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Red blood cell derived extracellular vesicles during the process of autologous blood doping

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Abstract

The purpose of this pilot study was to investigate the effects of the transfusion of one erythrocyte concentrate on the number of circulating red blood cell extracellular vesicles (RBC-EVs) and their clearance time. Six, healthy volunteers donated their blood and were transfused with their RBC concentrate after 35–36 days of storage. One K_2EDTA and one serum sample were collected before donation, at four timepoints after donation and at another six timepoints after transfusion. RBC-EVs were analyzed on a Cytek Aurora flow cytometer. A highly significant increase ($p < 0.001$) of RBC-EVs from an average of 60.1 ± 19.8 (10^3/μL) at baseline to 179.3 ± 84.7 (10^3/μL) in the first 1–3 h after transfusion could be observed. Individual differences in the response to transfusion became apparent with one volunteer showing no increase and another an increased concentration at one timepoint after donation due to an influenza infection. We concluded that in an individualized passport approach, increased RBC-EVs might be considered as additional evidence when interpreting suspicious Athletes Biological Passport (ABPs) but for this additional research related to sample collection and transport processes as well as method development and harmonization would be necessary.

KEYWORDS
biomarker, blood doping, extracellular vesicles, flow cytometry, transfusion

1 | INTRODUCTION

Autologous blood transfusion involves the collection and storage of blood and the retransfusion to the same person when required, whereas homologous transfusion relies on the transfusion of blood from a donor matched for major red blood cell (RBC) antigens.[1] The most common application of autologous blood transfusion is to correct blood loss in patients during major surgeries and to sustain oxygen transport. The advantage of autologous blood is the absence of the potential risk related to blood-borne diseases and immunoreactions, which are, although extremely low, associated with the transfusion of homologous blood. For patients with rare blood groups, autologous blood transfusion ensures that compatible blood is available in short time.[2]

Blood collected for transfusion is either stored under cooled conditions at approximately 4°C or cryopreserved at up to −80°C. While cryopreservation offers storage time of over 10 years, a disadvantage is the hemolysis rate and the loss of RBCs after thawing. The storage time for RBC concentrates produced from whole blood stored at 2–6°C is between 21 and 42 days depending on the storage medium.[3,4] A common procedure in blood banks is to store RBC concentrates in bags containing saline, adenine, glucose, and mannitol (SAGM). To produce SAGM-RBC concentrates, whole blood is initially collected in bags containing citrate, phosphate, adextrose (CPD) solution, which is further fractionated into plasma, platelets, leucocytes, and RBCs. RBC concentrates are then finally stored in bags containing SAGM solution and can be utilized for up to 42 days.[5] Independent of the storage medium, various components of the RBCs are subjected to a time-related degradation process, which is described as the development of "storage lesions."[6] The degradation is caused by a change in the concentration of certain metabolites, which are either accumulated (lactate) or depleted (ATP, 2,3-DPG, GSH, NADPH). In addition to this, the oxidation of proteins and lipids has also been reported to be involved in this mechanism. Consequently, the enzyme activity and antioxidant capacity of the cells are reduced, and the cell membrane and the cytoskeleton are damaged. The deformability of the cells decreases and becomes visible as an increase of echinocytes, spherocytes, and phosphatidylserine exposure on the cell surface and as an increase in the release/shearing of extracellular vesicles (EVs).[7]

Some endurance athletes abuse autologous blood transfusion to increase the maximum oxygen uptake and oxygen transport.[8] This procedure is forbidden in sports and mentioned as "prohibited at all times" in the World Anti-Doping Code International Standard Prohibited List. The transfusion of two bags of RBC concentrate, equivalent to the RBCs found in 900 mL of whole blood, has been described to result in meaningful improvements of endurance performance in athletes.[9,10] Recently, it has been reported that even 135-mL RBC concentrate corresponding to 225 mL of whole blood can have small performance enhancing effects.[11]

While homologous blood transfusion can be detected by flow cytometry when a difference in minor RBC antigens between donor and acceptor exists,[12–14] autologous blood transfusion can until now only be detected and sanctioned by the Athletes Biological Passport (ABP).[15,16] The ABP is an approach in which hematological parameters such as hemoglobin (Hb) concentration and reticulocyte percentage (Ret%) are recorded for an athlete on longitudinal basis at a central database administered by the World Anti-Doping Agency (WADA).[17] Fluctuations in the passport, which are out of the normal physiological variation and cannot be explained by pathological reasons, can potentially lead to a ban of the athlete from sports. Unfortunately, even the ABP has limitations such as being confounded by plasma volume variations or hypoxia, which make it difficult to distinguish between hematological changes caused by, for example, dehydration or small amounts of transfused blood.[18,19]

It is known from previous research that stored erythrocytes generate EVs, and they have been proposed as a potential biomarker to detect blood doping.[20–22] To the best of our knowledge, it is however not known to which extent these EVs will be visible after transfusion and for how long they will remain in the circulation until being removed by the reticuloendothelial system. In addition, it is also not known if RBCs transfused after storage is more fragile and could potentially generate more EVs during their remaining lifetime. Thus, the behavior of circulating RBC-derived EVs over a 6-week period that included the donation, storage, transfusion, and posttransfusion time was investigated in this study. The final goal was to obtain information to understand whether RBC-EVs could be a valuable biomarker for the detection of autologous blood doping.

2 | METHODOLOGY

In this study, RBC-derived EVs were a potential biomarker to detect autologous blood transfusion. In addition to the RBC-EVs, also the ABP related parameters Hb, Ret%, and OFF-hr Score were analyzed to be able to compare the effects of the transfusion biomarkers. Due to the extremely limited knowledge on the behavior of the RBC-EVs after transfusion, the study was performed with the character of a pilot study containing only six volunteers.

2.1 | Volunteers

Six male, healthy volunteers gave written consent to participate in this study. Details on the participants can be found in Table 1.

All volunteers were subjected to an initial health check in the hospital before the start of the study and had to fill a health-related online questionnaire required by the national blood donor center to confirm that they fulfilled all inclusion criteria. Five of the volunteers

TABLE 1 Information on the six study participants

Volunteer	Age (years)	Height (cm)	Weight (kg)	Blood group
1	42	176	86	A+
2	41	181	90	0+
3	41	190	89	A+
4	38	174	85	B+
5	31	178	72	A+
6	29	182	79	B+

practiced sports on a regular base three to five times a week, whereas one volunteer was physically not active.

The study was approved by the Ministry of Public Health registered Institutional Review Board of the Hamad Medical Corporation (Protocol No.: MRC-02-18-070) and registered at ClinicalTrials.gov (Identifier: NCT03548766).

2.2 | Study design

Each volunteer had to visit the clinical site 11 times (T). Baseline samples were collected on Day 0 (d 0) just before the donation of one bag of blood. To monitor the recovery from the donation and to create knowledge about the baseline variation of RBC-EVs in each individual, four additional samples were collected during timepoints 2–5. The effects of the transfusion were then monitored over 1 week during six further collections. Figure 1 describes the schedule of the sample collection timepoints.

2.3 | Blood collections for analytical procedures

Blood was collected via an antecubital vein puncture using a 21-gauge needle. The first tube was a 5-mL SST II serum tube (Becton Dickinson, USA) for the analysis of proteins, followed by two 3-mL K_2EDTA tubes (Becton Dickinson, USA) for the analysis of hematological parameters (Tube 1) and EVs (Tube 2). The EV tube was the last collected to avoid the contamination by vesiculation of platelets and release of vesicles upon venipuncture.

Tubes were directly stored in a cool box with an approximate temperature of 8–14°C as monitored by a thermometer. The tubes were then centrifuged within 1 h of collection by a Universal 320R centrifuge C (Hettich, Germany) at 1850*g* for 20 min at 8°. To reduce the risk of platelet and leukocyte contamination, the supernatant of tube 2 was collected at minimum 0.5 cm above the buffy coat. Two aliquots of 0.5-mL plasma were prepared in 1.5-mL Eppendorf Low-Protein Binding tubes and directly stored at −80°C.

2.4 | Blood donation and storage procedure

The standard blood donation procedure of the hospitals blood donor center for autologous blood donation was followed. Briefly, 450-mL blood was collected into a 600-mL bag containing 63-mL CPD solution (Terumo, Millbrok, UK) via an antecubital vein puncture using a 16-gauge needle. In a further process, the blood was leucocyte reduced, and RBCs were separated from plasma and buffy coat by an automated blood processing system (Reveos, Terumo, Millbrook, UK) and then gamma irradiated with an energy of 25 Gray (Gy) to eliminate potential pathogens and stored separately in 100-mL SAGM as is customary for regular whole blood donation.

2.5 | Blood transfusion

The transfusion of the RBC concentrate was performed either after 35 or 36 days of storage depending on the availability of the facilities. Hospital standard procedures that included the check of heartrate, blood pressure, and temperature as well as crossmatching of the blood groups for safety purposes were followed. The transfusion was then conducted via a 22G catheter at a flowrate of 150 mL/h over approximately 2 h. The first blood samples for the analytical procedures were then collected between 1 and 2 h after the transfusion finished.

2.6 | Analysis of ABP parameters

The analysis of the Hb concentration and reticulocyte percentage was done using a Sysmex XN1000 instrument (Sysmex, Kobe, Japan) following the ISO 17025 and WADA accredited standard operation procedure. Routinely, the analyzer is subjected to a monthly external quality assessment scheme (WADA, Centre Suisse de Controle de Qualite), and the laboratory also takes part in the proficiency tests of the College of American Pathologists, which is performed three times a year for the complete blood count and two times a year for

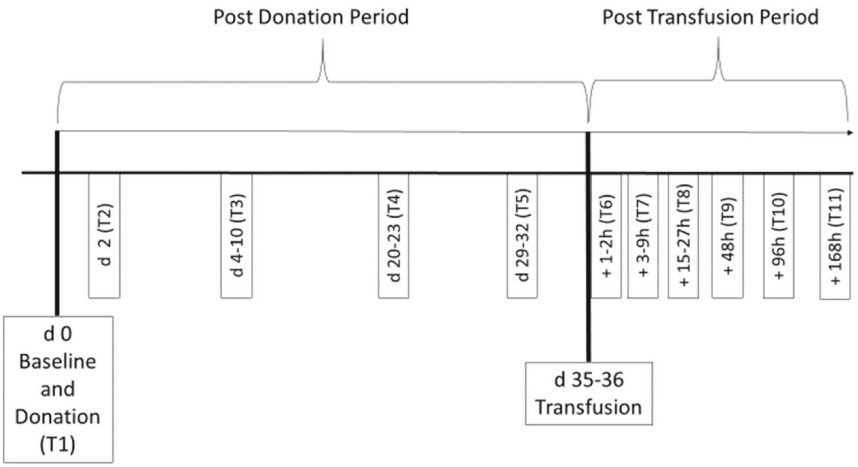

FIGURE 1 Timepoints (T) of blood collection. H = hours, d = day

reticulocyte parameters. Calibration is verified two times per month using the dedicated Sysmex XN-Cal material, which is a calibrator based on artificial blood. The XN Cal is supplied by Sysmex, and the results are monitored in the Sysmex network communication systems database, which enables a comparison between all anti-doping laboratories. All analyses were executed following the WADA Technical Document – TD2019BAR.[23] In brief, all samples were analyzed in duplicate following Sysmex QC material (XN-Checks Level 1–3). Analyses were repeated when the difference between measurements was above 0.1 g/dL for Hb or for Ret% when results were above 0.15% (Ret <1%) or 0.25% (Ret >1%). An analysis of XN-Checks at the end verified the validity of each run.

2.7 | Sample preparation for the RBC-EV analysis

The type-1 water used in this procedure was filtered before use by 30K Amicon filters (Merck Millipore, USA). All 5-mL FACS tubes (Becton Dickinson, USA) and 1.5-mL low-protein binding Eppendorf tubes used for this procedure were washed with 30K filtered type-1 water and air dried before use.

The phycoerythrin (PE)-conjugated mouse anti-human glycophorin A antibody (Cat. No. 340947, BD Biosciences, USA) was centrifuged in 1-mL aliquots for 5 min at 2000*g* to remove potential antibody aggregates, and the upper 900 uL were carefully transferred into a new tube to be used later for the staining of the EVs.

Frozen plasma was thawed over 3 h at room temperature (23 ± 2°C) and homogenized by gentle, manual up and down rotation of each tube; 10-μL plasma was then transferred into a 5-mL FACS tube, carefully mixed with 5-μL antibody, and incubated for 20 min at 23 ± 2°C protected from light.

A 985-μL type-1 water was added to the sample and carefully mixed by up and down pipetting; 100 μL of the sample was then further diluted with 900-μL type-1 water to achieve a final 1000-fold dilution.

2.8 | Cytometer controls and data acquisition for the EV analysis

The analysis of all samples was performed as a batch analysis on a Cytek Aurora flow cytometer (Cytek Biosciences, Fremont, CA, USA). The cytometer was set to trigger on the 405-nm SSC at a threshold of 1000 arbitrary units. The 488-nm laser was set at 70-mW power. The PE fluorescence of the labeling antibody and the green fluorescence of the Apogee latex calibration beads were collected using a diode array detector. The reference beads (Apogee, Hemel Hempstead, UK) were used to control the performance of the instrument and to estimate the size distribution of the RBC-MPs using 110- and 500-nm green fluorescent latex beads (refractive index = 1.59) and nonfluorescent silica (Si) beads with a diameter of 180, 240, and 300 nm (refractive index = 1.43). The flowrate was set to "Low," the data recording was started after 10–15 s of sample acquisition to stabilize the flowrate, and the recording volume was set to 15 μL. The exact recorded volume was monitored by the cytometers flow meter.

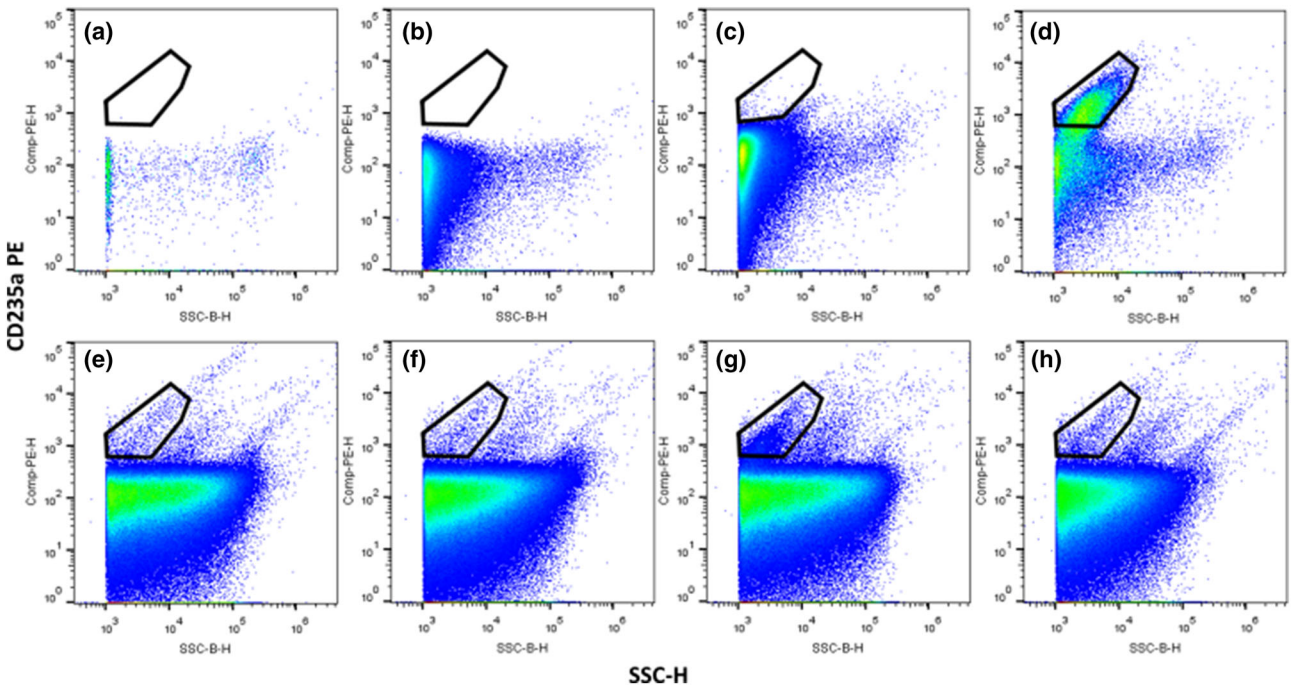

FIGURE 2 (a) Water, (b) unstained quality control (QC), (c) isotype QC, (d) stained QC, (e) volunteer at baseline, (f) volunteer 6 days before transfusion, (g) volunteer 2 h after transfusion, and (h) volunteer 9 h after transfusion. The *x*-axis presents the 405-nm sideward scatter and *y*-axis the phycoerythrin fluorescence intensity

2.9 | Assay controls

All controls were run at the same flow-cytometer acquisition settings (triggering threshold, voltages, and flowrate) as described above and the preparation of the QCs followed the sample preparation for the RBC-EV analysis as described in Section 2.7.

A 30K filtered type-1 water blank control was recorded to estimate the background signal. This water control had a count of 66 events per μL (Figure 2a).

Plasma from a previous blood bag storage study containing RBC-derived vesicles at a high concentration was used as stained and unstained quality control and to set the gates for all analyses.[22] The unstained plasma control had a count of 0 events and the stained control a count of 820,600 events per μL in the analysis gate (Figure 2b,d).

A Phycoerythrin Mouse IgG2b κ Isotype Control (Cat. No: 555743, BD Pharmingen) at the same concentration as used for the stained samples was recorded. This isotype control had a count of 14,172 events per μL in the analysis gate (Figure 2c).

2.10 | Statistics

Data were evaluated using Microsoft Excel and IBM SPSS 17. A one-way analysis of variance (ANOVA) with Bonferroni post hoc analysis was performed to test for significant differences between the collection timepoints. The level of significance was set to 0.05.

The OFF-hr score used within the ABP was calculated from the Hb concentration [Hb] as g/L and the reticulocyte percentage (Ret%) following the formula $([Hb] - 60\sqrt{Ret\%})$, as described by Gore et al.[24]

Timepoint 3 for Volunteer 2 was removed from all statistics due to an infection with high fever in the days before sample collection.

For the individual profiles, a three times standard deviation threshold for the RBC-EVs was calculated based on the mean and standard deviation of the data points of each volunteer collected before transfusion as described previously by Sieckmann et al.[25]

3 | RESULTS

In the present study, six volunteers were recruited to donate 450 mL of whole blood, which was processed to an erythrocyte concentrate and transfused after 35 or 36 days. In addition, all subjects provided regular blood samples for hematological analyses. All withdrawals and the donation procedure were well tolerated, and compliance to the protocol was 100% with 66 samples per matrix collected as planned.

Volunteer 2 contracted a viral infection after the donation between Timepoints 2 and 3, which was confirmed by the analysis of C-reactive protein (CPR) at Timepoint 3 (still 5.95 mg/L). The RBC-EV value at Timepoint 3 was removed from the statistics, but for information purposes, the RBC-EV value is reported in Table 4 with an asterisk.

3.1 | Cytometer controls and data acquisition for the EV analysis

The Apogee reference beads confirmed the performance of the instrument with a clear separation of beads in a size range of 110- to 500-nm green fluorescent latex beads and nonfluorescent silica (Si) beads with a diameter of 180, 240, and 300 nm (refractive index = 1.43).

The exact measured volume acquired for each sample as recorded by the cytometers flow meter varied between 15.0 and 15.29 μL and was later extracted for each individual sample from the flow cytometry standard (FCS) files to calculate the exact RBC-EV concentration per measured μL.

An example for the analysis of controls and samples with the cytometer is given in Figure 2, which shows density plots of filtered water for background noise estimation (a), an unstained plasma quality control (QC) sample (b), the same plasma sample incubated with an isotype-PE antibody (c), and the glycophorin-A-PE antibody (d). The plots e and f show the samples of a volunteer at baseline and 6 days before transfusion, and plots g and h show samples of the same volunteer 2 and 9 h after transfusion. The area of interest is defined by the black gate. The *x*-axis represents the sideward scatter signal of the violet laser and correlates to the size of the EVs, whereas the *y*-axis represents the PE fluorescence and as such defines the EVs derived from erythrocytes. Plots a–c confirm that the recorded signals are no artifacts or potential cross-reaction of vesicles with the antibody isotype. Plot d confirms the position of the gate, which contains stained RBC-EVs in high concentration. The increase of RBC-EVs after transfusion can be well observed in plots g and h when compared with the pretransfusion plots e and f.

3.2 | Hematological and RBC-EV data

The results of the ABP-related parameters Hb, Ret%, OFF-hr Score, and the RBC-EV concentrations are presented in Table 2 as mean and standard deviation of all volunteers. A nonsignificant decrease in the Hb concentration can be observed after donation (Timepoint 2), which returns to baseline values after transfusion (from Timepoint 8). The Ret% increases slightly and nonsignificantly at Timepoints 2–4 and shows the lowest value 7 days after donation (Timepoint 11). Significant changes can be observed for the OFF-hr score for which Timepoint 2 shows significantly lower values ($p < 0.05$) when compared with Timepoints 9–11. The Timepoint 3 score is also significantly lower ($p < 0.05$) compared with Timepoints 7–11 and Timepoint 4 significantly lower than Timepoint 11 ($p < 0.05$) (temporal views of the hematological values [Hb, Ret%, OFF-hr] for each volunteer available as Supplemental Material). For the RBC-EV concentration, the first point after transfusion (Timepoint 6) is highly significantly increased ($p < 0.001$) compared with Timepoints 1–5 and 8–11. Timepoint 7 is still increased but not significantly.

Table 3 shows the RBC-EV mean, standard deviation, coefficient of variation %, and a proposed individual threshold for each volunteer

TABLE 2 Mean concentration and standard deviation (SD) of the hemoglobin concentration (Hb), reticulocyte percentage (ret%), OFF-hr score, and CD235a positive extracellular vesicles (RBC-EV) of all volunteers at each sample collection timepoint

Timepoint	1	2	3	4	5	6	7	8	9	10	11
Hb (g/dL) Mean ± SD	15.0 ± 0.9	13.9 ± 1.0	13.7 ± 0.7	14.3 ± 0.8	14.2 ± 0.9	14.7 ± 1.2	14.7 ± 1.1	15.0 ± 1.0	15.0 ± 1.1	15.0 ± 1.0	15.2 ± 1.1
Ret (%) Mean ± SD	1.37 ± 0.36	1.57 ± 0.44	1.58 ± 0.57	1.60 ± 0.46	1.40 ± 0.37	1.26 ± 0.21	1.25 ± 0.23	1.33 ± 0.26	1.26 ± 0.29	1.23 ± 0.35	1.13 ± 0.36
OFF-hr (a.u.) Mean ± SD	80.5 ± 7.3	64.6 ± 9.8[a]	62.4 ± 15.5[b]	67.2 ± 7.2[c]	71.8 ± 8.1	79.6 ± 9.3	80.6 ± 7.0[b]	80.9 ± 6.0[b]	82.5 ± 5.1[a,b]	84.3 ± 5.8[a,b]	89.0 ± 8.2[a,b,c]
RBC-EV 10^3/μL Mean ± SD	60.1 ± 19.8	61.4 ± 29.5	54.6 ± 17.1	53.7 ± 11.6	62.6 ± 18.1	179.3 ± 84.7[d]	105.5 ± 49.3	64.8 ± 11.9	69.0 ± 16.2	59.4 ± 14.8	70.9 ± 24.0

Note: No significant differences for Hb or ret% between the timepoints could be found.

[a]For OFF-hr, Timepoint 2 is significantly lower ($p < 0.05$) compared with 9–11.

[b]Timepoint 3 is significantly lower ($p < 0.05$) compared with 7–11.

[c]Timepoint 4 is significantly lower than Timepoint 11.

[d]For the RBC-EVs, Timepoint 6, the first point after transfusion, is highly significant different ($p < 0.001$) compared with Timepoints 1–5 and 8–11.

calculated as described above and based on the timepoints before transfusion. Due to illness, Timepoint 3 was removed for Volunteer 2 so that his data are based on four instead of five pretransfusion timepoints. It becomes apparent that the baseline variation in each volunteer can be very different and that an individual threshold would be necessary when using RBC-EVs as a biomarker to detect doping by transfusion.

Table 4 shows the data on the RBC-EV concentrations for each volunteer at each timepoint. Violations of the proposed individual threshold are highlighted in cells with gray background and bold numbers. An asterisk at the data of Timepoint 3 in Volunteer 2 marks the sample, which was not included in the statistics due to illness. The data show that for Volunteer 5, even an individual threshold does not help to detect elevated RBC-EVs after transfusion. For the other volunteers, the detection time is highly variable, and concentrations above the threshold between 3 and up to 48 h can be observed. Volunteers 2 and 6 show at Timepoint 11 (7 days after transfusion) elevated RBC-EVs concentrations, which are however accompanied by elevated CRP concentrations (8.2 mg/L, 3.9 mg/L).

Figures 3–8 demonstrate the individual RBC-EV profiles for each volunteer on a time scale with the exact individual collection times. Timepoint 3, Volunteer 2 is not included (data available in Table 4). All apart from Volunteer 5 show a strong increase of RBC-EVs in the first sample after transfusion.

4 | DISCUSSION

This study reports the behavior of RBC-derived EVs in six male volunteers over a period of up to 43 days mimicking the whole process of autologous blood transfusion. The samples collected in each volunteer covered the time before blood donation, during the recovery from donation and up to 7 days after transfusion of the previously collected blood.

All volunteers showed the highest concentrations of circulating RBC-EVs at the Timepoints 6 or 7 corresponding to 1- to 2- and 3- to 8-h posttransfusion, respectively. After these timepoints, a return close to baseline could be observed. For cyclists in multiday competitions like the Tour de France, it has been reported that transfusions were given not only before the start of the Tour but also between different stages. As the clearance of RBC-EVs seems to be very fast, blood samples for the testing after a race stage that takes several hours would be probably too late. It would be also necessary to investigate the potential effects of exercise on the clearance of EVs as this would be an important information when planning a testing strategy. With the current knowledge, doping control samples would need to be collected unannounced in the night before and/or shortly before a race.

Another point to consider is that the EDTA blood samples in this study were centrifuged within 1 h, and plasma aliquots were prepared directly. In most cases where doping control samples are collected, the samples need to be shipped to a laboratory for analysis. This takes usually between 1 and 2 days. So far, it is however not known how

TABLE 3 Mean concentration, standard deviation (SD), and percentage of the coefficient of variation (CV %) of CD235a positive extracellular vesicles in EDTA plasma of each volunteer at each sample collection timepoint before transfusion (timepoints 1–5)

Volunteer	1	2	3	4	5	6
Mean (10^3/uL)	47.0	55.8	42.1	91.4	52.6	62.2
SD (10^3/uL)	1.5	15.2	4.7	19.4	3.7	5.5
CV (%)	3.3	27.2	11.1	21.3	7.1	8.8
Threshold (10^3/uL)	51.6	101.3	56.1	149.8	63.9	78.7

Note: A three standard deviation threshold was set for each volunteer based on the individual baseline data. Timepoint 3 in Volunteer 2 not included.

TABLE 4 Concentration (10^3/μL) of CD235a positive extracellular vesicles in EDTA plasma of each volunteer at each timepoint

Volunteer/timepoint (t)	1	2	3	4	5	6
1	49.0	58.3	42.9	98.8	56.2	55.1
2	44.7	43.5	41.9	119.1	55.3	63.9
3	47.2	203.3[a]	36.0	81.8	50.0	57.9
4	47.3	45.0	40.7	67.0	54.0	68.2
5	46.8	76.2	48.9	90.5	47.4	65.8
6	**89.6**	**213.2**	**244.4**	**235.2**	54.1	**239.5**
7	**61.0**	**144.1**	**64.2**	145.3	57.6	**161.0**
8	**54.2**	66.3	**56.9**	77.5	53.5	**80.2**
9	**58.9**	80.9	51.0	84.8	53.6	**85.0**
10	47.3	78.3	38.4	65.9	56.7	69.5
11	43.7	**104.0**	49.1	85.3	58.3	**85.1**

Note: Samples above the individual threshold highlighted in bold with gray background.
[a]Sample not included in all statistics as volunteer was sick.

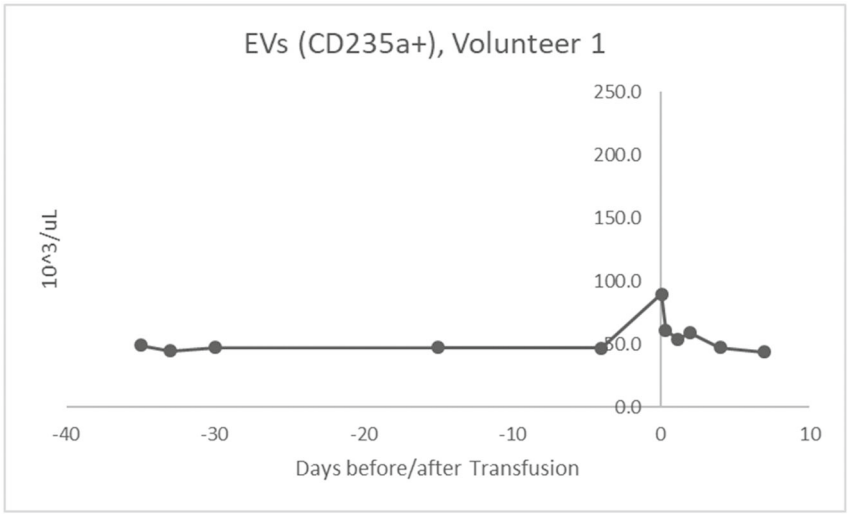

FIGURE 3 Concentration of CD235a positive extracellular vesicles in EDTA plasma of volunteer 1 before and after transfusion. Negative values describe points before transfusion, positive value samples collected after transfusion. Exact collection points were −35, −33, −30, −15, −4 days, +2 h, +8 h, +27 h, +2, +4, +7 days

long it takes until RBCs start to shed vesicles when collected in EDTA tubes and if different shipping temperatures could potentially affect RBC-EV numbers. These would be necessary points to evaluate before RBC-EVs could be considered as a good biomarker for the detection of blood doping. In the case that shipping time and temperature are confounding factors, a change in sampling protocols including a direct separation of plasma and cells at the point of collection or a different sampling matrix such as dried plasma spots could potentially represent a solution.

Due to ethical considerations only one bag of whole blood per volunteer was collected and transfused in this study. According to most publications in the relevant literature, it is more likely that athletes use two bags to achieve an increase in performance that is worthwhile.[26] Having in mind the higher performance related benefits of two bags of blood for an athlete, one might argue that this would have yielded also in a higher increase of RBC-EVs after transfusion. The identification of pre- and posttransfusion would in this case be even more clear, and the detection time maybe longer.

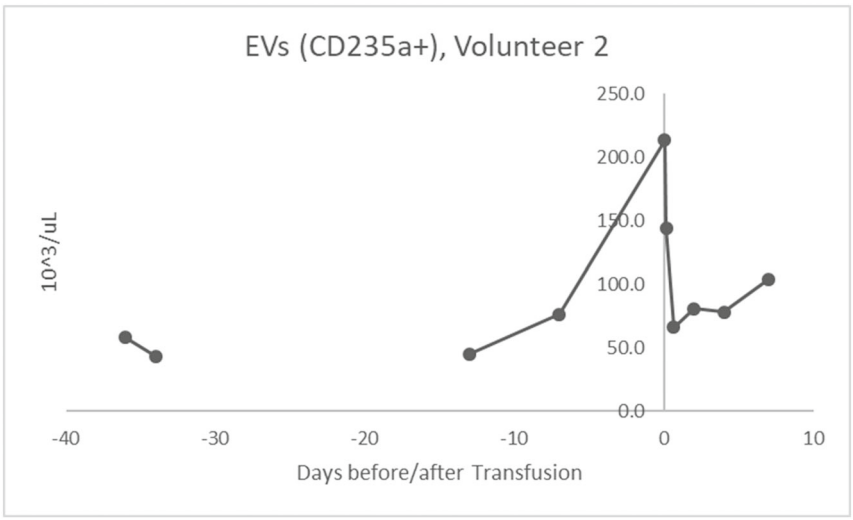

FIGURE 4 Concentration of CD235a positive extracellular vesicles in EDTA plasma of volunteer 2 before and after transfusion. Negative values describe points before transfusion, positive value samples collected after transfusion. Exact collection points were day −36, −34, −27, −13, −7, +1 h, +3 h, +15 h, +2, +4, +7 days. Timepoint −27 was removed due to viral infection

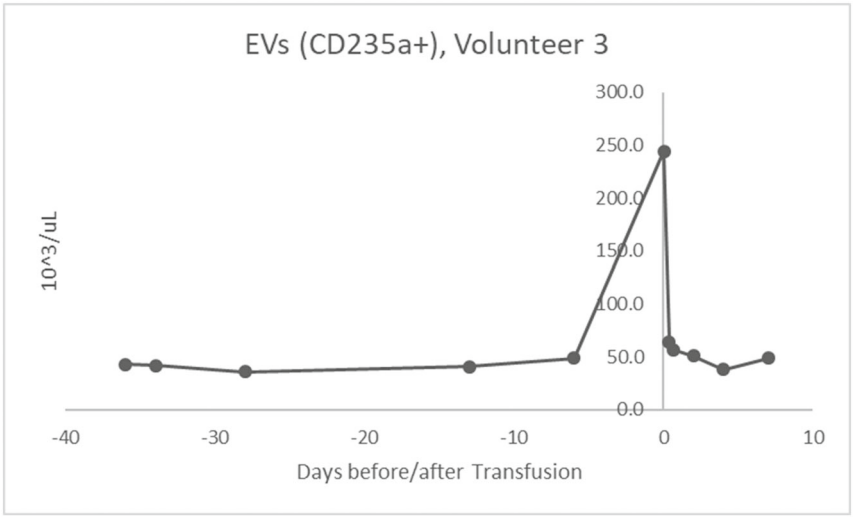

FIGURE 5 Concentration of CD235a positive extracellular vesicles in EDTA plasma of volunteer 3 before and after transfusion. Negative values describe points before transfusion, positive value samples collected after transfusion. Exact collection points were day −36, −34, −28, −13, −6, +2 h, +9 h, +15 h, +2, +4, +7 days

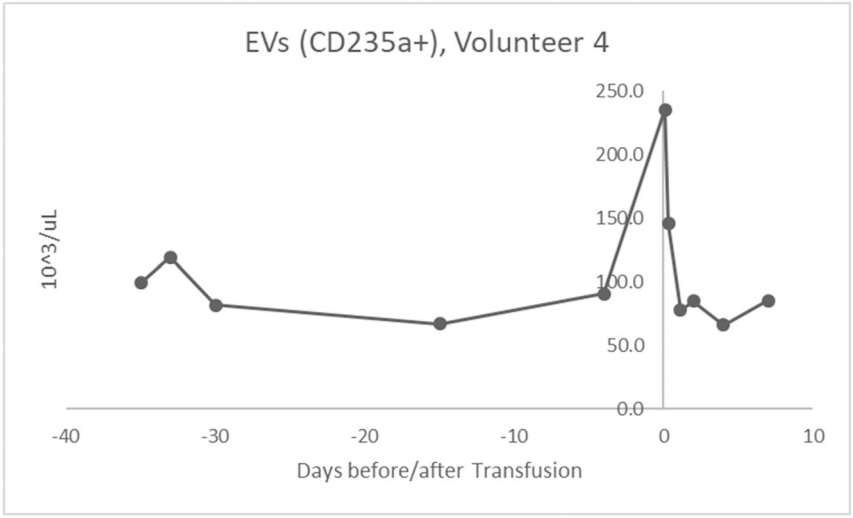

FIGURE 6 Concentration of CD235a positive extracellular vesicles in EDTA plasma of volunteer 4 before and after transfusion. Negative values describe points before transfusion, positive value samples collected after transfusion. Exact collection points were day −35, −33, −30, −15, −4, +2 h, +8 h, +27 h, +2, +4, +7 days

The variations of the RBC-EV concentration before transfusion and the response after transfusion were individually very different. The response of Volunteer 5 to the transfusion was too small to cross the calculated individual threshold so that the abuse of one bag of blood would have remained unrecognized.

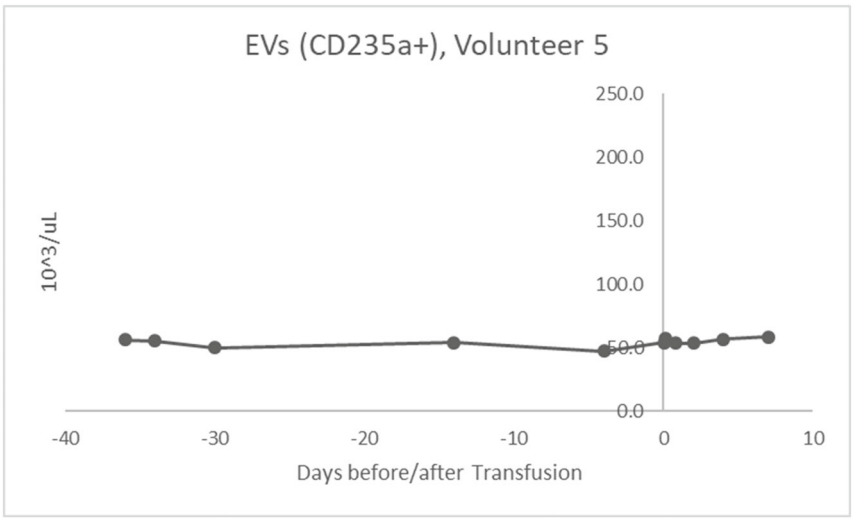

FIGURE 7 Concentration of CD235a positive extracellular vesicles in EDTA plasma of volunteer 5 before and after transfusion. Negative values describe points before transfusion, positive value samples collected after transfusion. Exact collection points were day −36, −34, −30, −14, −4, +1 h, +3 h, +19 h, +2, +4, +7 days

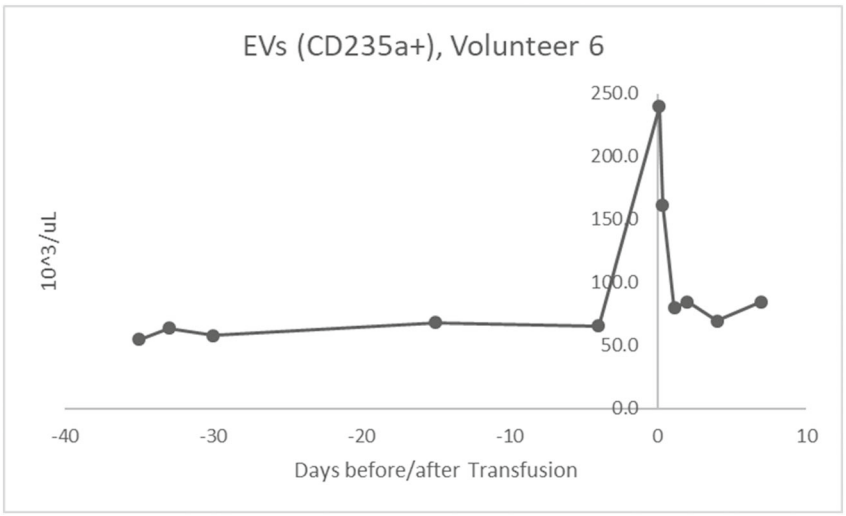

FIGURE 8 Concentration of CD235a positive extracellular vesicles in EDTA plasma of volunteer 6 before and after transfusion. Negative values describe points before transfusion, positive value samples collected after transfusion. Exact collection points were day −35, −33, −30, −15, −4, +2 h, +8 h, +27 h, +2, +4, +7 days

Another important observation was made for Volunteer 2 who contracted an infection. He felt ill 3 days after donation and had high fever for several days. After recovery (Timepoint 3, Day 10 after donation), he reported that his child was tested positive for influenza and that he probably got also infected. His reticulocyte percentage at this timepoint was substantially decreased compared with the two previously tested samples (0.7% compared with 1.37% and 1.67%), and the CPR remained elevated (5.95 mg/L). Interestingly the number of RBC-EVs was elevated to a level, which is comparable with the number detected after transfusion (203 vs. 213.2 (10^3/uL)). The plasma of the sample did however not show a red color, which could have been interpreted as a sign of hemolysis. With the information available, we can only speculate what could be the reason for the high number of RBC-EVs at this timepoint. One explanation could be the generation of RBC-EVs due to reactive oxygen species as, for example, generated by leukocytes as part of the immune response to the infection.[27] Another explanation could be linked to the fact that viruses bind to erythrocyte surfaces (e.g., via influenza hemagglutinin), which has been described to cause an adsorption of the virus by RBCs.[28,29] It has been proposed that erythrocyte vesiculation may represent a process of self-protection in which the pathogen is removed from the cell in form of generated vesicles, which either act as stimulant to cells of the innate immune system or which are removed by the reticuloendothelial system due to their phosphatidylserine exposure, which acts as an "eat-me" signal.[30] When evaluating the CRP concentration of all volunteers at all timepoints and comparing it with the number of RBC-EVs, no correlation could be found. Even when looking at elevated CRP concentrations, we could not observe a relation to elevated RBC-EVs. This suggests that inflammation per se does not mean an increased number of RBC-EVs.

It is also necessary to discuss the irradiation of the blood during the processing in the blood bank. It has been reported that irradiation by gamma rays can provoke damage to RBCs.[31] Studies investigated the irradiation with doses from 20 to 200 Gy,[32,33] which places the dose applied by the hospital in our study (25 Gy) on the lower end. It is however entirely possible that the observed increase of RBC-EVs could, in addition to the storage time, be also related to the irradiation. One might argue therefore that cheating athletes could avoid having a

suspicious test by not irradiating their blood. So far, no data on the effect of irradiation on RBC-EVs are available. One study used acetylcholinesterase activity as a substitute for the direct analysis of vesicles.[34] In this study, irradiated RBCs were exposed to 25 Gy and stored for 35 days, which is comparable with our study conditions. When compared with the control group, the irradiated group had on Day 35 an approximately 1.3-fold (approximately 500 U/L control vs. 650 U/L treated group) higher activity. The main effect on the acetylcholinesterase was however related to the storage time, which was responsible for an approximately 17-fold increase (approximately 30–500 U/L) as visible in the control group. Also, the results of a previous study, which was performed without any irradiation and in which RBC-EVs were directly measured, showed already after 35 days of blood storage a 133-fold increase of RBC-EVs.[22] Therefore, the potential impact of irradiation on the generation of RBC-EVs within the context of this study can be most likely considered as minor.

With the character of a "pilot study," this investigation was also conducted only in male volunteers to avoid too many potential confounding factors. In a study by Kanias et al., it was found that male RBCs stored in refrigerated conditions exhibited a higher susceptibility to hemolysis when compared with females.[35] This suggests also a possible gender effect on RBC vesiculation. Publications related to the menstrual cycle and to hormonal contraception show also a difference in RBC deformability.[36] Thus, if considering RBC-EVs as potential biomarker, investigations on possible gender effects would be necessary.

Only one antigen (Glycophorin A) was targeted in this study. Future studies could also investigate other markers that are related to the RBC itself or its aging process, such as band 3 protein or phosphatidylserine. This could help to obtain more information on the vesicle generation and vesicle clearance process.

5 | CONCLUSION

Elevated concentrations of RBC-EVs after transfusion of one RBC concentrate are mostly cleared within approximately 8 h. Viral infections could potentially also lead to elevated RBC-EVs, and future studies should try to find markers that enable to distinguish between an increase caused by transfusion or an increase by infection. To utilize RBC-EVs in the context of anti-doping testing, more research is necessary especially in regards of confounding factors such as exercise or hypoxic interventions. In an individualized passport approach, increased RBC-EVs might be considered as additional evidence when interpreting suspicious ABPs but for this additional research related to sample collection and transport processes as well as method development and harmonization would be necessary.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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