Red cell-derived microparticles (RMP) as haemostatic agent

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Summary
Among circulating cell-derived microparticles, those derived from red cells (RMP) have been least well investigated. To exploit potential haemostatic benefit of RMP, we developed a method of producing them in quantity, and here report on their haemostatic properties. High-pressure extrusion of washed RBC was employed to generate RMP. RMP were identified and enumerated by flow cytometry. Their size distribution was assessed by Doppler electrophoretic light scattering analysis (DELSA). Interaction with platelets was studied by platelet aggregometry, and shear-dependent adhesion by Diamed IMPACT-R. Thrombin generation and tissue factor (TF) expression was also measured. The effect of RMP on blood samples of patients with bleeding disorders was investigated ex vivo by thromboelastography (TEG). Haemostatic efficacy in vivo was assessed by measuring reduction of blood loss and bleeding time in rats and rabbits. RMP have mean diameter of 0.45 µm and 50% of them exhibit annexin V binding, a proxy for pro-coagulant phospholipids (PL). No TF could be detected by flow cytometry. At saturating concentrations of MPs, RMP generated thrombin robustly but after longer delay compared to PMP and EMP. RMP enhanced platelet adhesion and aggregation induced by low-dose ADP or AA. In TEG study, RMP corrected or improved haemostatic defects in blood of patients with platelet and coagulation disorders. RMP reduced bleeding time and blood loss in thrombocytopenic rabbits (bu-sulfan-treated) and in Plavix-treated rats. In conclusion, RMP has broad haemostatic activity, enhancing both primary (platelet) and secondary (coagulation) haemostasis, suggesting potential use as haemostatic agent for treatment of bleeding.

Keywords
Animal bleeding models, haemostatic agent, RMP–platelet interaction, red cell microparticles (RMP), thromboelastography

Introduction
Cell-derived microparticles (C-MP) are membranous vesicles released during cell activation and apoptosis (1). Release of C-MP is associated with reversal of membrane asymmetry, exposing procoagulant phospholipids such as phosphatidyl serine (PS) to the external medium. This provides negatively charged phospholipid membrane surface for the binding of clotting factors and assembly of tenase and prothrombinase, thereby accelerating thrombin generation and blood clotting (2). They are found in normal circulation at low levels and at higher levels in thrombotic, inflammatory, and cardiovascular disorders (3).

The role of C-MP in haemostasis and thrombosis has been receiving increased attention in recent years (3-5). Among the many species of circulating C-MP, those from red cells (RMP) have been investigated least in regard to their role in haemostasis and thrombosis. RBCs have traditionally been viewed as comparatively inert in haemostasis and thrombosis (6). However, a number of clinical and laboratory observations suggest a significant role in coagulation and inflammation (7, 8).

Platelet-derived MP (PMP) has been proposed as an infusible haemostatic agent for treatment of bleeding disorders (9-11) but development was apparently halted. Our studies in vitro, together with in vivo animal studies (12-15), suggested that RMP would have broad haemostatic activity and appear well suited for use as haemostatic agent.

In this paper, we report on indicators of haemostatic activity of RMP by in vitro and ex vivo methods, and also document in vivo efficacy in rats and rabbits.

Materials and methods
Preparation of RMP
Packed red blood cells (40 ml) were diluted 2.5-fold with isotonic saline, centrifuged at 650 x g for 10 minutes (min) and supernatant discarded. The RBCs were re-suspended in 40 ml saline and subjected to high-pressure extrusion via the TS2 Bench-top Cell Disruptor (Constant Systems, Kennesaw, GA, USA). The output was centrifuged at 250 x g for 10 min to remove large debris; the pellets and bottom 1 ml of each tube was discarded. The remaining super-

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natants containing the RMP were centrifuged at 41,000 x g for 45 min, and the RMP pellet was washed with 20 ml 0.9% saline and recentrifuged. The final pellet was suspended in 10 ml total of isotonic saline, homogenised with a 1 cm diameter Teflon pestle, and aliquoted into ten vials of 1 ml each and stored at -80°C.

**Preparation of PMP, EMP, and LMP**

Endothelial MP (EMP) were prepared from human brain microvascular EC tissue culture. Platelet MP (PMP) were prepared from fresh normal platelet-rich plasma (PRP) by exposure to calcium ionophore, 1 µmol/l. Leukocytes were isolated by Ficoll density centrifugation and were stimulated by lipopolysaccharide (LPS), 1 ng/ml, to release leukocyte MP (LMP), which were collected by centrifugation.

**Flow cytometric characterisation of MP**

For labelling of RMP, PMP, EMP, or LMP, 20 µl of MP samples were incubated with 4 µl of anti-CD235a-PE, CD41-FITC, CD105-PE, or CD45-PE mAb (Beckman Coulter, Fullerton, CA, USA), each separately. For labelling annexin V-positive MP, 20 µl of MP were incubated with 2 µl of annexin V-FITC (Sigma, St. Louis, MO, USA) plus 2.4 µl of 40 mM CaCl2. The samples were then gently shaken (60 rpm) for 20 min, then diluted with 500 µl of 0.9% NaCl plus 10 mM HEPES, pH 7.4. Flow cytometry was performed in a Beckman Coulter FC-500. The voltage and gain settings for forward scatter (FS), side scatter (SS), fluorescence 1 (FL1), fluorescence 2 (FL2), and gating of MP (<1 µm) were calibrated by Megamix beads based on the protocols recommended by the manufacturer (Biocytex, Marseille, France). The counting of MP was triggered by FL1 or FL2 rather than FS, for improved detection efficiency. The cut-off limits of fluorescence due to non-specific binding were determined by labelling MP with FITC- or PE-labelled non-immune isotypic mAb. The run time for each sample was 30 seconds (sec). The flow rate was determined by running known amount of diluted control RBC samples. The concentration of MP was calculated based on the following formula: (total MP count) x (1/run time) x (1/flow rate).

**Determination of size distribution of RMP produced by high pressure extrusion**

Size distribution of RMP was measured by Doppler electrohydrodynamic light scattering analysis (DELSA™ Nano, Beckman Coulter) at the IST Particle Characterisation Laboratory (Miramar, FL, USA). Measurements were performed under the following conditions: Temperature = 25°C, refractive index = 1.33 ± 0.02, viscosity = 0.88 ± 0.02 centipoise (cP), and scattering intensity = 9,500 – 11,500 cps. A representative histogram of RMP size distribution is shown in Figure 1A. A secondary method for size estimation was flow cytometry. Three sizes of Megamix beads cited above (0.5, 0.9, 3.0 µm) were run at the settings used for MP analysis, and log bead size was plotted vs log FS to yield a linear regression line: Log (particle size) = (0.4305)(log FS) + 2.4613, [R2 = 0.984]. By means of this relation, and using fluorescent trigger for event counting, we are able to extend the range of size detection to about 0.1 µm.

**Tissue factor (TF) expression**

Expression of TF on the four lineages of MP described above was measured by flow cytometry using FITC-labelled mouse anti-TF mAb (Clone VD8, Sekisui Diagnostics, Lexington, MA, USA). To minimise non-specific binding, aggregates of mAb in vial was avoided by first transferring supernatants of anti-TF mAb into another tube after low-speed centrifugation upon receipt, then pipetting from the supernatant without disturbing any sediment that may have later formed. Incubations were in presence of 1% BSA, then 2 hours (h) was allowed to elapse between dilution (1:20, from 50 µl incubation volume to 1.0 ml) and aspirating into flow cytometer, to favour dissociation of non-specific bound mAb. These conditions eliminated non-specific binding as judged by negligible binding on RMP.

**Thrombin generation**

Two methods were used to measure thrombin generation by MP. First, the fluorescence method of Hemker et al. (17) (Fluoroscan Ascent), termed the “Calibrated Automated Thrombin” (CAT) generation assay, adapted for MP-mediated thrombin generation as described (18). Briefly, to pooled normal plasma centrifuged to be nearly particle-free (PPF) was added a fixed concentration of MP, as measured by Ulex counts in flow cytometry, then calcium to initiate reaction. No thromboplastin was added since the aim of the test was to measure endogenous procoagulant activity.

We compared thrombin generation by RMP, PMP, and EMP at concentrations giving maximum activity. Preliminary dose-response experiments established that the quantity of RMP used (f.c., 1x10^8/ml) was saturating, i.e. gave maximal thrombin generation; the other MP required slightly less to achieve maximal response. Therefore, 1x10^7/ml was used for all three MP lineages.
A

B

C

D
Platelet adhesion

Platelet adhesion was measured using a cone-and-plate device (Diamed, model Impact-R, Cressier, Switzerland) which allows control of shear rate, and yields direct measurement of number and size of objects adhering to the plastic disc (mainly platelets) (12-14).

Platelet aggregation

Platelet aggregation was measured by a multi-channel aggregometer (Chrono-Log, Havertown, PA, USA). Platelet aggregation was initiated by adding low doses of adenosine diphosphate (ADP; 0.2 µM) or arachidonic acid (AA, 0.3 mM) into platelet-rich plasma (PRP) anticoagulated with heparin (10 U/ml) instead of sodium citrate. Platelet aggregation was measured in the absence and presence of RMP in two channels simultaneously (12-14).

Ex vivo studies by thromboelastography (TEG)

This method was used to investigate effect of RMP on blood samples of patients with platelet or coagulation disorders (12-14). Samples were run according to manufacturer’s instruction unless otherwise stated in text. Blood was drawn from volunteers or patients into blue-top Vacutainer® tubes (sodium citrate). The first 3 ml were discarded to avoid contamination by TF from the venipuncture. All donor blood was tested not more than 3 h post-venipuncture. To 330 µl whole blood (WB) was added 11 µl of either 0.15 M saline or RMP (f.c., 1 x 10^6/ml), mixed by pipette and incubated for 1 min at room temperature. The mixture was then added to a well containing 20 µl of 200 mM CaCl2 and run on the TEG to the achievement of maximal amplitude. The main parameters of TEG data, all in presence and absence of RMP, were as follows: R (lag to initial fibrin formation), K (time to amplitude 20mm), A (angle, initial rate), MA (maximum amplitude, reflecting platelet function), and G (torque, or shear elastic modulus, a measure of clot firmness). To study contact pathway in thrombin generation by RMP, procedure was modified by drawing blood in presence of corn trypsin inhibitor (CTI) as described in legend to ▶Table 1.

Animal model I: Rabbit

The rabbit ear BT was used (15), adapted from Blajchman et al. (19). In the present study animals were rendered thrombocytopenic by injection of busulfan 20 mg/kg on days 0 and 2, subcutaneously in the back between the shoulder blades, causing platelets to fall to nadir of 2-5 x 10^3/µl by day 7-12. Briefly, the ear of the anesthetised rabbit, with platelet count 30-60 x 10^3/µl, was pierced by no. 11 scalpel blade (6 mm wide) in a region of ear devoid of macroscopic (visible) vessels, maintained at 37°C, and bleeding time (BT) was defined as the elapsed time for absence of blood stain on dab of filter paper. Busulfan (Sigma) solution was prepared by making a slurry solution at approximately 100 mg/ml in polyethylene glycol (Molecular mass = 400) and stirring for 2 h at room temperature. This suspension was then brought to final concentration of 10 mg/ml in polyethylene glycol and heated at 75-80°C with stirring for 2 h to dissolve residual crystals of busulfan. Dose of RMP administered ranged from 1x10^4 to 9x10^6 per kg, in volume based on RMP stock concentration of 3x10^6 per ml. Concentrations were based on counts of CD235a-positive particles in flow cytometry. Thus for example, for a 3 kg rabbit, the injected volumes were 1.0 ml to 9.0 ml infused via rabbit marginal ear vein 5 min prior to initiation of bleeding, at rate of 2 ml/min.

Animal Model II: Rats

Male Sprague-Dawley rats were weighed (250-350 g) and anesthetised by placing in a sealed container with 1-2 ml Isoflurane. It was then intubated for maintaining anesthesia with isoflurane-oxygen by respirator. Temperature was monitored by rectal probe, and a heating pad under the surgical board maintained body temperature. The neck was shaved and an incision was made to find the jugular vein and carotid artery. Vessels were briefly tied off upstream of the insertion point, and a cannula was secured with suture thread. Minor bleeding is stopped with a cautery tool. The jugular vein is used for injections and the carotid for fluids (lactated Ringer’s) and to monitor heart rate. Bleeding was initiated by clipping 3 mm of toe (alternatively tail) and end-point was cessation of bleeding for at least 1 min. Total blood loss (TBL) was also measured. To prolong BT, Plavix 10 mg/kg was injected IV via jugular cannula 30 min prior to measuring BT, in presence vs absence of RMP (1 x 10^6/kg) bolus given intravenously at 5 min prior to initiation of bleeding. For a 300 g rat, the total injected volume of RMP was 300 µl, and the infusion time was 30 sec. [12,14]

Table 1: Effect of corn trypsin inhibitor (CTI) on response to RMP.

<table>
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<th>Variables</th>
<th>Lag time (R, min)</th>
<th>Angle (A, degree)</th>
<th>Max rate TG (mm/min)</th>
<th>Time to max rate TG (min)</th>
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<td>17.4</td>
<td>31.0</td>
<td>3.32</td>
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</tbody>
</table>

Results

General properties

Mean diameter of the RMP of eight different batches was 0.45 ± 0.09 µm, as measured by DELSA Nano (see Methods). A representative histogram of size distribution is shown in ▶Figure 1A. It...
Jy et al. RMP as haemostatic agent shows that about 95% of RMP have diameters between 50 nm and 3.0 µm. Size analysis by flow cytometry yielded somewhat larger mean diameters (0.67 ± 0.12 µm, n=8). This is expected since flow cytometry cannot detect particles in the smaller size range, < 0.1 µm.

About half of the RMP were positive for annexin V binding, a proxy for procoagulant phospholipids (PL) such as phosphatidyl serine (PS). Purity was assessed using markers of antigens specific for other lineages (platelets, endothelia, leukocytes) and was always <0.01% other cell types.

**TF expression and thrombin generation**

The percentages of TF-positive RMP and other MP are shown in Figure 1B, as measured by anti-TF mAb. Notice that RMP were unique in being devoid of detectable TF. A small fraction of PMP (= 2–4%) were positive for TF by this assay. Although TF associ-
ated with PMP has often been reported by many investigators (20, 21), other reports found no evidence of TF on PMP (22, 23). It has been suggested that some of the positive results may be due to non-specific binding of commercial mAb (22). Our study took precautions to minimise non-specific binding (see Methods).

In a series using the same MP isolates, we measured thrombin generation by RMP, PMP, and EMP (16). As seen in Figure 1C, RMP generated thrombin robustly but with a more extended lag time. In contrast, EMP produced an early but short-lived burst of thrombin generation. Thrombin generation by PMP began after a lag of about 10 min, rising to maximum amplitude ≈ 400 nM thrombin. The RMP curve began after prolonged delay, about 20 min post-calcium, and rose nearly as high as with PMP. These observations are consistent with absence of TF on RMP. Our data are consistent with the findings reported by Van der Meijden et al. (23) that RMP generate thrombin via intrinsic pathway.

To explore the question of contact activation by RMP, we looked at effects in blood drawn in presence of corn trypsin inhibitor (CTI), which blocks the contact pathway. Results are summarised in Table 1. It shows that in the control (RMP absent), clotting time R was nearly doubled by CTI, from 13.2 to 24.5 min, and maximal rate (MRTG) was halved, from 6.3 mm/min without CTI to 3.09 mm/min with CTI. We attribute the coagulation seen in presence of CTI to small amount of TF in the blood. Looking now at the effect of RMP in presence of CTI, notice that effect of RMP is not completely abolished: R is shortened by RMP, from 24.5 min to 17.4 min, and angle A is increased, from 27.7° to 31.0° by RMP. These experiments shows that RMP not only generate thrombin via contact pathway, but also can enhance coagulation independent of the contact pathway, probably via TF pathway by contributing procoagulant PL.

Thrombin generation by TEG method

Again using the TEG method, Figure 1D shows a representative tracing with normal whole blood in presence vs absence of RMP. The lag time is reduced, the initial rate is increased (angle of rise), and the peak amplitude is increased by presence of RMP.

Augmentation of platelet function by RMP

Platelet adhesion

Using a cone-and-plate shearing device (Impact-R, see Methods), we assessed effect of RMP on shear-induced adhesion. Representative fields of plates run in the absence (left, Figure 2A) and presence (right, Figure 2B) of RMP show that the presence of RMP results in about 20% larger aggregate size (“AS”) of the adhering objects (mostly platelets). The number of adhering objects (“OB”) was correspondingly reduced by RMP. The percent surface coverage (“SC”) was unchanged by RMP. Results of five replicate experiments are summarised in Figure 2C. Interestingly, the second level structure is "Platelet adhesion". 

Figure 3: RMP effect on TEG of patient blood samples. Meaning of parameters: R, Lag time to initiate fibrin formation. MA, Maximum Amplitude. A, Angle, reflects early of fibrin formation. Black TEG tracing is without RMP; red is in presence of RMP. Note that RMP shorten R, increase A and MA. A) Aplastic anaemia. The TEG tracing shown is representative of several TEG run on other patients with same disorder. In the example shown, patient had severe pancytopenia from aplastic anemia with platelet count ≈ 3,000. Notice the almost flat response in absence of RMP, and correction to nearly normal of R, A, and MA by RMP. B) Acetyl salicylic acid (ASA, aspirin) therapy. In these patients, platelet dysfunction was induced by antiplatelet therapy. Note that addition of RMP largely corrected abnormal parameters, most notably initial rate (A, angle of rise) and normalization of lag time, R. C) Plavix therapy. Plavix also induces platelet dysfunction but by a mechanism distinct from ASA, yet RMP correct the TEG abnormalities similarly. D) Haemophilia A with inhibitor. Patient with haemophilia with mild inhibitor. Blood sample was obtained in stable condition without active clinical bleeding. Note prolonged R, decreased A and MA. Addition of RMP normalised these parameters. E) Warfarin (Coumadin) therapy. Coagulopathy induced by Coumadin therapy (Prolonged R, decreased A and MA) is largely corrected by RMP, seen by shortening or R, increase in A and MA. F) Lovenox therapy (low-molecular-weight heparin, LMWH). Coagulopathy induced by LMWH is corrected by addition of RMP ex vivo. Similar correction by RMP was observed in coagulopathy induced by dabigatran (not shown).
Figure 4: Rabbit ear bleeding model. A) Thrombocytopenia induced by busulfan. Figure shows the time-course of platelet drop following busulfan treatment and its recovery. To maximise consistency, testing was performed at platelet count 40–80 $\times 10^3/\mu l$ range. B) Rabbit ear bleeding time: Relation to platelets. Rabbit ear bleeding time depends mainly on platelet counts. In normal platelet counts, bleeding time is too short to evaluate effect of RMP. Therefore study was performed at lower platelet counts which prolong bleeding. C) Rabbit ear bleeding time (BT) response to RMP intervention. Left bar is BT of untreated animals and next bar shows prolongation of BT to about 500 sec by busulfan pre-treatment. Remaining bars show dose-dependent reduction of prolonged BT by RMP infusion. RMP dosages were increased three times from $1 \times 10^9$ to $3 \times 10^9$ and $9 \times 10^9/kg$. Note statistically significant shortening of BT at each higher dosages of RMP.
20% increase in AS due to presence of RMP (p<0.02) was consistently seen but only at shear rate of 1,800 s⁻¹, not at lower (1,200 s⁻¹) or higher (2,700 s⁻¹) rates. Since 1,800 s⁻¹ is close to the shear rate in normal arteriole blood flow this result suggests that C-MP are tailored specifically to enhance platelet deposition in injured small vessels.

**RMP on platelet aggregation**

The effect of RMP at therapeutic dose (determined in animal studies) on platelet aggregation induced by threshold concentrations of two different agonists are studied. As shown in Figure 2D (for ADP) and Figure 2E (for AA), RMP acted to largely prevent recovery of platelets, i.e. prevented dissociation. This effect was seen only at threshold concentrations of agonists (0.2 μM ADP, 0.3 mM AA), which are more physiologically realistic than the saturating levels used in standard lab tests (10 μM ADP, 3 mM AA). Therefore, these results could be critically important *in vivo*.

**Ex vivo study on patient blood samples**

The effect of RMP added to fresh blood samples from patients with bleeding disorders are shown in Figure 3A-F. Each tracing is representative of several different patients having the same or similar disorder. In all figures of this series, black lines are in absence and red lines in presence of RMP. Correction or improvement of abnormal parameters in patients with severe thrombocytopenia (platelet count = 3,000) from aplastic anaemia is shown in Figure 3A. Effect of RMP on platelet dysfunction induced by aspirin is shown in Figure 3B, and by Plavix in Figure 3C. Correction is signified mainly in reduction of lag time ("R") and steeper angle ("A") of initial rate in presence of RMP. Thus, RMP correct haemostatic aberration due to both deficiency of platelet number or function.

RMP also improved coagulopathy arising from congenital coagulation disorder (FVIII with mild inhibitor), shown in Figure 3D, and acquired coagulopathy due to anticoagulant therapy with warfarin (Figure 3E) and low-molecular-weight heparin, Lovenox (Figure 3F).

**Haemostatic efficacy *in vivo* (I): Rabbit**

In order to assess efficacy of RMP *in vivo*, we studied an ear bleeding model (15). Rabbit ear BT was prolonged by pre-treatment of the animals with busulfan as described in Methods. In these initial studies, bleeding was induced by standardised incision of the ear of anesthetised animals (see Methods). Figure 4A shows the time-course of platelet decline in the rabbit following busulfan treatment, reaching nadir after about 10 days. Figure 4B shows the relation of platelet count to the BT. The studies were performed at platelet count of 30-60 x 10⁶/μl; in these thrombocytopenic animals, the BT was prolonged to 580 ± 130 sec (platelet count 30-60 x 10⁶/μl).

Injection of RMP at 1 min post-injury induced significant shortening of BT from mean 580 sec to 440 ± 90 sec (n = 8) at dose 1 x 10⁹ RMP/kg (p<0.01); to 280 ± 65 sec (n = 8) at dose 3 x 10⁹ RMP/kg (p < 0.001) and to 230 ± 55 sec (n = 6) at dose 9 x 10⁹ RMP/kg (p < 0.001) (Figure 4C). When platelet counts dropped much below 20,000/μl and BT >15 min, RMP had greatly reduced efficacy. The haemostatic effects also largely disappeared if >30 min elapsed between RMP pre-infusion and making the incision, indicating a short half-life of RMP in circulation.

**Haemostatic efficacy *in vivo* (II): Rat**

In normal (untreated) rats, the BT was 110 ± 45 sec and total blood loss was 0.14 ± 0.05 ml. As shown in Figure 5, Plavix treatment (10 mg/kg) prolonged the BT to 640 ± 90 sec and increased the blood loss to 2.3 ± 0.8 ml. Injection of RMP at 1 x 10⁹/kg to the Plavix-treated rats significantly shortened the BT, to 270 ± 50 sec (Figure 5A) and reduced blood loss to 0.32 ± 0.14 ml (Figure 5B).

**Discussion**

It has recently been emerging that RMP can activate the contact pathway, as well as support thrombin generation in both the TF and contact pathways (23, 24). Our clinical observations suggest...
that RMP play an important role in haemostasis. For example, we observed that patients with ITP with elevated levels of RMP had reduced bleeding compared to a cohort with similar platelet counts but low RMP (25). This led us to investigate the procoagulant properties of RMP for possible use as haemostatic agent. High-pressure extrusion was found to be a practical method for the large-scale production of RMP, as required for assessing haemostatic efficacy in animals. This paper summarises data of RMP as insufusable haemostatic agents (12-15).

Other types of cell-derived MP have been proposed as haemostatic agents, notably PMP (9-11). However, RMP offer a number of advantages. First, of all blood cells, only red cells are available in sufficient abundance for practical production by high-pressure extrusion. This abundance will allow production of RMP from a patient’s own blood (autologous option) in anticipation of surgical or invasive procedures. Second, RMP are free of TF; making them less likely to be thrombogenic. Third, it is well known that transfusion of platelets or leukocytes are more often associated with adverse reactions. For this reason, current practice minimises these cells in packed RBC (leuko-reduced). It is reasonable to expect that MP derived from these cell types will have safety profiles similar to their parent cells, suggesting that RMP will be least likely to cause complications. However, this expectation must be confirmed by rigorous experimental data.

The mechanisms of haemostatic action of RMP remain to be fully elucidated. It appears that RMP work in at least three ways, as shown in this report: i) by supplying procoagulant phospholipids (PL) to propagate coagulation, ii) by contact activation, and iii) by augmenting platelet function. Regarding i), we find that about 50% of RMP are positive for annexin V binding, a proxy for PL-bearing platelets. Such PL support coagulation by serving as sites for assembly of the coagulation factors, thereby accelerating thrombin generation.

Regarding ii), data in this report support the hypothesis that an important aspect of RMP-mediated haemostasis is via the contact pathway, reportedly by activation of FXII (23, 24). Thrombin generation was markedly inhibited by blockade of the contact pathway with CTI (Table 1). Even in presence of CTI, however, RMP accelerated coagulation, presumably by their PL contribution. In studies not yet published, we found that RMP largely restore coagulation in plasma depleted by as much as 95% of FXII.

In regard to iii), we observed that RMP significantly augment platelet function, as reported herein. The mechanism of this effect remains unclear, but may be mediated by a putative “GPIIb/IIIa-like” integrin recently reported on RBC (26, 27).

This dual action of RMP, working on both primary (platelet) and secondary (coagulation) haemostasis, is further supported by our findings that RMP improve or correct haemostatic defect of plaques from patients with coagulopathy (e.g. haemophilia with inhibitor, or induced by anticoagulants) and from patients with thrombocytopения or thrombocytopathy induced by antiplatelet drugs; see Figure 3. This dual action of RMP fulfills criteria for a “universal” haemostatic agent (12, 14).

The RMP studied in this report are not necessarily identical to those arising in circulation, or those released from RBC in storage. The latter appear to be pro-inflammatory and thrombogenic, and may account for some complications associated with transfusion (28). The natural release of MP is caused by entry of Ca$^{2+}$ into the cell, which can be modelled by exposure to calcium ionophore. However, we and others have observed that only a limited number of MP can be released from each cell in this way, <5% of the cell membrane area. In contrast, nearly 100% of RBC membrane is converted to RMP by our method of high-pressure extrusion. The restricted shedding of MP by calcium ionophore appears to be limited by the detergent-insoluble membrane fraction termed “lipid-raft” proteins, which are enriched in MP generated by ionophores and natural means (29). Some complications of transfusion have been attributed to raft-bearing MP (29-31). High concentrations of raft proteins are expected to be hazardous in an infusible product, since they carry many potentially noxious ligands and signalling complexes (30, 31).

In summary, data of this report support the suitability of RMP as a potential haemostatic agent. More rigorous documentation of safety and efficacy in animals, and then in humans, is warranted. Research on mechanisms of actions, immunogenicity, interactions with other cells and MP, and comparisons with other available haemostatic agents remains to be done, but will be helpful in guiding further improvement of the product.

Conflicts of interest
None declared.

References