# Future of platelet formulations with improved clotting profile: a short review on human safety and efficacy data

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**BACKGROUND:** Platelet (PLT) transfusion is a widely used therapy in treating or preventing bleeding and hemorrhage in patients with thrombocytopenia or trauma. Compared to the relative ease of PLT transfusion, current practice for PLT storage at room temperature (RT) for up to 5 to 7 days is inefficient, costly, wasteful, and relatively unsafe.

**STUDY DESIGN AND METHODS:** This study was a review of major advances in PLT derivative products with improved hemostatic potential and safety feature.

**RESULTS:** Recent progress in understanding the PLT activation and host clearance mechanisms has led to reassessments of current and new storage conditions that employ refrigeration and/or cryopreservation to overcome storage lesions and significantly extend shelf life of PLTs with reduced risk of pathogen contamination.

**DISCUSSION:** It is anticipated that future PLT preservation involving cold, frozen, and/or pathogen reduction strategies in proper PLT additive solutions will enable longer term and safer PLT storage.

Five million platelet concentrate (PC) doses are estimated to be administered every year in United States and Europe. In United States, where approximately 40% of all platelet (PLT) doses are administered, the average dose for an adult transfusion consists of 300 to 400 billion PLTs, equivalent to the amount in four to five whole blood–derived collections or one apheresis collection.[1]

Platelets are collected in three different ways: they can be centrifuged from PLT-rich plasma, isolated from buffy coats, or collected directly from the bloodstream by apheresis. There is evidence that the buffy coat and apheresis methods provide better PLTs, because there is a likelihood that centrifuging PLTs from the PLT-rich plasma can lead to partial or complete activation of some of the PLTs.[2] The current prevalent method for PLT storage is at room temperature (RT) with continuous agitation (RT-PLTs). A practical shelf life of only 3 days, due to 2-day sequestration in testing, together with highly variable and unpredictable future usage creates challenges in inventory management.[3,4] To minimize the impact of short-life products, a common strategy employed by many blood centers is to aim for an outdated rate for apheresis PLTs of approximately 10% of total units produced, to allow for a surplus buffer so that there is always enough PLT units on the shelf for unanticipated patient demand.[5] This wastage and inefficient production is a necessary precaution to have

**ABBREVIATIONS:** PC(S) = platelet concentrate(s); PMP(s) = platelet microparticles; RT = room temperature; RT-PLTs = room temperature–stored platelets.

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enough products on hand at all times to meet the highly variable and unpredictable patient demand.

The Determination of the Optimal Platelet Dose Strategy to Prevent Bleeding in Thrombocytopenic Patients (PLADO) clinical trial[6] was conducted to determine whether bleeding outcomes after transfusion of RT-PLTs differed among different age groups, or between any pediatric age group versus adults. This post hoc analysis of the pediatric population demonstrated large and significant differences between age groups for the primary endpoint, which was the percentage of patients having at least 1 day of Grade 2 or higher bleeding after PLT administration. Lack of bleeding response occurred in 86% of patients ages 0 to 5 years, 88% of patients ages 6 to 12 years, 77% of patients ages 13 to 18 years, and 67% of adults. No effect was observed for prophylactic PLT transfusion doses in any age group. Children undergoing stem cell transplantation were found to be at higher risk of bleeding than the same type of adult patients over a wide range of morning PLT counts. Compared with adults, a higher percentage of children experienced oral, nasal, and gastrointestinal bleeding, but a lower percentage experienced skin, soft tissue, and musculoskeletal bleeding. These data suggest that RT-PLTs are not effectively potent in the hemostasis of at least some groups of patients.

Uncontrolled bleeding is considered one of the most likely causes of preventable death in those less than 65 years of age[8] and in the battlefield.[9] The analysis of the medical records from battlefield casualties indicates that 91% of the potentially preventable deaths that occurred after arrival at a hospital were from hemorrhage. Several randomized clinical trials have demonstrated that the early infusion of plasma[10,11] and PLTs[11] in ratios close to 1:1 with red blood cells (RBCs) results in improved survival.

Interestingly, modifications in the collection, storage, and/or processing of PLT products may result in increased bleeding control potency. This review intends to provide a brief overview of PLT product alternatives that may be superior in their activities to control bleeding in the context of traumatic injuries.

## COLD PLTS

Storage of PLTs at 4°C was largely abandoned in the 1970s and 1980s in favor of the longer circulation time of RT-PLT.[12] Cold-stored PLTs (COLD-PLTs) are cleared rapidly from the circulation by hepatocytes and macrophages upon transfusion, resulting in a half-life of approximately 1.3 days compared to 4 days of RT-PLTs. COLD-PLTs undergo an additional number of cytoskeletal changes collectively termed as the cold storage lesion.[13] Different lesions occur early on in COLD-PLTs. These are manifested as an irreversible disc-to-sphere shape change,[14] and an irreversible activation, including increased thromboxane A2 production and an increased surface expression of P-selectin (reviewed in Winokur and Hartwig[15]).

Previous studies have found that cold-stored PLTs could induce clot formation faster, although this was associated with increased microplatelet formation.[16] Research conducted by multiple laboratories in recent years has renewed interest in the storage of PLTs at refrigerated temperatures (1-6°C).[17–19] Cold-stored PLTs aggregate better in vitro than RT-PLTs, form stronger clots, and preserve mitochondrial function better than RT-PLTs; they can be inhibited by physiologic antagonists including nitric oxide and prostacyclin, and are functionally primed in static and flow assays. In addition, the refrigeration of PLTs often inhibits cytokine secretion and contributes to decreased cytokine-associated febrile transfusion reactions.[20] RT-PLTs, on the other hand, have increased PLT counts, well-preserved recovery/survival properties, and reduced aggregate formation activity during storage compared to COLD-PLTs when both are stored in 100% plasma.[17–19]

In the United States, the refrigerated storage of PLTs complies with the US Food and Drug Administration (FDA) Code of Federal Regulations sections 21CFR610.53, 640.24, and 640.25.[21] From the 1960s to the mid-1980s, COLD-PLTs were a standard component for PLT transfusions, before being abandoned for RT-PLTs which had increased in vivo survival.[22,23] However, RT-PLTs have an increased risk of bacterial contamination resulting in sepsis and death[24–27] and may not be effective in rapid hemostatic function in vivo.[28] Cold storage would significantly ameliorate the concern regarding bacterial growth in PLT products. Recently, the US FDA granted an exception to the Code of Federal Regulations (21CFR606.65 [e] and 610.53[c]) to permit sites "to store apheresis platelets at refrigerator temperature (1°-6°C), without agitation for up to 3 days." These cold-stored PLTs may only be used in the resuscitation of actively bleeding patients. Because the data regarding PLTs stored in cold indicated that these PLTs aggregate better and have stronger clot formation than RT-PLTs.[17–19,29] Laboratory research has indicated that PLTs can be stored for longer than 3 days at refrigeration temperatures and maintain many of the properties needed for aggregation and clot formation.[17–19] A small number of clinical studies support the efficacy of refrigerated PLT-containing blood components.[28,30,31] Filip and Aster[30] showed that COLD-PLTs corrected bleeding time better than RT-PLTs.[28] This study in aspirin-treated adults showed significantly better improvement of bleeding times after COLD-PLTs compared to RT-PLTs. In fact, RT-PLTs were shown to have delayed and/or minimal effects on bleeding time in this study. A double-blind study compared the postoperative blood loss in children undergoing open heart surgery with cardiopulmonary bypass with either COLD fresh whole blood (or close to fresh, within 48 hours postcollection), or conventionally stored, reconstituted (RBCs, PLTs, and plasma) whole blood. This study found that the children receiving COLD "fresh" whole blood was associated with significantly less postoperative blood loss than transfusion of reconstituted whole blood, and suggested that the this association may be at least partly due to the presence of better functioning "cold" PLTs.[31] A prospective, randomized, unblinded,

noninferiority, two-arm study of leukoreduced COLD-PLTs in 60 patients undergoing cardiopulmonary bypass is currently ongoing in Norway (ClinicalTrials.gov ID: NCT02495506). In this trial, preliminary data indicate an approximately 30% reduction in chest drain output after chest closure when using COLD-PLTs, with no significant differences in the presence of thromboembolic events.[32]

The current approach to reduce the PLT lesion of COLD-PLTs uses a combination of plasma and PLT additive solution (PAS). The advantages of such a combination include reducing the frequency of transfusion-associated circulatory overload and transfusion-related acute lung injury due to a reduction in plasma volume and, inflammatory mediators. This also releases plasma to be used in other blood derivatives such as fresh-frozen plasma or plasma fractionation products. An additional advantage of the use of PAS in COLD-PLTs is the ability to add novel additives to the PLT storage medium to support regimens such as pathogen inactivation, cryopreservation, or inhibition of the cold-induced lesion. These additives are expected to attenuate the development of the storage lesion by reversibly inhibiting activation or preventing the changes in pH and lactate associated with glycolysis, or reducing the risk associated with pathogen contamination.

A variety of PASs have been developed. Standardized nomenclature labels PAS as PAS-A to PAS-G.[33,34] All these solutions contain varying combinations of citrate, phosphate, acetate, magnesium, potassium, gluconate, and glucose, and the ingredients synergistically act to provide anticoagulation, membrane stabilization, metabolic substrates, buffering activity, apoptosis inhibition, and activation inhibition.[33,35] Recent data has noted that COLD-PLTs collected and stored in PAS-F may be significantly superior in in vitro functional assays than PAS-C–stored COLD-PLTs, especially when analyzed for their ability to adhere to collagen under flow conditions,[35] although their survival may be inferior to the PAS-C–stored COLD-PLTs.[36]

Several novel additives to PLT storage media are under investigation. These solutions aim to attenuate the storage lesions by reversibly inhibiting PLT activation, preventing the changes in pH and lactate associated with glycolysis, or reducing the risk of pathogen contamination. Whether these solutions prevent the storage lesion while retaining the hemostatic activity of cold PLTs is an area of active investigation.

## CRYOPRESERVED PLTS

Cryopreservation of PLTs using 6% dimethyl sulfoxide (DMSO) was developed by Valeri's group at the US Navy in the 1970s.[37] The initial approach involved freezing PLTs suspended in DMSO with postthaw removal of the DMSO. Removing most of the DMSO before freezing, allowing omission of any postthaw processing, was found to produce a comparable product[38] with obvious advantages in rapidly delivering the product to the patient with minimal equipment and training required in hospital blood banks. These studies have been extensively reviewed.[39] A modification of this technique for which thawed PLTs were resuspended in plasma rather than saline was used by the Dutch military in Bosnia and Afghanistan and adopted by other groups.[40,41]

In vitro and ex vivo studies of cryopreserved PLTs have shown promising results. In a baboon model, 54% of thawed cryopreserved PLTs were recovered 2 hours after transfusion, more than that for liquid PLTs stored for 5 days.[42] Cryopreserved human PLTs have a higher capacity to bind Factor (F) V[43] than liquid-stored PLTs and produce more thromboxane A2 after ADP stimulation.[38] In a Phase I human study involving a total of 32 healthy volunteers, cryopreserved PLTs obtained by apheresis and stored at less than 65°C were compared with fresh liquid-stored apheresis PLTs. Radiolabeling allowed assessment of posttransfusion viability in 24 healthy volunteers where cryopreserved PLTs showed approximately 50% reduced 24-hour recovery while the remaining PLTS persisted in circulation for an almost normal life span.[44] More recently, a three-center, dose-escalation, Phase I clinical trial in 28 bleeding (Grades II-IV of the World Health Organization), hematology/oncology patients with thrombocytopenia has been reported. In this study, no thrombotic events were identified and anecdotal experience of hemostatic bleeding response was reported, especially in patients with intracranial hemorrhage.[45]

In a trauma setting, 868 patients have received 1679 cryopreserved PLT units, which worked effectively and without adverse events.[39] Massively transfused trauma patients may be the population that most benefits from early, aggressive treatment with cryopreserved PLTs. In a prospective audit of 46 patients who received massive transfusions, and 234 patients who received less-than-massive transfusions in a NATO military hospital in Afghanistan, receipt of a high ratio of cryopreserved PLTs to RBCs (≥1:8) compared to a lower ratio was associated with increased survival in the massive transfusion group (74% vs. 50%).[46] The single controlled clinical trial of thawed frozen PLTs involved 73 randomized patients receiving cryopreserved or liquid-stored PLTs for treatment of bleeding after cardiac surgery.[47] Blood loss in 24 of the patients who received cryopreserved PLTs was significantly less than in the 29 patients who received liquid-stored PLTs, despite lower posttransfusion PLT increments and a tendency toward decreased PLT survival. There was no observable difference in adverse effects between the groups. These findings are currently being tested in a prospective clinical trial in cardiac surgery patients in Australia,[48] and a similar study is in preparation in United States, funded by the US Department of Defense. Whether the Australian DMSO-cryopreserved PLT preparations result in similar efficacy and/or safety profiles than the preparations in the United States and Europe remains unclear since their in vitro properties seem to be different.[49]

## LYOPHILIZED AND FREEZE-DRIED PLTS

Studies with rehydrated lyophilized PLTs were initially carried out in the 1950s. However, studies in experimental animals with the preparations available then failed to show any hemostatic efficacy in vivo. In the 1990s, washed PLTs treated with 1.8% paraformaldehyde, frozen in 5% albumin, and subsequently lyophilized were used in preclinical trials.[50] Rehydrated lyophilized PLTs are structurally similar under electron microscopy to fresh PLTs, and have been shown to express most of the PLT glycoproteins, albeit at a decreased concentration/density. The membranes of reconstituted, lyophilized PLTs appear capable of supporting thrombin generation and increasing fibrin deposition on exposed subendothelium in vascular perfusion models.[51] In vivo, rehydrated lyophilized PLTs have been evaluated in animal models and have been shown to be hemostatically effective in animals with thrombocytopenia. The duration of their hemostatic activity in vivo is short (approx. 4-6 hr) however, and depends on their interaction with FVIIa.[52–59]

Freeze-dried PLTs are usually prepared from a pool[5–10] of group O in-date leukoreduced apheresis PLT units in the presence of trehalose as a cryoprotectant.[60,61] They are provided in freeze-dried form, can be prepared for use in 5 to 10 minutes by the addition of sterile water, and have demonstrated conservation of the essential characteristics required to form a clot, primary adhesion at the site of injury leading to aggregation, thrombin production, fibrinogen binding, formation of fibrin, and wound closure.[61] Preliminary safety analysis using extremely small doses of freeze-dried PLTs have not identified any significant safety signal (manuscript under revision). An open-label, Phase I, dose-escalation safety study on clinically relevant doses is currently undergoing (NCT03394755) in hematology/oncology patients with thrombocytopenia. While this is a primary safety study, some efficacy variables are assessed in the secondary endpoint analyses.

## PLT MICROPARTICLES AND INFUSIBLE PLT MEMBRANES

A postulated mechanism for greater hemostatic efficacy seen in cryopreservation is that the cryopreservation/thawing process can "preactivate" the PLTs so that they may bound more rapidly to the damaged endothelium after transfusion. Cryopreserved PLTs produce more thromboxane A2 and display more procoagulant activity on their surface in response to stimulation which allows the PLTs to activate coagulation.[38,43] The formation of PS-exposed and tissue factor–expressing PLT microparticles (PMPs) has been recognized as a significant contributor to the hemostatic effect of cryopreserved PLTs.[62–64] Thawed cryopreserved PLT units contain a 15-fold higher concentration of functional PMPs compared to fresh and 5-day RT-PLTs.[62,64] The PMP-containing supernatant of cryopreserved PLTs reduces clotting time and stimulates a twofold increase in phosphatidylserine and tissue factor–induced peak thrombin generation compared to fresh PLT supernatant.[62,64] Interestingly, the clotting time of PMP-filtered cryopreserved supernatant is similar to cryopreserved PC, strongly suggesting that PMPs are the main mediator of the procoagulant activity of cryopreserved PLTs.[63]

A human PMP preparation in the form of infusible PLT membranes (IPMs) from outdated PCs was tested in clinical trials. These IPMs, which consist of spherical vesicles with a diameter of approximately 0.6 μm, have been shown to contain various procoagulant phospholipids. IPMs have been shown in vivo to shorten the prolonged ear bleeding time in rabbits with thrombocytopenia for at least 6 hours postinfusion. However, by 24 hours their hemostatic activity is no longer detectable.[65] Toxicity studies in experimental animals have not found any pathologic thrombogenicity or the potentiation of disseminated intravascular coagulation in endotoxin-treated rabbits. IPMs have been successfully infused into normal human volunteers in Phase I and II clinical trials. The Phase II trials have been conducted in bleeding refractory thrombocytopenic patients and have provided indications of improvement or cessation of bleeding in some cases,[66] but difficulties in demonstrating its efficacy in Phase III clinical trials (reviewed in Nasiri [67] have hampered the regulatory path toward their approval and use in bleeding patients.

## POSSIBLE HAZARDS TRANSFUSING ACTIVATED PLTS

The PLT derivative products mentioned earlier have a common denominator. Their efficacy is associated with PLT activation. Transfusion of currently licensed PCs containing activated PLTs and mediators of inflammation and cellular injury has been linked to PLT transfusion–associated adverse events. Khorana and colleagues[68] examined the medical records of 15,237 hospitalized cancer patients who received PLT transfusions between 1995 and 2003 at 60 different medical centers. Interestingly, they found that patients who received PLT transfusions had increased risks of venous and arterial thromboembolism as well as death. De Boer and coworkers[69] found that PLT transfusion and outcome had a negative effect on patient survival in a retrospective study of 433 adult patients undergoing first-time orthotopic liver transplant. In this study, increasing PLT transfusion was linked to worse survival in a dose-dependent fashion. PLT transfusion has also been linked to coronary stent thrombosis in several studies. Cornet and coworkers[70] reported three patients with gastrointestinal bleeding who received PLT transfusions early in their course of treatment after stenting and were diagnosed with stent occlusion 6 to 17 hours after PLT transfusion.[70] Similarly, Shin and colleagues[71] reported on a patient with aplastic anemia who received a PLT transfusion and subsequently

developed a late stent thrombosis in a drug-eluting stent. These studies emphasize the risk of PLT transfusion in patients with both early and late coronary stents for thrombotic occlusion. However, the retrospective, uncontrolled nature of these studies prevent the identification of the true risk of PLT transfusion-associated thrombosis.

In fact, other studies have failed to identify the same issue in patients undergoing cardiac surgery. Ninkovic and coworkers[72] found that after confounders were adjusted for, PLT transfusion was not associated with an increased risk of 30-day mortality or infective complications. PLT transfusion was associated with higher rates of return to the operating room, and interestingly, a decreased risk of thromboembolic events. It is worth noting that all these were retrospective studies using RT-PLTs. In all cases, the nature and the severity of adverse events appear to be recipient specific and highly associated with patients with pro-thrombotic diseases. To illustrate this effect, PLT transfusions in patients with thrombotic thrombocytopenic purpura and heparin-induced thrombocytopenia, but not patients with immune thrombocytopenia, appear to have higher odds of arterial thrombosis and mortality rate, and are considered contraindications for PLT transfusion.[73] In hospitalized cancer patients, PLT transfusion has been linked to a higher risk of venous and arterial thromboembolism as well as an increased in-hospital mortality rate.[68] Activation of neutrophils by PLT sCD40L accumulated during PC storage contributes to TRALI.[74] TRALI also appears to be responsible for increased postoperative mortality in patients receiving PLTs during liver transplantation.[75]

Whether the aforementioned alternative PLT products result in risks that are similar, lower than, or higher than the current expected risks from licensed PLT products also remains unclear, and at this time, a source of mere speculation. In any event, the possible sources of adverse effects of activated PLT transfusion may be mitigated in the future through the incorporation of methods and/or additives currently under investigation that may result in prevention of PLT activation during their manufacturing and storage.

## CONCLUSIONS

In accompanying the development of new PLT products with increased clotting potency, the concerns about PLT activation and shortened survival in vivo due to storage lesions have hampered their application in human therapy. Further improvements in the composition of current PASs to extend the life span of PLT products and reduce their activation, while maintaining an enhanced proclotting capability are desirable. The PLT preservation strategies discussed above could complement current storage technologies, and together they may result in products with increased potency in bleeding therapy and prophylaxis. The safety profile of these new PLT products remains unknown at this time, but based on their profiles, we should expect that they are likely to be different from the safety profile of currently licensed PLT products.

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### CONFLICTS OF INTEREST

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