

## Evaluation of Blood Storage Lesions in Leuko-depleted Red Blood Cell Units

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### Abstract

**Background:** Red blood cells (RBCs) undergo biochemical and morphologic alterations during storage that are known as the storage lesions causing decreased RBC quality and are correlated with transfusion reactions in certain groups especially in infants and critically ill patients. Microvesicles (MVs) as one of storage lesions may be derived from various cell types and have key roles in several biological processes. The aim of the study was to evaluate various storage quality measures in RBC concentrates during storage under blood bank condition.

**Materials and Methods:** In this descriptive study, twenty leuko-depleted packed RBCs bags from healthy donors were prepared and stored at 4°C for up to 42 days. Samples were withdrawn at seven different times and evaluated for various hematological, biochemical, and hemolysis measures. In addition, red blood cell microvesicles (RBC-MVs) were separated and characterized based on the expression of Glycophorin A (Gly.A) antigen.

**Results:** The assessment of RBCs during cold storage showed significant increase in hemolysis rate, hematocrit (Hct), free hemoglobin, plasma potassium, and plasma lactate ( $p < 0.0001$ ), while a significant decrease in plasma sodium and glucose ( $p < 0.0001$ ) was observed. Significant increase was also identified in the RBC osmotic fragility ( $p < 0.001$ ). During the storage of RBCs in Saline- Adenine- Glucose- Mannitol (SAGM), the MVs count increased significantly. The majority of MVs had positive staining for Gly.A (74%) and it correlated with the changes in hemolysis rate ( $r = 0.77$ ;  $p < 0.001$ ).

**Conclusion:** Storage of RBC was associated with important changes which influence biochemical parameters, hemolysis, and microvesiculation process that generally affected the product quality and may contribute to the negative post-transfusion outcome.

**Key Words:** Glycophorin, Leuko-depleted, Microvesicle, Red blood cell, Storage lesion

### Introduction

Red blood cells (RBCs) concentrates are the most transfused blood component worldwide (1). Nowadays for having a sufficient and available blood supply, blood can be stored in a solution of Saline-Adenine- Glucose- Mannitol (SAGM) as a combined anticoagulant and energy source for up to six weeks at +4°C, although the storage may affect the safety and efficacy of blood components (2). Despite recent advances in improvement of blood storage conditions, a wide variety of harmful

molecular, biochemical, and physical alterations have been identified during storage that are collectively referred to as “storage lesions” (1, 3). Generally, these changes affect the quality of long-term stored RBCs. Transfusions of aged RBCs in certain patient groups are related to higher morbidity and mortality rates, especially in newborns and infants following massive transfusions (2-4). Transfusions of RBC containing increased levels of potassium have been related with myocardial hyperkalemia and neonatal arrhythmia. Although randomized clinical

prospective trials are still missing or incomplete, it is now widely accepted that long storage affects a wide range of biological and biochemical properties of RBCs (3, 4).

Storage damages are manifested by the alteration in cell flexibility and reduction in glucose, di-phosphoglycerate and adenosine triphosphate content, as well as acidosis takes place as a result of lactic acid accumulation. The sodium/potassium ATPase pump is deactivated causing a decrease in sodium with an increase in potassium levels in plasma. The most common changes in the biochemical structure of the red blood cells occur due to anaerobic glycolysis and are relative to the storage period. Furthermore, the susceptibility of stored erythrocytes to lysis triggers the formation of hemoglobin-containing microvesicles and the release of free hemoglobin which may add to transfusion side effects (3-6). Oxidative damages to lipids, proteins and carbohydrates and other morphological and molecular changes also occurred and led to sphero-echinocyte morphology. Compared with normal discoid shape, spherical cells have a lower surface area to volume ratio which leads to increased osmotic fragility and reduced deformability and viability of cells (4).

During cold storage, RBCs loses their membrane in form of vesicles and also as a consequence of membrane protein failure, small vesicles detach from the cell membrane by blebbing (microvesicle formation) (7).

In the context of transfusion medicine, Microvesicles (MVs) are considered as a storage lesion. Several experiments have shown that the number of MVs in RBC concentrate gradually increases over time, suggesting that MVs might have been responsible for some adverse transfusion reactions. Since, all of MVs activities are not fully understood, so further investigation of MVs in stored RBCs seems to be important (8).

Microvesicles (MVs) are particles around 0.1–1.0  $\mu\text{m}$  in diameter that shed from different cell types (7). MVs composition contains a subset of proteins and phospholipids that derived from their parent cells. MVs play important roles in many biological and physiological processes, including hemostasis (9), inflammation (10), vascular (dys-) function and angiogenesis (11). Through the expression of phosphatidylserine on the outer leaflet of the membrane, MVs offer a procoagulant surface that is essential for activation of the coagulation cascade and microvesiculation process (9). MVs can be found at a low level in the circulation of healthy individuals and are also elevated in various diseases states. It has been demonstrated that MVs can be used as biomarkers of diseases but before that they need to be identified to a greater extend (12).

This study aimed to assess various storage quality measures in RBC concentrates, to isolate and enumerate MVs based on flow cytometry method and finally, to determine the correlations between MVs count with hemolysis in packed RBC products in order to better understand the storage lesion and in the long time to develop strategies to prevent it.

## Materials and Methods

### *RBC preparation and sampling*

In this descriptive study, 20 healthy volunteers without having any known coagulation disorder were selected randomly. At first, consent form was signed by all donors and 450  $\pm$  45 ml whole blood was collected into blood bags (Fresenius Kabi, Homburg, Germany) containing CPD anticoagulant solution (63 ml). The units were centrifuged at 2000g for 20 minutes at 4°C to extract plasma from the original blood donation bag. Erythrocytes were then suspended in 100 ml of SAGM additive solution.

In addition, leucocytes and platelets were removed by filtration through an integrated filter. In these conditions, RBC concentrates can be stored up to 6 weeks at 4 °C. At each testing point (days 0, 7, 14, 21, 28, 35, and 42), units were thoroughly mixed and samples were aseptically taken from the bags by a sterile sampling coupler (Macopharma, Italy).

#### ***Erythrocyte Indices, hemolysis, and biochemical measurements***

Erythrocyte indices, including RBC count, the mean corpuscular volume (MCV), hemoglobin (Hb) and hematocrit (HCT) were determined using a hematology analyzer (Sysmex XT 2000i, Diamond Diagnostics, USA). Packed RBCs were centrifuged at 2000g for 10 minutes at 4°C, plasma removed and used for hemolysis index and biochemical parameters. Free hemoglobin content in plasma was determined based on the cyanmethemoglobin method. To perform this method, samples were centrifuged to obtain the supernatant containing free hemoglobin. Then, supernatants (20µl) were diluted with a drabkin solution (5ml) containing potassium ferri-cyanide and potassium cyanide. Drabkin solution oxidizes all hemoglobin forms (except sulf-Hb) to methemoglobin, and then generates cyanmethemoglobin. After incubation for 10 min at RT, we read produced color absorbance in a spectrophotometer at 540 nm. Following determination of total Hb, HCT, and plasma Hb, the percent of hemolysis was calculated as described below: Hemolysis percent =  $\frac{\text{plasma Hb (g dl}^{-1}) \times (100 - \text{HCT})}{\text{total Hb (g dl}^{-1})}$ .

For biochemical analysis, plasma lactate and glucose were measured enzymatically (Hitachi 911 chemistry analyzer, Roche, Germany) and plasma sodium/potassium were measured by a flame photometer (Eppendorf, Hamburg, Germany). In addition, extracellular pH was determined by a pH meter at RT (Mettler Toledo, Germany).

#### ***RBCs osmotic fragility assay***

Osmotic fragility test is a method that determines the resistance of red blood cells to lysis when they are exposed to the hypotonic environment. RBCs were placed in a series of saline solutions with increasing dilution ranging from 0.0–0.9% at pH 7.4. After the suspension of the blood sample (0.1 ml) in NaCl content solutions (10 ml), the tubes were inverted several times, and allowed to stand for 20 minutes at RT. Following incubation, tubes were centrifuged for 10 minutes at 2000g and then supernatants were collected. The percent hemolysis was measured in the supernatant by spectrophotometer at 540 nm for each saline solution and plotted against saline concentrations. The final result of the osmotic fragility was reported as the mean corpuscular fragility (MCF), which corresponds to the saline concentration inducing 50% of hemolysis.

#### ***MVs isolation***

RBC concentrates were evaluated for the presence of microvesicles. As seen in previous reports (13-14), MVs were isolated from samples with centrifugation. For this purpose, 10 ml of RBC concentrate mixed with 2 ml of phosphate buffer saline (pH 7.4) and centrifuged at 2000g for 10 minutes at 10°C, the supernatant was centrifuged again with this protocol to exclude the residual RBCs. The top two-thirds of the double centrifuged plasma were then removed, and used for flow cytometric analysis. Flow cytometry analysis of MVs was immediately performed within 1 hour of sampling.

#### ***MVs quantification by Flow cytometry***

MV counts and phenotypes were determined as described previously (13-14). In this study, measurement of microvesicles in samples was performed on the cy flow space flow cytometry (Partec PAS, Germany) and collected data were analyzed by Flomax software. Both

forward scatter (FSC) and sideward scatter (SSC) had a logarithmic gain. Phycoerythrin (PE) anti-human Gly.A (BD Pharmingen, San Diego, CA, USA) was used as a specific marker for RBC-derived MVs. In addition, conjugate isotype-matched immunoglobulin (PE-Mouse IgG2b k, BD Pharmingen) was used as a negative control to differentiate the background noise of the cytometric analysis. Using Carboxylate Microspheres 1.0  $\mu\text{m}$  (Polysciences, Warrington, Philadelphia), size events were defined. Bead tubes contain a standardized number of fluorescent beads which allow us to compare measured numbers of MVs between samples. Briefly, samples were incubated with anti-Gly.A antibody (5  $\mu\text{l}$ ) and IgG2b isotype control (5  $\mu\text{l}$ ) for 30 min at 4  $^{\circ}\text{C}$  in the dark. Before analysis, 5  $\mu\text{l}$  of well-mixed 1.0  $\mu\text{m}$  beads which diluted 1:500 in distilled water were added to each sample. Samples were analyzed at a low flow rate

The concentration of MVs was calculated by comparison to the bead concentration. Actually, the number of MVs was measured in relation to 10,000 beads events. In order to discriminate true events from background noise, MVs were defined by size (less than 0.1  $\mu\text{m}$ ) and RBC origin (Gly.A+).

### **Statistical analyses**

Data are expressed as mean (SD) values. Variables were assessed statistically using the repeated measure ANOVA. Correlations analysis were made using Pearson correlation test. Stata software, version 13 (Stata Corp, College Station, TX, USA) was used for all statistical analysis. Graphs were depicted by Excel and Stata software. The statistical significant level was accepted at  $p < 0.05$ .

### **Results**

In the leuko-depleted packed RBC units, we measured several variables that have been shown to change (Table I). The progressive increase in HCT and plasma

Hb concentration over the storage period was statistically significant ( $p < 0.0001$  by ANOVA). As shown, the mean corpuscular volume (MCV) also increased ( $p < 0.001$ ). The RBC count parameter remained fairly stable during this study period, hence it was not statistically significant ( $p = 0.06$ ).

The result also showed that the change in biochemical parameters during storage was statistically significant ( $p < 0.0001$ ) which clearly indicates an increase in plasma level of potassium and lactate and in contrast plasma sodium and glucose decreased in all units over time (Table 1). Biochemical variables changed early with cold storage of RBCs and were statistically significant at day 42 compared to day 0 ( $p < 0.0001$ ). Moreover, the extracellular pH of RBC concentrates reduced rapidly during cold storage (Table 1,  $p < 0.001$  by ANOVA). Stored RBCs started with a pH of 6.99 and declined to 6.55 after 42 days of storage.

Hemolysis is one of the main RBC storage lesions and its clinical implication for transfused patients is very serious. As expected, hemolysis of packed RBCs was increased slowly during storage (Fig.1;  $p < 0.0001$  by ANOVA). The hemolysis rate of all units (apart from one sample on day 42) remained below the maximum acceptance limit of 0.8% stated by the Council of Europe guideline.

The osmotic fragility of packed RBC with standard method was also evaluated. As depicted (Fig.2A), the leftmost curve represents the osmotic fragility of fresh blood on day 0 with the least susceptibility to increasing dilutions of saline. As storage progressed, the osmotic fragility increased and caused fragility sigmoidal curves to shift towards the right. The mean corpuscular fragility which associated with the NaCl concentration that produced 50% cell lysis, increased late during storage. The differences in MCF were statistically significant at day 42 compared to day 0 (Fig.2B,  $p < 0.001$ ). Large donor variability observed in hemolysis and osmotic

fragility when RBCs units from different volunteers are stored under similar conditions.

### Flow cytometry

The population of MVs was first gated according to their forward and side scattering profile in comparison to the beads (Figure 3A, 3B). The identified MVs population was then analyzed for Gly.A expression in order to consider MVs that had released from RBC (Fig.3C). RBC-MVs were seen as the population that had lower light scattering compared to 1.0  $\mu\text{m}$  beads.

We observed that the number of total MVs increased significantly during storage. Moreover, differences between the number of MVs in Day 42 ( $51087.5 \pm 11181.4 /\mu\text{l}$ ) compared to Day 0 ( $3976.1 \pm 1186.3 /\mu\text{l}$ )

were statistically significant and increased approximately 12.8 times (Fig.4A;  $p < 0.001$ ). There was a significant variation of the MVs counted between samples that obtained from different donors.

Measured numbers of Gly.A+ MVs which represents RBC-derived MVs was observed from approximately  $2650.7 \pm 851 /\mu\text{l}$  in fresh samples to approximately  $40823.2 \pm 8491.7 /\mu\text{l}$  in aged erythrocyte concentrates which statistically significant (Fig.4A;  $p < 0.001$ ). As expected, the majority of total MVs were of RBC origin and had positive staining for Gly.A (74%). Moreover, the result showed a significant relationship between RBC-MVs count and hemolysis rate (Fig.4B,  $r=0.77$ ;  $p < 0.001$  by Pearson's test), indicating that the level of hemolysis correlated with the number of RBC-MVs detected.

Table I: Main hematological and biochemical characteristic of packed RBC stored for six weeks

Assay	Days of storage							p-value
	0	7	14	21	28	35	42	
pH	$6.99 \pm 0.15$	$6.88 \pm 0.08$	$6.79 \pm 0.07$	$6.73 \pm 0.05$	$6.65 \pm 0.08$	$6.61 \pm 0.05$	$6.54 \pm 0.06$	0.001
Lac (mg/dl)	$88.5 \pm 9.2$	$118.9 \pm 9.7$	$174.6 \pm 13.7$	$219.8 \pm 10.4$	$263.03 \pm 10.8$	$284.6 \pm 12.6$	$313.2 \pm 15.7$	0.0001
Glu (mg/dl)	$549.4 \pm 13.5$	$416.6 \pm 17.4$	$359.2 \pm 24.6$	$307.9 \pm 26.1$	$262.8 \pm 19.2$	$243.2 \pm 19.05$	$210.7 \pm 22.3$	0.0001
Na <sup>+</sup> (mEq/l)	$155.2 \pm 5.8$	$142.8 \pm 8.1$	$126.5 \pm 8.4$	$112.55 \pm 7.1$	$106.95 \pm 6.5$	$101.6 \pm 6.4$	$94.75 \pm 5.5$	0.0001
K <sup>+</sup> (mEq/l)	$1.8 \pm 0.7$	$10.5 \pm 1.6$	$15.4 \pm 1.8$	$18.1 \pm 1.6$	$20.9 \pm 1.9$	$23.5 \pm 2.6$	$26.07 \pm 3.7$	0.0001
Hb (gr/dl)	$18.03 \pm 0.53$	$18.5 \pm 0.54\ddagger$	$19.04 \pm 0.54\ddagger$	$19.67 \pm 0.52\ddagger$	$20.8 \pm 0.68$	$21.9 \pm 0.81$	$21.04 \pm 0.97$	0.01
Hct (%)	$53.54 \pm 1.6$	$54.49 \pm 1.9\ddagger$	$55.15 \pm 2.11\ddagger$	$56.32 \pm 1.55\ddagger$	$59.65 \pm 1.42$	$60.88 \pm 1.93$	$62.91 \pm 1.96$	0.0001
RBC ( $\times 10^6 /\mu\text{l}$ )	$5.97 \pm 0.34$	$5.93 \pm 0.22\ddagger$	$6.00 \pm 0.36\ddagger$	$6.05 \pm 0.23\ddagger$	$6.25 \pm 0.28\ddagger$	$6.33 \pm 0.42\ddagger$	$6.41 \pm 0.47\ddagger$	0.06
MCV (fL)	$89.6 \pm 4.36$	$91.9 \pm 2.98\ddagger$	$92.08 \pm 3.4\ddagger$	$93.05 \pm 4.45\ddagger$	$95.5 \pm 4.45$	$96.3 \pm 4.32$	$98.14 \pm 5.55$	0.001
Free Hb (gr/dl)	$0.056 \pm 0.01$	$0.09 \pm 0.02\ddagger$	$0.125 \pm 0.02$	$0.174 \pm 0.03$	$0.231 \pm 0.04$	$0.283 \pm 0.09$	$0.337 \pm 0.06$	0.0001

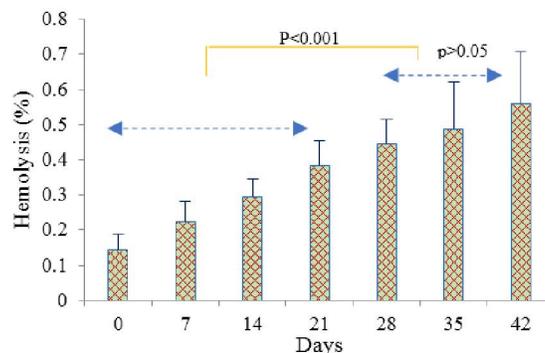


Figure 1. Assessment of hemolysis rate during storage of packed RBCs in different days (n=20). RBCs lysis increased significantly in all units during storage ( $p < 0.001$  by ANOVA).

## Discussion

The effect of storage lesions on leuko-depleted RBC concentrate is incomplete and the clinical aspects of the safety and effectiveness of stored RBC are still under evaluation (15).

The finding showed that the biochemical parameters changed rapidly over the first week of storage. During RBC storage, biochemical changes are generally related to alteration in energy metabolism which is associated with increased extracellular lactate and potassium concentration and in contrast plasma glucose and sodium decreased over time. Reduction of glucose and increase of lactate concentration indicates RBC glycolysis (12). A recent study by D'Alessandro et al (15), published in detail on the time-course of changes in metabolites during RBC storage. Their findings showed that concentration of glycolytic metabolites increased during the first week of storage, suggesting that at this early stage of storage glycolysis is proceeding. The leakage of potassium from cells in to surrounding milieu may be responsible for the drastic progression in potassium increase in the present study. It has been documented that ill infants requiring surgery or due to hemorrhage may have to be transfused with large volume of stored red blood cell and transfusions of RBC containing increased levels of potassium have been associated with myocardial hyperkalemia and neonatal arrhythmia (16).

Moreover, the extracellular pH of RBC concentrates was significantly reduced during cold storage. It has been shown that a decrease in pH level and increase in lactate concentration may happen within the first days of storage (5, 15).

In agreement with the work done by Hess et al (2), a progressive increase in HCT was also observed from day 0 to day 42. Automated hematology analyzers do not

directly measure HCT, but rather calculate hematocrit from measurements of individual cell sizes (MCV) and counts (RBC), which alteration in each one of them may affect the HCT (17). Indeed, the MCV of RBCs stored in SAGM increases steadily from day 1 and throughout storage. It is possible that cation gradient changes in stored RBC affect efficiently cell volume and shape (18).

In addition, free Hb in plasma as an indicator of RBC lysis was determined using drabkin method and seemed to accumulate in plasma as storage progressed and is in accordance with previous reports (3, 19).

Storage of red cells causes a progressive increase in hemolysis. It was observed that RBC lysis occurred late during storage in contrast to biochemical parameters. Hemolysis is considered as an important marker for evaluating the quality of stored RBCs (20). In assessing the effect of storage period on hemolysis, it was observed that percentage hemolysis increase significantly over several weeks of cold storage as also reported by other researchers (19-20). Some degree of hemolysis is acceptable and expected. The Council of Europe standard defined acceptable levels of hemolysis in RBC components as  $< 0.8\%$  at expiry (21). With the exception of one unit that showed hemolysis more than the permissible limits at day 42, in other red cell units' hemolysis was within the acceptable level. Hemolysis may have happened as a result of irreversible changes in cell membrane leading to membrane loss by microvesiculation. Furthermore, hemolysis is known to occur during sampling, processing, storage, and also during transportation (20).

The osmotic fragility of stored RBC was also evaluated and significant increases in the MCF were observed during storage. The susceptibility to osmotic lysis is

mainly affected by cell morphology and the surface area to volume ratio of erythrocytes. As the storage time increases, old RBCs lose their normal discoid shape and change to irreversible spherocyte form, with reduced surface area to volume ratio. Spherocytes have higher osmotic fragility which can hemolyze in hypotonic solution earlier than normal discoid shaped RBC. In this study, a significant increase in the osmotic fragility was determined on day 42, that showed the highest osmotic susceptibility to lysis with shifting the curve towards the right, while fresher RBCs on day 0 showed the lowest osmotic fragility. Blasi et al (4), have also reported similar findings. In their study conducted on leuko-depleted packed RBC, prolonged storage induces an increase in the osmotic fragility and causes the sigmoid curve to shift to the right.

Beside these measurements, RBC-MVs were also quantified in stored RBCs. The clinical importance of RBC-MVs in blood components has been increasingly recognized, emphasizing the need to identify their biological properties and their roles in pathological situation (11).

Despite recent advances in order to identify MVs but generally, there is still no standardized method to quantify them (11, 14). In this study, RBC-MVs in stored RBCs were detected using flow cytometry with specific antibody which bind to glycophorin A, a protein widely presented on the erythrocyte membrane.

Microvesicles were quantified on the basis of their size and density that were confirmed using 1.0  $\mu\text{m}$  beads and then were further identified by their ability to bind fluorescent specific antibodies as previously described (22). The findings of current study showed a gradual increment in the total count of MVs and also RBC-derived MVs, which had been previously reported by Grisendi et al (13). Totally, RBC-MVs that expressed Gly.A on their surface represented about 74 % of the total MVs. Noteworthy, differences between

fresh and 42 days old samples varied significantly.

This study had a number of limitations. It seems that MVs located near to the background noise and cell debris. It should be noted that the small size of MVs is the most common limitation that challenges the sensitivity of flow cytometry method (23). Currently used flow cytometer only detect vesicles above the detection limit of the instrument, so its use to characterize MVs could underestimate the total number of vesicles in samples.

Considerable differences in the numbers of RBC-MVs were also identified between donors. The exact reason for this variation is unknown, but it appears that factors like age, sex and ABO blood group have effects and should be investigated (14). MVs variability in samples also arises from technical factors which referred to as pre-analytical issues such as sample preparation, centrifugation, and storage (13). Centrifugation procedure considerably affect MVs measurements, mainly through shear stress, an important mechanical factor inducing MVs release from RBCs and other cells (14). A careful standardization of sampling, process, and storage was set up in order to reduce the pre-analytical variations.

Researchers suggest that MVs changes in blood products may be clinically important (24), and further investigations are needed to determine how these changes relate to the excess morbidity and mortality associated with aged blood transfusion. As shown by Koch et al (25), there is a link between duration of RBC storage and adverse side effects after cardiac surgery. Based on previous studies, transfusion of aged erythrocytes in patients undergoing cardiac surgery strongly correlates with the risk of postoperative complications and reduces survival time. However, this subject is still debatable and some of the recently published prospective clinical trials have failed to replicate these findings and limited this link only to particular situations (26)

The correct enumeration of MVs in blood components is very important. In this study, the number of RBC-MVs was correlated with hemolysis rate that occurred during storage. The number of RBC-MVs correlates significantly with the hemolysis level. As suggested, it is somewhat related to the capacity of MVs to enclose free hemoglobin (13) and clear explanation of this correlation requires further investigations. This study aimed to correlate MVs count with hemolysis induced by storage as a possible novel step in the quality control of preserved blood products.

### Conclusion

It can be concluded that storage of RBC concentrate under blood bank condition is associated with important storage lesions. The length of storage time influences biochemical parameters, hemolysis, osmotic fragility, and also MVs shedding that generally affect the blood product quality and may contribute to post-transfusion complications. Correct quantification of MVs throughout storage time as an indirect index of RBC preservation may be clinically important to provide information about adverse clinical outcomes in transfused recipients. However, there is a general need for clinical studies to determine how the MVs present in all blood components might affect the transfusion recipients.

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### Conflict of interest

The Authors declare no conflicts of interest.

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