease causing surface tension effects. Nevertheless, the model gives statistically significant correlation between the shear velocity at the inner wall of the needle and the concentration of NVs in isolates (Table 7.2). It is therefore indicated that besides the needle thickness also its length and the pressure difference affect the concentration of NVs in the isolates.

Blood sampling technique and equipment can influence the results of various blood tests [37]. Lippi *et al.* [38] studied the effect of the needle thickness on platelet count and routine coagulation testing. They found that thinner needles yielded on the average smaller number of platelets in the samples; the differences were small (less than 6%) but statistically significant ($p < 0.01$). Also we have obtained smaller concentration of NVs in the isolates when the needles with smaller inner radii were used. This is in agreement with the results of Lippi *et al.* [37], considering that the source of NVs in the isolates are platelets that reside in the supernatant after the (first) low-speed centrifugation. If there are less platelets in the supernatant plasma, less NVs are formed from them.

Figure 7.2 shows that scattering of the measured time of flow of a given volume of blood is rather strong. Further study is needed to determine which parameters influence the yield of NVs in isolates and to elaborate protocols for blood sampling. However, also other elements of the procedure should be improved [36], for example, determination of NVs by flow cytometry [39,40].

To conclude, our results indicate that if least activation of platelets is required, a thin and long needle should be used; however, the flux through the needle should remain continuous. From the needles tested in this work, the 38 mm, 21-gauge needle seems the best choice.

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