TRANSFUSION MEDICINE

Original article

Achieving haemostasis in thrombocytopenia in remote settings: an *in vitro* comparison of frozen and lyophilized products

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Background - Platelet concentrates have a limited shelf life due to room temperature storage and therefore, are not kept in regional centres where turnover is low. Cryopreserved platelets have been proposed as an alternative to platelet transfusion in austere circumstances and fibrinogen concentrate has improved thromboelastometry parameters in thrombocytopenia. This study compared the ability of stored haemostatic products and platelets to correct thromboelastometry parameters in thrombocytopenia.

Materials and methods - Blood from eight patients with severe thrombocytopenia was combined with platelet concentrates, cryoprecipitate, fibrinogen concentrate, factor VIII, factor XIII and cryopreserved platelets in ratios equivalent to transfusion. Tissue factor initiated thromboelastometry (EXTEM) was compared between the products.

Results - EXTEM amplitude at 20 minutes (A20) improved by 13.1 mm with platelets ($p<0.01$). The 5mm increase in A20 seen with cryoprecipitate ($p=0.06$) was not statistically different from platelets ($p=0.19$). No improvement in A20 was observed with cryopreserved platelets or factor concentrates. EXTEM clotting times (CT) improved with cryopreserved platelets (19.4 s, $p=0.001$) and cryoprecipitate (24.1 s, $p<0.05$), but not fibrinogen, and both were superior to platelets (9.9 s, $p<0.05$). Clotting concentrates did not improve EXTEM parameters although further studies suggested the improvement in A20 was largely driven by higher fibrinogen concentrations in cryoprecipitate.

Discussion - These results suggest that cryopreserved platelets enhance clot initiation but do not contribute to clot strength in thrombocytopenia. When platelets are not available for transfusion, cryoprecipitate may be of value, however this requires further clinical studies.

Keywords: *thrombocytopenia, cryoprecipitate, cryopreserved platelets, platelets.*

INTRODUCTION

Platelet concentrates are stored at room temperature to prevent activation and improve post-transfusion increments; however, this limits storage duration due to the risk of bacterial contamination, increasing the complexity of stock management. Cryopreserved platelets (CPP) have been proposed as an alternative to room temperature (RT) stored

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platelets as they can be stored for long periods and may, therefore be available in austere and regional sites where RT platelets are not stored. However, equivalence of CPP to RT-stored platelets in transfusion settings has not been shown.

Preliminary studies in hematological malignancy[1] and cardiac surgery[2] have suggested that CPP may assist in bleeding cessation. However, prior studies have shown that CPP transfusion results in minimal platelet increments following transfusion. Cryopreservation and thawing results in activation and fragmentation of platelets leading to an increased number of microparticles, expression of activation markers such as P-selectin and externalization of phosphatidylserine[3,4]. While RT-stored platelets increase clot firmness, CPP have minimal impact, but do increase thrombin potential and reduce the time to initiation of clot[5].

The promotion of haemostasis by CPP appears, therefore, to be different to that seen with RT-stored platelets. Other frozen and lyophilized products may have similar effects on clot initiation, particularly cryoprecipitate and the lyophilized concentrates fibrinogen and factor VIII (FVIII). This study compared the effect of these readily available products with CPP and RT-stored platelets.

MATERIALS AND METHODS

The study was approved by the Australian Capital Territory Health and Australian National University Human Research Ethics Committees and conducted in accordance with the Declaration of Helsinki. It recruited haematological malignancy patients with severe thrombocytopenia (platelets <20×10^9/L). Following informed consent, 5.4 mL of blood was collected into 3.2% sodium citrate. Patients with active infection, defined as a fever within the preceding 48 hours, or immune mediated thrombocytopenia, were excluded. Citrated blood was mixed with RT platelets at day 6-7 of age, cryopreserved platelets, cryoprecipitate (Australian Red Cross LifeBlood), fibrinogen concentrate, FXIII or FVIII/VWF concentrate (CSL Behring, Broadmeadows, Australia). The doses were calculated to be equivalent to 1 pooled platelet transfusion or 100 mg/kg fibrinogen, based on prior studies[6,7]. Equivalent doses of fibrinogen were used to determine cryoprecipitate doses, then FVIII and FXIII doses from the expected concentrations in cryoprecipitate[8]. The reconstituted products and mixing volumes are shown in **Table I**.

Cryopreserved platelets were prepared from a unit of Group O pooled platelets on day 6 of storage to match the age of RT stored platelets using a method modified from Johnson *et al.* to create single use aliquots[9]. Excess volume was removed from the platelet bag to reduce the volume to 196 mL then dimethylsulfoxide (DMSO) 25% in 0.9% saline 40 mL added and gently mixed. Platelets were then aliquoted into 1.8 mL cryopreservation vials then centrifuged at 2,485 × *g* for 10 minutes. Excess supernatant was removed before freezing at –80°C. When required, cryopreserved platelets were thawed in a 37°C water bath and reconstituted to 1.8 mL with thawed plasma. Plasma aliquots were prepared from extended life plasma at expiry (5 days after thawing FFP), frozen at –20°C until required. Vials were thawed as needed as per cryopreserved platelets.

Each patient sample was tested both neat and with added plasma or platelet products, by tissue factor activated (EXTEM) rotational thromboelastometry (ROTEM, Werfen, Barcelona, Spain) for at least 30 minutes. The primary analysis was EXTEM amplitude at 20 minutes (A20). The amplitude at 5 minutes (A5), maximum clot firmness (MCF), clotting time (CT), clot formation time (CFT) and alpha angle (AA) were also measured. While conventional tests of coagulation were not part of the protocol, results were collected when available for clinically indicated tests of prothrombin time (PT, Recombiplastin), activated partial thromboplastin time (APTT, APTT-SS) and fibrinogen (QFA Thrombin) all performed on an ACL TOP instrument (Werfen). The full blood counts (DXH800, Beckman Coulter, Brea, CA, USA) performed to assess study entry was also collected.

Table I - *Ex vivo mixing study products and ratios*

Product	Product volume (µL)	Citrated blood volume (µL)
Neat		330
RT Platelets	22	308
Cryopreserved platelets	22	308
Cryoprecipitate	46	284
Fibrinogen concentrate (20mg/mL)	23	307
FVIII/VWF (50/130IU/mL)	3.9	326
FXIII (62.5IU/mL)	2.1	328

Statistical analysis

The primary endpoint was the change in EXTEM amplitude at 20 minutes (A20). With an expected mean EXTEM amplitude at 20 minutes (A20) of 27 mm, a standard deviation of 10 mm and an expected increment to a mean of 48 mm following the addition of platelets[7], 8 patients were expected to be required to demonstrate a difference at $p<0.05$ with 80% power using a 2-sided paired τ test. Subsequent analyses to compare treatments between the ex vivo or additional ROTEM parameters were considered significant with $p<0.05$. Paired t tests were preferred to compare differences, unless plotted data inspection showed obviously non-parametric data when the Wilcoxin signed rank test was used. Data were analyzed using Prism statistical and graphing software (v6, GraphPad, San Diego, CA, USA).

RESULTS

There were eight (four female) patients recruited, with a median age of 63.5 years (range 36 to 68) who had received cytotoxic chemotherapy for hematological malignancy, with a median platelet count of 13×10^9/L (range 10-16). The haematological malignancies were acute myeloid leukaemia in five patients and one each of acute lymphoblastic leukaemia, non-Hodgkin lymphoma and plasma cell myeloma. Coagulation studies were available in six patients, with four having elevated fibrinogen levels (median 5.1, range 2 to 7.8 g/L). All had a normal APTT and normal to mildly elevated PT (median 13.5 s, range 12-18 s). Baseline EXTEM values were impaired with a mean clotting time (CT) of 80.5 s (Normal range [NR]: 42-74 s) and A20 of 36.5 mm (NR: 50-69 mm). MCF values are shown in **Figure 1A**, however as not all samples reached

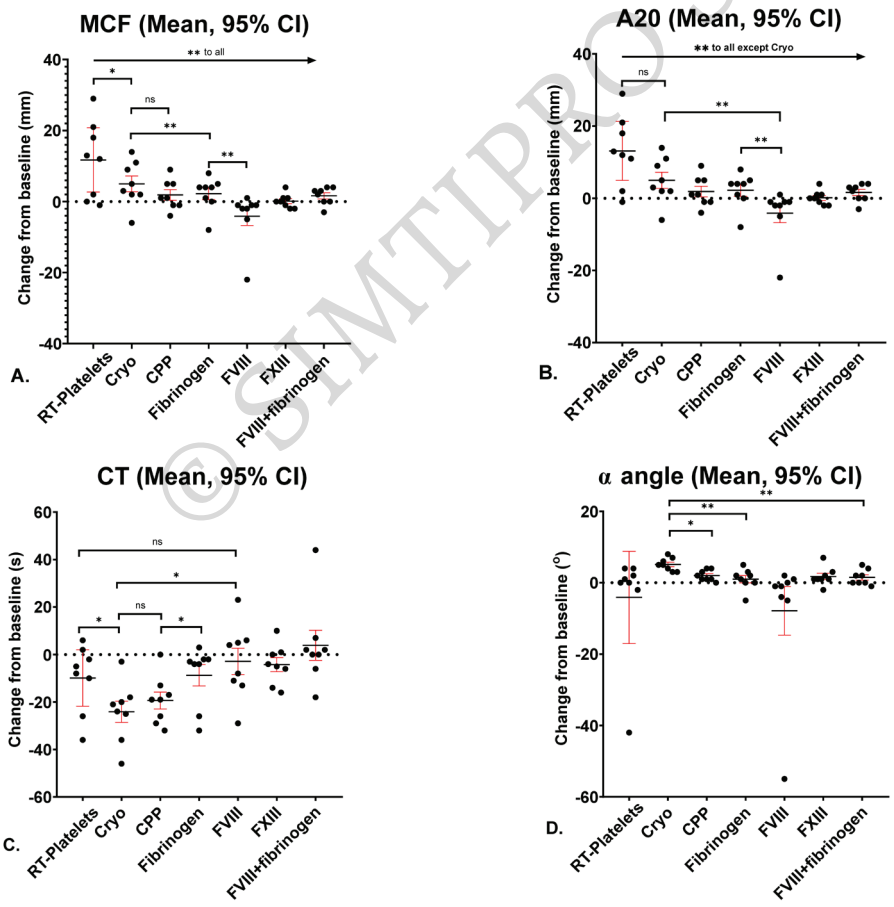

Figure 1 - Change of thromboelastometry values from baseline after addition of plasma and coagulation products *in vitro*
Data points represent values from samples of n=8 individuals, red line and error bars represent means ± SD. MCF includes some values extrapolated by the ROTEM. *p<0.05, **p<0.01, ***p<0.001. MFC: maximum clot firmness; Cryo: cryoprecipitate; CPP: cryopreserved platelets, data were analyzed by 2-sided paired τ test; NS: not significant.

the MCF, this graph includes device software extrapolated values. The A20 showed a significant improvement of 13.1 mm from baseline (95% CI: 5-21 mm) p<0.01) following the addition of RT-stored platelets (**Figure 1B**), but there was no statistically significant improvement in CT (**Figure 1C**), AA (**Figure 1D**) or clot formation time (CFT).

There was a trend towards an improved A20 after treatment with cryoprecipitate (5 mm, 95% CI −0.25-10 mm, p=0.06). This was not statistically different from the change seen with platelet addition (p=0.19, **Figure 1B**). With cryoprecipitate, the CT reduced by a mean of 24.1s (95% CI 13-35 s, p<0.05, **Figure 1C**), which was 14.3 s shorter than RT-stored platelets (p<0.05). The AA increased by 5.1° (95% CI 3.6-6.6°, p=0.001, **Figure 1D**), which resulted in a mean level of 81.8°, just above the normal reference range (63-81°). The CFT was shortened with cryoprecipitate by 82.6 s (95% CI 6.1-159 s, p<0.05), which was not statistically different to the improvement observed following RT-stored platelets.

Fibrinogen concentrate addition did not improve any of the reported parameters when compared with baseline values. RT-stored platelets were superior to fibrinogen in increasing the A20 with a 10.9 mm difference in the mean (p=0.002). The A20 was not significantly different between cryoprecipitate and fibrinogen concentrate (**Figure 1B**), however, cryoprecipitate was superior to fibrinogen with respect to clot initiation as measured by the CT (p<0.05, **Figure 1C**) and AA (p<0.01, **Figure 1D**).

Cryopreserved platelets addition reduced the CT by a mean of 19.4s (95% CI: 11-28 s, p=0.001) and increased the AA by 2° (95% CI: 0.7-3.3°, p<0.01) when compared

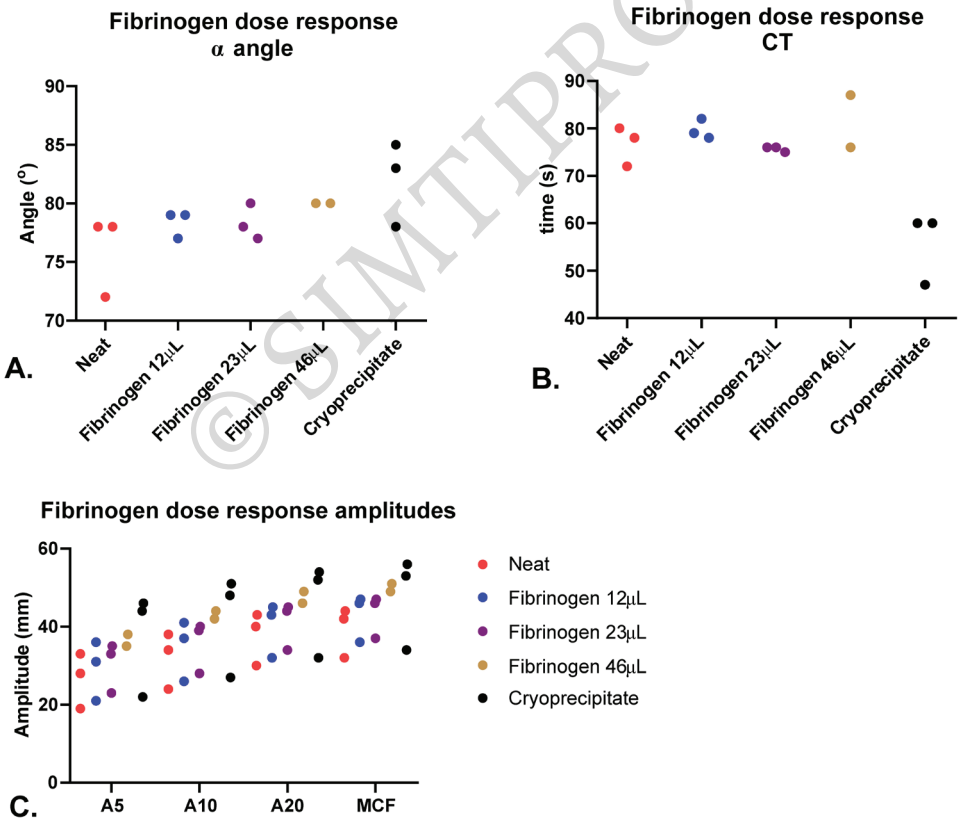

Figure 2 – Viscoelastic parameters with addition of fibrinogen
Increasing concentrations of fibrinogen were added to samples (patients 6-8) which then showed a dose response curve for the AA (**A**) and amplitudes (**C**) parameters, suggesting the benefit of cryoprecipitate over fibrinogen may be consistent with increased fibrinogen concentration alone. By contrast, there was no dose-response curve with CT (**B**), suggesting the unique effect of cryoprecipitate on CT was not simply due to increased fibrinogen concentration.

with baseline, indicating an effect on clot initiation, with this improvement being not statistically different to RT-stored platelets (p=0.07). The effect of cryopreserved platelets on AA was 3.1° less than that of cryoprecipitate (p<0.02) but the CT was not statistically different. There was no impact of cryopreserved platelets on the A20 or any other clot amplitudes.

Addition of either FVIII/VWF or FXIII did not have any significant effect on thromboelastometry parameters. As there was a difference between fibrinogen and cryoprecipitate, the addition of FVIII/VWF concentrate to fibrinogen was compared with fibrinogen alone and with cryoprecipitate, with no additional impact on thromboelastometry parameters observed over that of fibrinogen alone (**Figure 1 A-D**).

Due to the differences in thromboelastometry measurements between cryoprecipitate and fibrinogen, additional dilutions of fibrinogen were tested and the fibrinogen concentration in cryoprecipitate determined in a subset of samples. The fibrinogen concentration in cryoprecipitate was higher than anticipated (34 g/L). The dilutions showed a dose-response to fibrinogen for the AA (**Figure 2A**), but not the CT (**Figure 2B**), which was shortened by cryoprecipitate, indicating a fibrinogen independent effect. Amplitudes at all time-points also showed dose-response relationships (**Figure 2C**). The final fibrinogen concentrations were determined for those samples with known fibrinogen levels. Fibrinogen correlated with A20 (R^2=0.70) and AA (R^2=0.67). There was a negative logarithmic correlation with fibrinogen and CFT (R^2=0.80), but no correlation with CT (**Figure 3**).

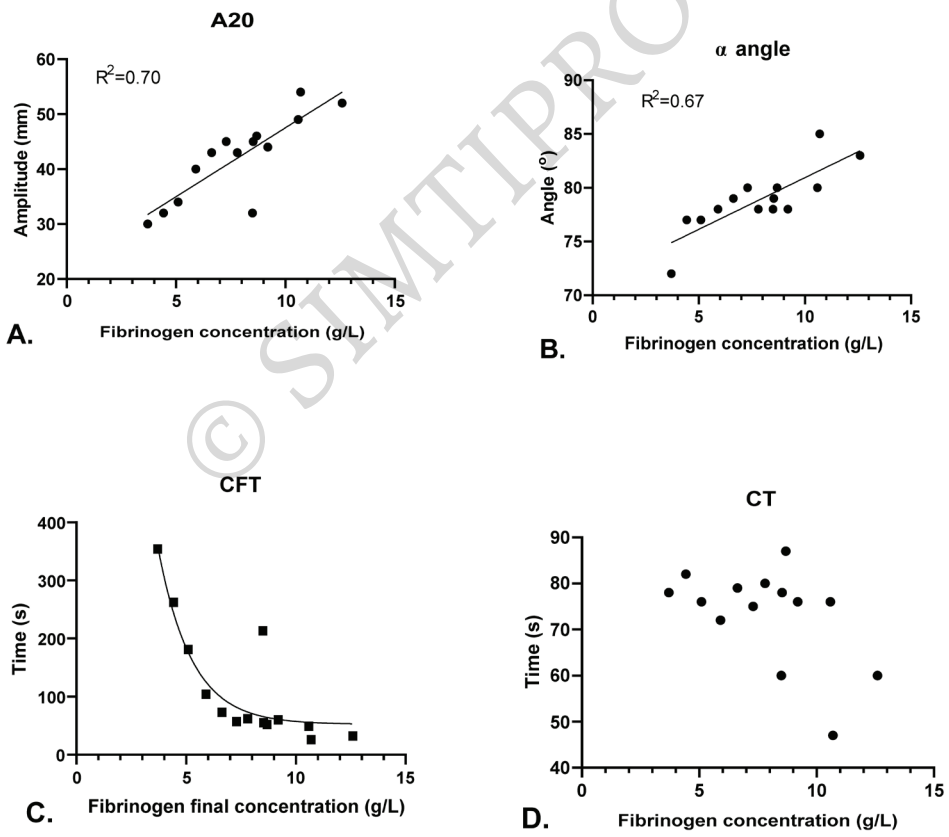

Figure 3 – Viscoelastic test parameters with increasing fibrinogen
Correlations between the final calculated fibrinogen concentrations within the samples and EXTEM parameters, for samples 6-8 where escalating fibrinogen dosing was performed. Correlations between A20 (**A**) and AA (**B**) were linear. CFT showed a strong logarithmic correlation (**C**), but CT was not affected by the final fibrinogen concentration.

DISCUSSION

Managing bleeding thrombocytopenia patients can be difficult in rural and remote areas where room temperature stored platelets are not kept in inventory due to their limited shelf life. Cryopreserved platelets may be an option in these circumstances, however in this ex-vivo study, cryoprecipitate, a product that is widely available and has a long shelf life, was found to be the most effective in improving haemostatic parameters measured by ROTEM. Although there was a trend to the addition of platelets having the greatest absolute EXTEM A20 increment, this change was not statistically different to that seen with cryoprecipitate. While this improvement appeared proportional to fibrinogen increases, cryoprecipitate improved the CT and AA whereas fibrinogen concentrate did not. The CT and AA were improved with cryopreserved platelets; however, this improvement was inferior to that seen with cryoprecipitate.

The fibrinogen dose-related improvement in EXTEM amplitudes in thrombocytopenia previously reported[10] was confirmed in this study. While the addition of fibrinogen was designed to match an infusion of 100 mg/kg, as previously shown to have a similar effect to the transfusion of platelets[6,7], our ex vivo study did not confirm the equivalence of platelets and fibrinogen at this dose. This may be due to our ex vivo platelet addition assuming 100% platelet recovery, which is higher than seen with transfusion[11,12]. Therefore the ex vivo platelet dose may have had a greater effect than the equivalent transfused dose due to early platelet clearance *in vivo*.

The superiority of cryoprecipitate over fibrinogen to improve thromboelastometry was in part due to the higher-than-expected fibrinogen concentration in cryoprecipitate. However, the reduction in CT was not seen with fibrinogen alone, consistent with prior studies[6,7], and suggests a potential advantage of cryoprecipitate over fibrinogen concentrate in clot initiation. This was further explored through the addition of FVIII/VWF and FXIII, neither of which showed a significant effect on thromboelastometry parameters. The lack of benefit from additional FXIII is consistent with the findings from an in vitro model of dilutional coagulopathy with massive bleeding, showing no benefit from FXIII replacement[13].

Cryopreserved platelets have been advocated as a replacement for RT-stored platelets when appropriate platelet concentrates may be difficult to source, such as in patients with human leukocyte antigen antibodies[1,2,14,15]. Treatment with cryopreserved platelets reduced the time to initiation of clot formation, a finding previously reported by others[1,4,16] but there was no effect on the overall clot strength. While some groups have found a difference in viscoelastometry amplitudes[1,16], this has not been consistently observed[17]. Our results also show minimal, if any effect on clot amplitudes.

The significant fragmentation and activation of cryopreserved platelets ensures their availability as a source of phospholipid in the coagulation cascade[5], but at the expense of cell surface glycoproteins (GP) GPIba, GPVI and aIIbβ3 (GPIIbIIIa) which contribute markedly to clot strength via engagement of ligands VWF, fibrinogen and fibrin[4,18]. The lack of improvement in thromboelastometry amplitudes suggests that the cryopreserved platelets contribute minimally to the fibrin-platelet meshwork in the final thrombus. While cryopreserved platelets may be pro-haemostatic, they appear to have a different mechanism of action to that of RT-stored platelets with a greater effect on clot initiation time than on clot firmness. Importantly, they may not be the best substitute for platelets in severe thrombocytopenia and consideration should be given to transfusion roles for CPP other than as a substitute for RT-stored platelets.

Limitations of this study include that it is entirely in vitro, and that it measures clotting outcomes only by viscoelastic testing, which may not detect all aspects of platelet function. In addition, the use of small aliquots of cryopreserved platelets is different to the storage of cryopreserved platelets for clinical use. This is a standard practice, for example with cryopreserved stem cells, however an effect on cryopreserved platelet function cannot be excluded.

Cryopreserved platelet transfusions result in minimal increments in platelet counts on standard impedance blood analyzers[1]. Despite this, the potential to improve thrombus formation and reduce bleeding has been postulated, mediated by pro-haemostatic microparticles not included in automated platelet counts. However, there is no clear method for detecting the effect of cryopreserved platelets following transfusion, although functional global coagulation assays are preferred[19]. There have been several clinical studies undertaken with cryopreserved

platelets, but further studies will be needed to establish the hemostatic potential of cryopreserved platelets and their clinical role[1,2,15,20].

CONCLUSIONS

These results indicate that cryoprecipitate is likely to be at least as effective if not superior to fibrinogen concentrate or cryopreserved platelets, in severe thrombocytopenia. Transfusion of these frozen or lyophilised products may be of value in centres where RT-stored platelets are not available.

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AUTHORSHIP CONTRIBUTIONS

PC, LC and EEG conceived and designed the study, interpreted the results and wrote the manuscript. PC recruited patients and conducted the experiments.
Data sharing statement: original data are available from the corresponding author.

DISCLOSURE OF CONFLICTS OF INTEREST

PC has received support from CSL Behring for research consumables and travel. There are no other conflicts of interest to declare.

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