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# Biomolecular Consequences of Platelet Pathogen Inactivation Methods

Hendrik B. Feys [a,b,*], Britt Van Aelst [a,c], Veerle Compernolle [a,b,c]

[a] Transfusion Research Center, Belgian Red Cross-Flanders, Ghent, Belgium
[b] Faculty of Medicine and Health Sciences, Ghent University, Ghent, Belgium
[c] Blood Service of the Belgian Red Cross-Flanders, Mechelen, Belgium

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ABSTRACT

Pathogen inactivation (PI) for platelet concentrates (PC) is a fairly recent development in transfusion medicine that is intended to decrease infectious disease transmission from the donor to the receiving patient. Effective inactivation of viruses, bacteria and eukaryotic parasites adds a layer of safety, protecting the blood supply against customary and emerging pathogens. Three PI methods have been described for platelets. These are based on photochemical damage of nucleic acids which prevents replication of most infectious pathogens and contaminating donor leukocytes. Because platelets do not replicate, the collateral damage to platelet function is considered low to non-existing. This is disputable however because photochemistry is not specific for nucleic acids and significantly affects platelet biomolecules as well. The impact of these biomolecular changes on platelet function and hemostasis is not well understood, but is increasingly being studied. The results of these studies can help explain current and future clinical observations with PI platelets, including the impact on transfusion yield and bleeding. This review summarizes the biomolecular effects of PI treatment on platelets. We conclude that despite a comparable principle of photochemical inactivation, all three methods affect platelets in different ways. This knowledge can help blood banks and transfusion specialists to guide their choice when considering the implementation or clinical use of PI treated platelets.



**Contents**



Pathogen inactivation (PI) has been in routine use for decades to prevent transmission of pathogens in plasma. The past years, similar technologies have been developed for platelet concentrates (PC) and recently were adopted in routine use in several countries worldwide. These technologies have been shown to inactivate bacteria, viruses and parasites that were experimentally added to PC in laboratory settings. The inactivation efficiency is variable and depends on the PI method, the type and number of contaminating pathogens [1-4]. The supplementary inhibition of donor leukocytes could render gamma irradiation for prevention of transfusion associated Graft-*versus*-Host-

* Corresponding author at: Hendrik B. Feys, Ottergemsesteenweg 413, 9000 Gent, Belgium.
*E-mail address:* hendrik.feys@rodekruis.be (H.B. Feys).

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Disease, obsolete. The primary goal however is to decrease potential transmission of pathogens from the donor to the thrombocytopenic patient during PC transfusion. Patients that require platelet transfusion sometimes are immunocompromised to varying degrees and therefore the inactivation of both pathogens and donor leukocytes offers a great potential for reducing adverse events related to disease transmission [5]. Current PI technologies target the replication machinery of pathogens by directly damaging or modifying the organism's nucleic acids (DNA and RNA) with photochemical modification.

Our Belgian (EU) national parliament was the first in the world to pass a bill in 2009 that mandates nationwide PI for all PC distributed to hospitals for transfusion. The law came into force in 2013 following additional recommendations to the blood establishments. These included a minimal platelet dose criterion of 3.0 X $10^{11}$ per PC and a limitation of the shelf life from seven to five days following concerns over PI platelet efficacy after storage. The implementation in our blood service was furthermore hampered by risks of aseptic breaches, arising from perforations of PI bag systems.

For platelets, three methods have been developed of which two are currently commercially available. The first available technology (AS-PCT) uses a proprietary psoralen derivative called amotosalen as a photosensitizer in combination with ultraviolet (UV) A light and was developed by Cerus Corporation (Concord, CA) [6, 7]. It is used in more than 40 countries including ours. The second technology uses riboflavin (RF-PRT) in combination with a broad wavelength spectrum of UV light (TerumoBCT, Lakewood, CO) [8]. It is used in over 15 countries including the Czech Republic, Poland and Egypt. The latest technology (UV C) does not include a photosensitizer but exploits the inherent high energy of narrow band shortwave UV C light (Macopharma, Tourcoing, France) [9]. This technology is not yet in routine use.

The *in vitro* quality of PC treated with each of these PI methods has been investigated by several groups over the past years. Most data are available for AS-PCT and the least for UV C, in order of their respective chronologic tracks. In all cases *in vitro* platelet studies indicate anything from low to normal platelet quality depending on the study design, the projected acceptance criteria and the experimental approach. In addition, primary product type and product composition often differ by country or by blood institution. Factors like plasma content, white and red cell contamination, total product volume, bag plastics, and platelet content all influence the outcome of longitudinal quality control studies and therefore represent important variables. The recent clinical study by Garban et al [10] confirmed this by showing the importance of plasma as a contributing factor when comparing AS-PCT treated PC with untreated control PC in patients with hematologic malignancies.

Platelets are small anucleate and discoid cells. They are produced by megakaryocytes which reside in bone marrow [11] and lung [12]. About $10^{11}$ fresh platelets are released daily in a healthy circulation. A normal platelet concentration is between 1.5 and 4.5 X$10^{8}$ cells per mL of blood. During hemostasis, platelets initially adhere to injured blood vessels through interactions of the platelet specific GPIbα-IX-V receptors with von Willebrand factor. Subsequent firm arrest of platelets is mostly through integrin interactions with the denuded subendothelial matrix [13]. During this process platelets become activated, change morphology and recruit additional platelets as well as coagulation factors to eventually stop bleeding through an insoluble platelet and fibrin plug at the injured site. Hemostasis significantly slows down in the absence of platelets, leading to potentially fatal hemorrhage if not corrected by a platelet transfusion. A typical transfusion delivers 3.0 X $10^{11}$ stored donor platelets to the thrombocytopenic patient. Storage is short (4–7 days) because even in optimal conditions a gradual decline in platelet function is found. This storage lesion is a complex biochemical process which is not related to intrinsic apoptosis [14].

Excellent reviews are available in literature describing our current understanding of platelet storage lesion [15] as well as several aspects of the available PI methods [16-18]. The reader is referred to these for details on PI methodology, inactivation potency and clinical use. This review will however focus on the biochemical effects of PI and present current understanding of the impact that PI can have on platelets.

## Amotosalen and UV A Light

Amotosalen (or S59) is a furanocoumarin that can intercalate in helical regions of DNA and RNA [19]. The AS-PCT process uses approximately 150 μM amotosalen with 3.9 J/$cm^2$ of UV A light. Upon photoexcitation of amotosalen in the vicinity of nucleic acids, covalent monoadducts can be formed with thymidine bases (Figure) [20]. These chemical modifications efficiently inhibit subsequent DNA or RNA (reverse) transcription, thereby preventing replication of many pathogens and leukocytes [21]. Because the functional target is biological proliferation and because platelets do not replicate, the technology seemingly affects dividing life forms only, and not platelets. However, several lines of research including ours show that platelets readily react with photoexcited amotosalen during AS-PCT and this is independent of pathogen contamination.

### *Modifications of Nucleic Acids*

Although platelets may not replicate [22], they do carry significant amounts of nucleic acid in diverse RNA forms, including short mRNAs and miRNA but also long non-coding RNA and ribosomal RNA [23]. Consequently, amotosalen chemistry with platelet RNA will inevitably take place upon photoexcitation and modify the platelet transcriptome. This was confirmed by RNAseq based genome-wide differential expression analysis, showing that 147 genes become deregulated by ≥2-fold following AS-PCT [24]. Functional clustering analysis of these genes suggested significant changes in AS-PCT platelet membrane function, transport, metabolism and structure compared to untreated control. Some uncertainty still exists around platelet RNA function [25, 26], but biochemical evidence for protein translation [27, 28], pre-mRNA splicing [29], processing of miRNA precursors [30], functional miRNA [31] suggests significant roles for RNA beyond being a mere remnant from thrombopoiesis [32]. For instance, a decrease in the mRNA levels for the pro-survival protein Bcl-XL suggests that platelets treated with AS-PCT may turn to apoptosis quicker than untreated platelets [33].

The AS-PCT method also modifies platelet mitochondrial DNA (mtDNA). Mitochondria in platelets logically deliver energy equivalents, but also play a role in hemostasis and necrosis [34, 35]. In addition, the release of mtDNA during storage has recently been linked to transfusion adverse events [36, 37]. Approximately 4.0 ± 1.2 (n = 6) amotosalen adducts per 1000 base pairs of mtDNA are found in AS-PCT treated platelets. This is less than the 12 ± 3.0 adducts per 1000 base pairs found in nuclear DNA, but still sufficient to prevent mtDNA replication [38]. The mtDNA modifications in platelets are currently investigated as a form of quality control for AS-PCT treatment using real-time polymerase chain reaction [39]. The biomolecular changes in mtDNA do not lead to mitochondrial depolarization [40, 41] implying that AS-PCT does not alter the pH gradient across the mitochondrial membrane. It therefore remains to be demonstrated that the changes to the platelet transcriptome (RNA) and platelet mtDNA account for downstream effects like functional decline [42, 43] or reduced survival after transfusion [17].

### *Modifications to the Platelet Membrane Leading to Signal Transduction Defects*

Our initial study of AS-PCT platelets in additive solution was in microfluidic flow chambers with real-time fluorescence microscopy [44]. The data showed significantly reduced platelet thrombus formation kinetics to immobilized collagen for AS-PCT treated platelets compared to paired untreated controls [42]. This was an acute defect, evident immediately after AS-PCT treatment which did not recover

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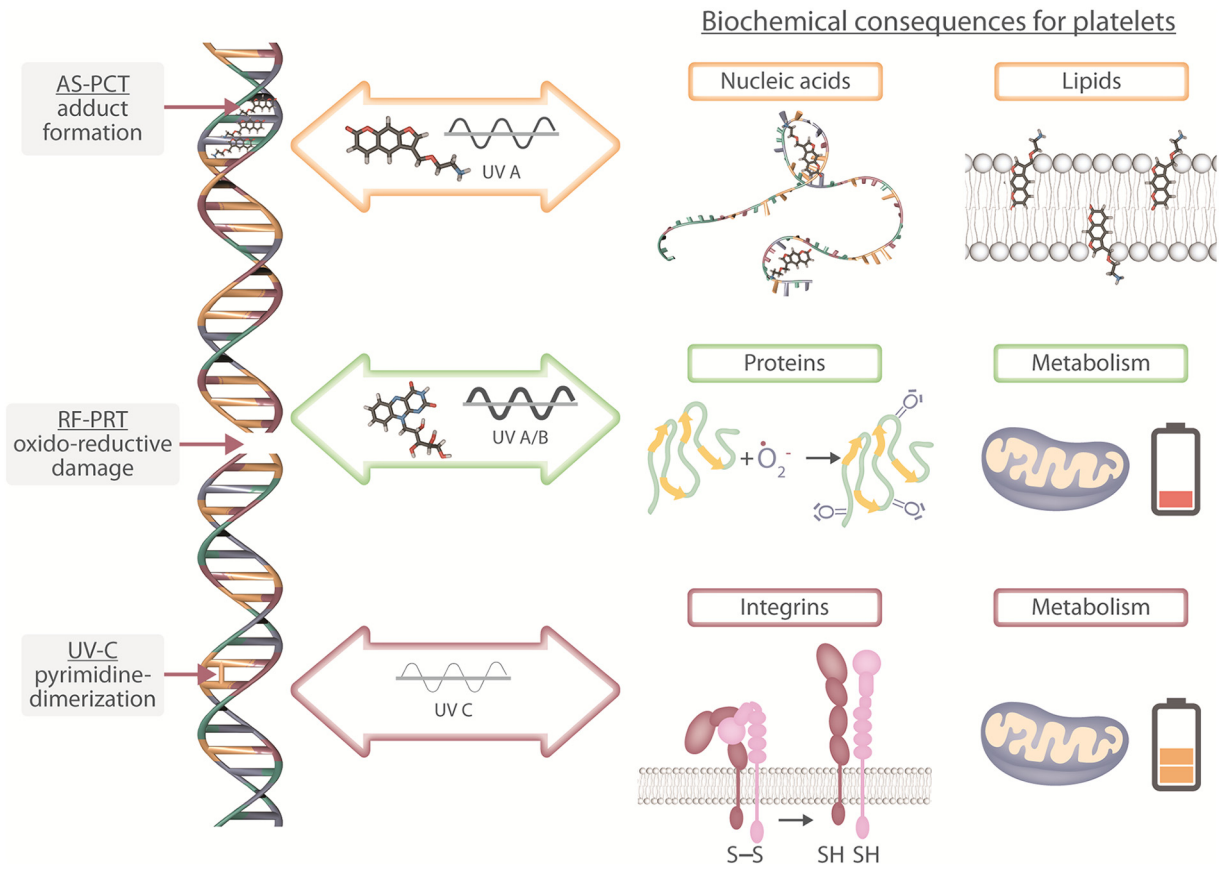


**Figure.** The impact of three PI methods for platelets. Current PI methods are amotosalen photochemical treatment (AS-PCT) or the *Intercept* method (orange); riboflavin pathogen reduction technology (RF-PRT) or the *Mirasol* method (green) and ultraviolet C (UV-C) or the *Theraflex UVC* method (purple). The targeted damage to (pathogen) proliferation is summarized on a common DNA triple helix to the left. The molecular structure of photosensitizers and UV light wavelengths are given in the arrows. To the right, specific biomolecular consequences for platelets are depicted; AS-PCT damages nucleic acids like RNA and mitochondrial DNA by covalent modification. Lipid molecules are also targeted by AS-PCT leading to changes in membrane packing and subsequent defects in platelet signal transduction. RF-PRT modifies proteins including labile proteins like FVIII by oxidative mechanisms. Platelet metabolism increases leading to increased rates of lactic acid following RF-PRT. UV-C impacts integrin structure and increases in metabolism are also found.

during subsequent storage under normal blood banking conditions. In addition, activation of the fibrinogen receptor integrin $\alpha_{IIb}\beta_3$ was significantly impaired when platelets were stimulated *via* the thrombin receptor, PAR1. In fact, the agonist dose needed to achieve a comparable response to untreated controls was twofold [45]. These findings inevitably became part of the inconsistent literature on AS-PCT platelet quality, so we next questioned why AS-PCT platelets are less responsive to agonist stimulation.

To this purpose, all major known signal transduction pathways in platelets were interrogated. Significant decreases were found only for Akt phosphorylation, pointing to defective phosphatidylinositol-tris-kinase (PI3K) signaling in AS-PCT treated platelets [45]. Surprisingly, the enzyme product of PI3K, phosphatidylinositol(3,4,5)-tris-phosphate (PIP3) was not decreased. This PIP3 inositide is generated in the inner cell membrane leaflet in response to cell activation, providing a major nucleation site for downstream signal transduction. Key effector molecules Akt and Bruton's tyrosine kinase (Btk) become phosphorylated at these sites, but much less in AS-PCT treated platelets. Signal transduction through the PI3K axis is important for platelet granule secretion, platelet aggregation and thrombus formation [46-48]. Impaired Akt and Btk phosphorylation but normal PIP3 levels implied inhibition of Akt/Btk binding to PIP3 rather than a malfunctioning PI3K enzyme reaction.

Because the PI3K axis requires normal membrane interactions and because psoralens are known to biophysically partition to membranes [49], we investigated platelet membrane composition next. Using targeted lipidomics, amotosalen adducts were readily found in all classes of membrane phospholipids with unsaturated fatty acyl side chains [45]. The latter is important because unsaturated carbon side chains cause pivotal structural membrane features called *lipid packing defects* (LPD). These are dynamic gaps in the hydrophilic sheet that tops each bilayer leaflet [50]. The gaps accommodate protein-membrane interactions [51]. Chemical modification of unsaturated phospholipids with amotosalen indeed caused fewer LPD, preventing the interaction of model proteins like the amphipathic-lipid-packing-sensor motif (ALPS) and α-synuclein with AS-PCT treated liposomes. Discerning between membrane-bound and cytoplasmic Akt allowed to subsequently demonstrate that binding of Akt to the amotosalen modified membrane indeed was inhibited in the AS-PCT treated platelet [45]. These data provide a mechanistic explanation for why platelet activation by signaling pathways that include the PI3K axis are impaired after AS-PCT treatment (Figure). Whether the changes in membrane composition also cause the increased clearance rates of transfused AS-PCT platelets needs further investigation.

Our findings furthermore imply that amotosalen (photoproducts) remains bound to AS-PCT treated platelets and are transfused. Indeed, it is estimated that 4 mg of amotosalen remains in a standard PC after AS-PCT [52], of which half is bound to platelets. These levels have not been found to be toxic in animal studies and clinical trials in patients [52-54]. Based on these and available lipidomics data [55], we estimate that maximally 4% of all membrane lipids are in complex with amotosalen. But, the photochemical reaction of amotosalen with lipids is selective for phospholipids with unsaturations so the modified fraction of that subspecies will be larger. The consequences are nonetheless large enough to render the cytoplasmic membrane less accessible for membrane binding kinases.

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## Riboflavin and UV A/B light

Riboflavin is a water soluble essential vitamin (B2). The molecule is therefore considered safe for injection, thereby avoiding the cumbersome adsorption step required for AS-PCT to remove photosensitizer. In RF-PRT practice, on average 50 μM of riboflavin is added to a PC and illuminated with broad spectrum UV light (280–400 nm) at a dose of 6.2 J/mL [56]. The delivered light energy in this case is dependent on the exact product volume implying that every PC is weighed before photo-treatment. The photochemical pathogen inactivation of RF-PRT is governed by several possible redox reactions. Guanine bases in DNA or RNA may accept electrons directly from photosensitized riboflavin, but in the presence of dissolved molecular oxygen the reaction shifts towards substantive reactive oxygen species (ROS) formation [57]. Oxygen species like singlet oxygen, superoxide anion and hydroxyl radicals strongly damage biomolecules, including DNA. These reaction products then efficiently prevent replication and proliferation of many pathogens [58] and leukocytes.

### *Modifications to proteins by reactive oxygen species*

In a comparable study as for AS-PCT, microfluidic flow chambers with real-time fluorescence microscopy were used to measure hemostasis of RF-PRT platelets in reconstituted blood perfused over immobilized collagen. Similar to AS-PCT, the RF-PRT treatment significantly and immediately reduced platelet thrombus formation compared to controls [42, 59]. In addition, platelet aggregation *via* thrombin receptor activation was significantly decreased. Premature platelet degranulation was moreover increased. Aggregation defects and premature degranulation were not observed in AS-PCT treated platelets in our hands [42], but a direct comparison with paired RF-PRT treated samples was never performed.

Although we were unable to dissect the biochemical consequences of RF-PRT technology on platelets in detail, earlier data from our team showed that superoxide anion is a dominant ROS formed during RF-PRT of plasma [60] (Figure). This study followed a comparative analysis of plasma PI methods that found significantly reduced activities for FVIII, ADAMTS13 and fibrinogen in RF-PRT treated plasma compared to AS-PCT and methylene blue photo-treatment [61]. Proteins in RF-PRT plasma were significantly carbonylated, which is a toxic endpoint modification of oxidative damage in proteins. These findings were confirmed in RF-PRT treated platelets by Johnson and Marks [62]. Of note, by performing RF-PRT in experimental conditions of hypoxia, the damage to labile plasma proteins could be rescued [60] proving that oxidative damage indeed underlies the functional decline of plasma treated with RF-PRT. It is not clear if these hypoxic conditions which are useful to safeguard plasma proteins, would equally safeguard platelet quality and retain sufficient photochemical potential to kill pathogens at the same time.

Indirect modifications to proteins following RF-PRT were also reported multiple times. In particular, increased phosphorylation was found for the cytoskeleton regulator VASP [63], the stress response molecules p38MAPK [64], NF-κB and IκBα [62]. The changes to p38MAPK were shown to be relevant, because these could be linked to increases in the expression of pro-apoptotic proteins Bax and Bak in RF-PRT platelets [65]. In this study inhibition of p38MAPK with SB203580 reversed most of the pro-apoptotic responses including caspase action. The same researchers also showed that RF-PRT treatment can be successfully postponed with a beneficial effect on platelet quality in comparison to PC treated immediately after component preparation [66]. Whether this is sufficiently practical remains to be determined, but the fact that there is no photosensitizer removal step makes this modification worthwhile investigating.

### *Modifications to Anaerobic Respiration Rates*

There is consensus that RF-PRT specifically increases platelet anaerobic metabolism rates. In biochemical terms, this observation is not necessarily similar to an acceleration of platelet storage lesion *per se* as recently demonstrated by Salunkhe et al [67]. Most studies nonetheless find increased lactic acid production rates in RF-PRT treated PC [42, 68-70]. This is relevant, because lactic acid production rate is one of the few *in vitro* platelet measures that correlates reasonably well with transfusion success [71]. Despite the obvious glycolytic shift, depolarization of mitochondria in RF-PRT treated platelets is not [69, 72] or just slightly increased [70, 73]. Therefore, other mechanisms must cause the heightened level of glycolysis in RF-PRT platelets. All studies have focused on mitochondrial polarization using probes like JC-1 and flow cytometry. Other more delicate aspects of platelet mitochondrial respiration can be investigated nowadays using more advanced techniques [34] to fully understand the metabolic consequence of RF-PRT. For instance, Dahiya et al demonstrated that changes to miRNA's during platelet storage may indirectly affect respiration by changing the transcription of a subunit of the $F_0$ATPase complex [74]. Data from the Devine team suggest a link between changes in mitochondrial respiration, p38MAPK signaling and apoptosis of RF-PRT platelets [65, 73]. This deserves further attention because RF-PRT technology could be used for inactivating whole blood [58]. The latter is very relevant in endemic regions where the platelet quality is in balance with risk for transmission of infectious disease. A better insight into the mechanisms of altered platelet metabolism may one day help to take measures to protect platelets or even red blood cells from the consequences of RF-PRT in PC and in whole blood, respectively.

In our institute, RF-PRT was not implemented because >10% of products had unacceptable swirling before expiration, including pH <6.4 for some of these. The Belgian requirement for producing PC that contain more than $3.0 \times 10^{11}$ platelets pushed the lower boundaries of platelet content to levels that were incompatible with the RF-PRT method [75]. The metabolic impact was too large for assured quality of most products until day 5. It should be noted that our data furthermore indicate that also for AS-PCT, platelet content combined with primary product type (apheresis *versus* buffy coat) can change the rates of storage lesion [76]. A high platelet content in apheresis products is more likely to have increased storage lesion rates than a low content, in the context of AS-PCT.

## UV C Light

The UV C method is a one-step illumination with UV C light. The PC is transferred to a specially designed large transparent container. The large size increases the total surface area and renders the volume column less high. Combined with constant vigorous agitation, efficient UV C light penetrance is achieved. The energy delivered is 0.2 J/cm$^2$ from both sides of the bag for about 30 to 60 seconds [4, 9]. The highest intensity of incoming light is delivered at a peak wavelength of 254 nm, but there is no information on additional wavelengths illuminating the PC. A laser is required to achieve absolute monochromatic light and this is not part of the Theraflex UV C equipment. Therefore, lower intensity light of other unknown wavelengths is delivered to PC during treatment in the illuminator.

The advantage of this technology is the absence of exogenously added photosensitizers and their photoproducts that are generated during excitation with UV light. The biochemical mechanism of nucleic acid damage by UV C is well described [77]. Pyrimidine dimers are generated which prevent replication of the genetic material and effectively inactivate pathogen proliferation. The method is not in routine use, but a phase I trial has indicated good tolerance in healthy volunteers receiving autologous transfusion [78]. A phase III clinical trial is under way (EUDRACT n° 2015–001035-20).

Similar to the other two PI methods, *in vitro* platelet concentrate quality has been studied for UV C as well [4, 79, 80]. In our microfluidic model system for hemostasis, thrombus formation kinetics of UV C platelets was significantly decreased after storage (day 5) compared

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to untreated control [81]. Because UV C illumination of protein solutions generates ROS [82], the UV C procedure was repeated in hypoxic conditions but this could not rescue the decreased thrombus formation after storage. Unlike the other two methods, the impact on thrombus formation under flow was less obvious immediately after treatment.

What differs from AS-PCT and RF-PRT is that UV C PI induces a conformational change in platelet integrin $\alpha_{IIb}\beta_3$, reminiscent of receptor activation [80, 81, 83]. Seminal work from Verhaar et al suggests that UV C illumination can directly reduce disulfide bonds by photolysis [84]. Disulfide bonds are crucial for biomolecular control over integrins, including $\alpha_{IIb}\beta_3$ conformation [85, 86]. Direct photolysis is believed to trigger dissociation of the intracellular tails of $\alpha_{IIb}\beta_3$ thereby releasing the forces that hold the receptor in a closed conformation. The opened conformation thus binds $\alpha_{IIb}\beta_3$ ligands like fibrinogen, which is present in plasma and platelet α-granules. The influence of UV C on protein disulfide bonds may also be related to changes on the proteome level because Mohr et al showed alterations in the protein disulfide isomerase (PDI) isoform ERp72 [4]. A direct link between integrin activation and PDI has been shown in genetically modified mice [87] suggesting that changes in these enzymes levels can contribute to the observation.

The immediate effect on platelet quality after UV C treatment is less than for AS-PCT and RF-PRT in our hands. However, storage of UV C platelets causes increased lactic acid production rates, P-selectin exposure and phosphatidylserine exposure compared to untreated control [81]. The open conformation of some integrin $\alpha_{IIb}\beta_3$ receptors may cause low but constitutive fibrinogen binding. This in turn may render (some) UV C treated platelets "primed" for activation increasing consumption of energy and reaching exhaustion faster. Experiments in the presence of a potent integrin $\alpha_{IIb}\beta_3$ inhibitor should confirm or refute this hypothesis.

## Conclusion

Recent clinical trials have assessed bleeding in hematology-oncology patients with thrombocytopenia, both for AS-PCT (EFFIPAP) [10] and RF-PRT (PREPAReS) [88] compared to their respective untreated controls. The results indicate that non-inferiority of PI treated platelet concentrates is questionable, at least for this endpoint and patient population. These clinical data are accompanied by the multitude of laboratory data that have indicated low to substantial platelet damage following PI *in vitro*, or at least found changes in platelet function during PC storage. From the limited basic biochemical studies available we can conclude that the molecular details behind this damage probably differ per PI method. This adds to the complexity of the field. Additional research to the basic phenomena that lead to platelet damage after PI should be conducted to explain the observations in the clinic. The indisputable advantage of mitigating transfusion transmitted infectious disease by PI must be compatible with a high quality platelet that functions as near as possible to an untreated one. This will require a better understanding of the platelet's reaction to PI methods and the development of creative solutions to counteract these.

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## Conflict of Interest

None.

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