# Troubleshooting in platelet storage temperature and new perspectives through proteomics

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## Introduction

Platelets represent a key cellular blood component under physiological conditions, due to their implications in the maintenance of vascular integrity and prevention of haemorrhagic phenomena[1]. Dysfunctions associated with thrombocytopenia constitute a significant threat to patients' health. So far, administration of platelet concentrates (PCs) from healthy donors is the only known strategy for medical care of patients with active bleeding, thrombocytopenia caused by bone marrow dysfunctions or due to chemotherapy for treatments of malignancies, or upon preliminary treatments prior to stem cell transplantation. Qualitative and quantitative changes in platelets could also occur following coronary artery bypass surgery and trauma, and may represent a key indicator of likely thrombotic complications[2]. Platelets are basically collected from donors in two ways: extraction from whole blood throughout centrifugation (buffy-coat) or through apheresis. Both methods are currently employed and have bright sides and disadvantages. Analogously to other blood components, techniques to improve the shelf life of platelets (currently, only five days), while maintaining their safety and effectiveness, are under constant investigation worldwide. Indeed, blood transfusion services are always in shortage of PCs, because of the impossibility to prolong their storage in like fashion to hypothermic storage of erythrocyte concentrates or frozen storage of fresh frozen plasma. In order to improve storage of PCs, lowering temperature has been attempted as well, although results were not as positive as expected, since platelets do not tolerate refrigeration. Hypothermic (4 °C) storage conditions cause deep modifications in platelet shape and functionality. These are relevant issues which compromise viability of cold stored platelets, as they exert their physiological role through their ability to change shape and activate under various conditions. Low temperature appears to be a triggering factor for activation as well, thus yielding yet activated platelets as unviable blood product at the end of the storage.

This review focuses on platelet storage temperatures and the main issues which affect both current (22 °C) and alternative (4 °C) storage protocols for PCs. In particular, we herein discuss the initial events which trigger morphological and physiological changes upon cold activation, partially distinguishable from physiological activation. Moreover, a glance will be given at recent proteomic approaches, which promise to improve current knowledge on blood components of transfusion interest, mainly addressing current technical hurdles (such as the analysis of membrane proteins and their reciprocal interaction).

## PCs: storage temperature and major issues

### Current guidelines for storage of platelet concentrates: shortcomings and perspectives.

Since 1960's, platelets are stored at 22-24 °C as PCs, a methodology that had significantly improved their availability. PCs are stored under continuous gentle agitation in plasticized polyvinylchloride bags with di-(2-ethylhexyl) phthalate (DEHP), which are permeable to oxygen, in order to promote aerobic metabolism instead of glycolysis. This prevents pH drop which would render PCs acidic. However, their shelf life stops at the fifth day of storage because of the risk of bacterial and viral contamination, on the one hand, and the occurrence of structural lesions, on the other.

As far as contamination is concerned, a series of studies have been conducted in the USA as to quantify the risk of bacterial contamination associated with

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platelet transfusion, which resulted to be limited to one over 1000-3000 platelet/unit[3]. Although rare, this event has elevated probability to cause sepsis in recipients[4].

In parallel, stored platelets undergo a series of shape and functional modifications, which are commonly referred to as platelet storage lesions (PSLs). This term indicates the progressive decrease of functionality that accompanies storage of platelet at 22 °C, and in particular represents the final detrimental effect caused by a series of events happening during storage. PSLs include morphological changes with loss of the quiescence-related discoidal shape, release of granule contents, exocytosis of cytosolic proteins, increase of procoagulant properties, modification of glycoprotein patterns[5]. Notably enough, these features are also typical of platelet activation as well, suggesting for shared molecular pathways between PSLs and activation itself.

In conclusion, basic clinical goals to be fulfilled include both the enhancement of platelet shelf life and the development of cheap, fast and reliable pathogen inactivation procedures, which are currently under evaluation. International projects are currently *in fieri* which pursue quality assessment through proteomics upon pathogen inactivation/reduction processes on PCs (for example, the Italian platelet technology assessment study -IPTAS).

## Cold storage

Storage at refrigerating temperatures would prevent many unwanted processes which take place at room temperature, at least under a molecular perspective. Lowering storage temperature has several benefits, among which a bacteriostatic effect reducing likelihood of bacterial infections and thus decreasing the potential for sepsis. In this view, it is not cursory to conclude that the lower the temperature, the stronger is this effect. Unfortunately, platelets stored at temperatures below 15 °C perform very poorly *in vivo*, mainly due to an elevated percentage of cold stored platelet being rapidly cleared from the bloodstream of the recipients[6]. Galactosylation has been proposed to tackle this issue in cold-stored mouse platelets, although it only showed relevant results *in vitro*[7], while yielding poor survival in *in vivo* studies on human counterparts[8,9]. Removal of refrigerated platelets from the circulation appears to be partly mediated by recognition of clustered beta-N-acetylglucosamine on platelet surface glycoproteins by the alphaMbeta2 hepatic lectin receptor. Capping the exposed beta-N-acetylglucosamine residues by enzymatic galactosylation restored the circulation of short-term chilled murine platelets, introducing a novel method that allows for cold storage of platelet[10]. Nonetheless, galactosylation is not sufficient to restore circulation of long-term refrigerated platelets. Additional data indicate that differential carbohydrate-mediated mechanisms may exist for clearance of short-term and long-term cold-stored platelets[8,9].

Refrigeration could also inhibit accumulation of contaminating white cell products, such as cytokines, and contribute alleviating cytokine-associated febrile transfusion reactions[11].

Protocols involving storage of PCs at 4 °C may allow extension of the storage limit beyond 5 days, prolonging platelet shelf life and thus solving current shortages in transfusion services. However, although lowering temperature would prevent occurrence of several PSLs, cold storage seems to trigger different lesions, which end up impairing platelet integrity and functionality.

### *Cold-induced storage lesions*

The sum of untoward effects occurring upon platelet storage in the cold are generally termed cold-induced storage lesions. One of the first visible effects of platelet impairment is the irreversible loss of the discoid morphology towards a spherical shape, and the appearance of spiny projections on the surface due to calcium dependent gelsolin activation and phosphoinositide-mediated actin polymerization[12].

Under physiological conditions, platelet change in shape is a crucial event for their proper functioning: this phenomenon consists in a global remodelling of actin cytoskeleton, with destruction of pre-existing actin filaments and concomitant assembly of new monomeric actin units. This event leads to the conversion of discoid platelets to spiny globes.

The morphological changes induced in platelets by low temperatures have been observed from 1950's; when platelets are exposed to temperature lower than 20 °C, they undergo fast modifications in shape[13], notably increase intracellular calcium levels[14] and actin polymerization degree. Moreover, stored platelets

secrete alpha granule and lysosomal contents[15], and reorganize the microtubule coil lying under the plasma membrane through depolymerization processes[13].

Many efforts have been put forward in order to avoid platelet functional impairment during cold storage. In this respect, additive solutions have been recently developed, such as ThromboSol which is characterized by second messenger effectors (amiloride, sodium nitroprusside and adenosine). Additive solutions have been ideated to biochemically stabilize platelets against cold storage lesions[16]. Experimental applications of ThromboSol to the cryopreservation of platelets gave optimal performance in terms of retention of cell number, *in vitro* functional activity[17], and *in vivo* percent recovery[18]. Several authors have demonstrated effectiveness of ThromboSol in inhibiting bacterial growth and decreasing accumulation of contaminating white blood cells-derived cytokines interleukin (IL) 6, IL-1 and tumour necrosis factor á in platelets kept at 4 °C[11]. Moreover, experimental approaches demonstrated that, whereas control PCs without treatment displayed a time-dependent increase in the plasma concentration of IL-6, IL-1β, and tumor necrosis factor á, as well as a time-dependent increase in the bacterial titer, treated PCs stored at 4 °C displayed no accumulation of these cytokines in the plasma fraction and no increase in bacterial titer above the initial inoculation[19].

***Cold activation***

Some features of cold-stored platelets closely recall platelet activation processes. Storage of whole blood at 4 °C for 6 h has been demonstrated to induce platelet activation similar to that of patients with cardiovascular diseases[20].

The molecular basis underlying platelet activation during storage at 4 °C have been extensively studied, but not yet fully characterized. Cold storage does not cause an increase of glycoprotein (GpIb, GpIIb/IIIa) and platelet activation markers (CD62p and CD63), upon comparison with storage at 22 °C[21].

Clinical approaches imply platelet aggregation assays, expression of CD40 ligand (CD40L), plasma levels of soluble form of CD40L (sCD40L), analysis of platelet-leukocyte aggregates.

Cold-stored platelets have been demonstrated to be more sensitive to agonist-induced aggregation with respect to platelets stored at room temperature. This feature has been validated through the analysis of platelet response to signal transduction inhibitors on fibrinogen binding, aggregation, the activation state of GP IIb-IIIa, and cytosolic calcium levels. Moreover, cold-stored platelets show a higher aggregation response (in response to ADP and epinephrine) and a major resistance to disaggregating agents (promethazine, prostaglandin D2, yohimbine, and echistatin) when compared to their counterparts stored at room temperature[22].

Prolonging PC shelf life at 22 °C for more than 5 days, other than holding an increased bacterial contamination potential, could also result in morphological changes that impair platelet functionality. Cold storage stresses occurrence and gravity of theses untoward effects[23]. These are important effects to take into account in clinical practice[24]. Actually, it is controversial whether the effects of slight decrease in temperatures during hypothermia in cardiac surgery would result in improved or impaired platelet functions: several authors have noticed the enhancement of platelet function at slightly lower-than-physiological temperatures, particularly in the presence of agonists such as ADP[25]. The decrease in temperature from 37 °C to 28 °C has been demonstrated to promote platelet spontaneous aggregation both in citrated and hirudinised blood, also increasing agonist-induced aggregation[25]. It is nevertheless questionable whether this represents an actual trend, correlating higher activation likelihood to lower temperatures. Some efforts, aimed at preventing platelet activation in the cold, start from the assumption that metabolic suppression could lead to a better maintenance of a quiescent status and, insodoing, avoid platelet activation during storage[26]. In fact, platelets have been demonstrated to maintain their ability to aggregate and secrete granule contents after a brief period of metabolic suppression via administration of glucose-free media[27-29]. This treatment has been tested in association with antimycin A, an inhibitor of mitochondrial ATP synthesis, as to avoid anaerobic energy generation[30]. This double blockade of the glycolytic flux (through removal of glucose) and aerobic metabolism (through addition of antimycin A) is enough to impair platelet energetic balance, since glycogenolysis alone does not succeed in satisfying

cellular requests. Afterwards, this transient blockade can be removed by addition of glucose to the suspension medium, restoring complete platelet functionality. Moreover, transient metabolic suppression has also been demonstrated to reduce platelet binding and phagocytosis by macrophages[31]. Differences between aggregation and disaggregation responses of cold- and room temperature-stored platelets suggest that cold-stored platelets may have different mechanisms to stabilize platelet aggregates during their formation, as it has been extensively documented[6-9].

## Agonist-induced versus cold-induced platelet activation

Cold-stored platelets set up a process similar to agonist-induced activation: however, the similarity between these two events seems to be restricted to the final steps of the signalling cascade, making knowledge of cold activation intriguing and challenging. In contrast to physiological platelet activation, which is triggered by the binding of an agonist to a membrane receptor, the initial event leading to activation of cold-stored platelets is only partially known. The change in platelet shape is the first visible sign of activation, and it is apparent both in agonist-induced and cold-activated platelets. Although the molecular sequence of platelet activation has yet to be fully elucidated, some of the basic mechanisms that finally lead to their physiological haemostatic action are quite well established. It is well known that exposure of platelets to damaged endothelium triggers their activation: in particular, collagen is the most relevant thrombogenic molecule that is exposed to injury sites and acts as a major ligand for platelets[32]. Platelets possess membrane receptors responsible for priming activation process. These are intrinsic membrane proteins, highly glycosylated in their extracellular side, that are responsible for binding collagen and other protein products. Integrin $\alpha 2\beta 1$ allows platelets to adhere to collagen, then glycoprotein VI triggers a signalling cascade[33] that involves a series of secondary intra-cellular messengers, including inositol 1,4,5-trisphosphate, 1,2-diacylglycerol[34] and calcium[35]. 1,2-Diacylglycerol and calcium mediate the characteristic platelet activation responses: shape change, granule secretion, and aggregation. Calcium is clearly an important mediator of activation, since its levels during this process raise up several folds with respect to the resting platelet levels: this increase turns on gelsolin severing activity on actin filaments leading to their dissociation and thus to cytoskeletal reorganization (Figure 1). Another crucial factor responsible for physiological activation is the *de novo* synthesis of phosphoinositides that exert three fundamental actions: i) they act as actin nucleation sites; ii) they release barbed end capping proteins that inhibit actin

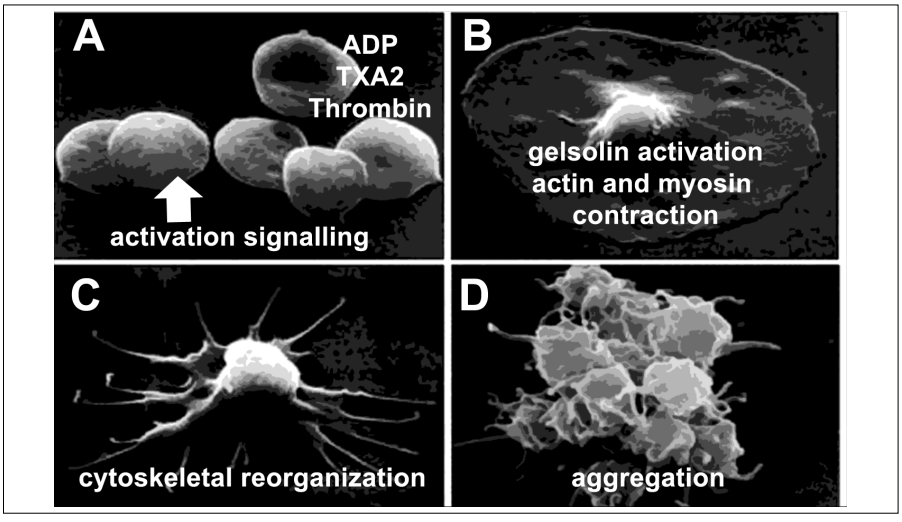


**Figure 1 -** Graphic representation: panels from A to D show gradual activation and aggregation of platelets.

polymerization; and iii) they branch barbed end nucleation at the cell cortex through activation of interaction between WASp and Arp2/3 complex leading to cell motion[37].

Most of the events described above have been demonstrated to happen in cold-stored platelets as well. In particular, morphological changes have been recorded by several research groups exposing platelet to low temperatures. The increase in cytosolic calcium during chilling has been pointed as the major factor responsible for depolymerization of pre-exhisting actin filaments, since platelet microtubule skeleton seems to disappear with exposure to chilling temperatures[38]. Furthermore, it has been also proven the existence of a phosphoinositide-dependent actin assembly, that justifies cytoskeleton reorganization and change in shape of chilled platelets[39-42]. Some signalling events appear to occur during activation, independently from the nature of the onset element, such as the increase in cytosolic calcium and an induction of tyrosine phosphorylation of many cytosolic proteins. Most seemingly, it appears that chilled (mouse) platelets undergo clustering of von Willebrand factor (vWf) receptors (glycoprotein Ib), eliciting recognition of mouse and human platelets by hepatic macrophage complement type 3 (CR3) receptors, a phenomenon which triggers chilled platelet rapid elimination upon transfusion[6] (Figure 2). Indeed, CR3-expressing but not CR3-deficient mice exposed to cold rapidly decrease platelet counts. As cooling seems to prime platelets for activation, it has been proposed that platelets could act as thermosensors, primed at peripheral sites where most injuries occurred throughout evolution. Their clearance prevents pathologic thrombosis by primed platelets. Chilled platelets bind vWf and function normally in vitro and ex vivo after transfusion into CR3-deficient mice[6]. Therefore, GPIb modification might permit cold platelet storage.

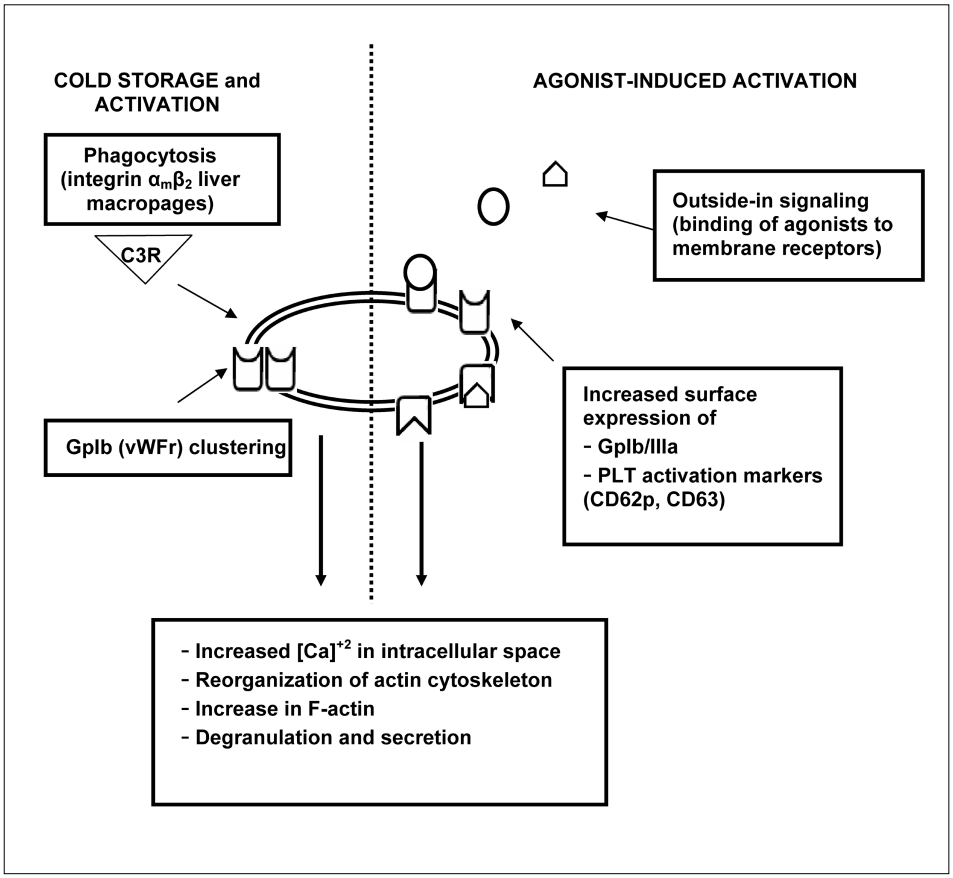


**Figure 2 -** Schematic representation of common and distinguishing features of cold and agonist-induced platelet activation.

## New proteomic applications: delving into platelet physiology

Proteomic techniques, such as bidimensional electrophoresis (2D-E), have been widely applied on platelets[43-46]; basic approaches range from the characterisation of proteomic changes occurring upon platelet activation to the undisclosure of the molecular basis of hereditary diseases.

In 2000, Marcus and colleagues performed the first characterization of the platelet cytosol coupling 2D-E with mass spectrometry, which yielded identification of 186 protein products[43]. Two years later, the platelet proteome was further expanded by O'Neill et al. (2,300 protein spots)[46]. Many other groups contributed to the global mapping of the platelet proteomic asset, such as Garcia and coworkers[47], which separately focussed the pI 4–5 region and pI 5–11 regions of the human platelet proteome in the first dimension, identifying 311 gene products. Later on, as it often occurs after exploring a basic theme, the aim of the researchers progressively moved from the holistic characterization of the total platelet proteome to the deepening of a particular facet of the topic. To this end, the study of platelet signalling network leading to their activation represents a challenging issue to expand: at the protein level, this phenomenon has been studied through comparison between proteomes from resting versus agonist-activated platelets (through the use of thrombin-receptor activating peptide). Differences have been detected both in platelet cytosolic[48,49] and secreted (secretome[50,51]) proteomes. These strategies turned out useful for the characterization of platelet proteomes upon different treatments. However, these basic gel-based proteomic approaches have significant technical limitations. First, 2D-E does not provide functional information (protein-protein interactions); second, its use is positively biased towards hydrophilic proteins, whereas hydrophobic, high-molecular weight and basic products are more difficult to detect through the 2D-E approach. Since platelet activation (both cold and agonist-induced) is associated with signalling via the plasma membrane, proteomic dissection of platelet cold activation is therefore problematic, being membrane proteins highly hydrophobic in nature. Thus, other proteomic approaches to the analysis of this mechanism must be adopted. Nowadays, new strategies have been proposed to ameliorate the analysis of membrane proteins, among which the use of blue native (BN)-PAGE. This technique was initially applied on mitochondrial membrane protein complexes, to shed light on oxidative phosphorylation mechanism[52]. The power of this approach is the maintenance of the protein physiological status, including native protein-protein associations. Electrophoretic mobility is ensured by the use of Coomassie Blue G250, which confers a negative charge to the proteins. The association of Blue Native-PAGE with SDS gel electrophoresis is called 2D- BN/SDS PAGE; this technique allows the separation of monomeric and multimeric proteins in their native state in the first dimension and their subsequent denaturation in the second dimension. This approach has been shown to be effective in the separation of several individual subunits of the resolved complexes, allowing the characterization of membrane proteins and their reciprocal interactions. Many researchers have successfully applied native techniques to dissect human pathological diseases from a proteomic point of view, from the characterization of oxidative phosphorylation complexes in mitochondrial encephalomyopathies[53], Parkinson disease[54], and Alzheimer disease[55], to the study of cytochrome c oxydase deficiency, confirming the validity of proteomic techniques in revealing the molecular bases of human pathologies. Beyond the analysis of purified mitochondria membrane fractions, 2D-BN/SDS-PAGE has also been applied to the analysis of raft domains[56] and of endoplasmic reticulum[57]. Claeys and colleagues have recently published an application of 2D-BN/SDS-PAGE on the cytosolic and microsomal membrane fractions from platelets[58]. From these assumptions, the use of native techniques may allow the extraction of intact protein complexes from platelet membranes, thus clarifying the initial events of platelet activation upon storage at refrigerating temperatures.

## Future perspectives in platelet proteomics

Proteomics is emerging as a powerful tool, which has yet partially fulfilled its promise to enrich our current knowledge about many biological processes, among which normal and pathological health conditions. This is particularly true for platelets, since these cellular blood components are enucleated and contain meagre amounts of mRNA from their

nucleated precursors, thus vanishing mRNA transcript oriented approaches.

While proteomics has already made its way through the academic setting, massive translation of the proteomics know/how to the clinical endeavour still needs standardization of each very phase of the proteomic workflow-streamline, as to guarantee reliable and comparable data, while ameliorating and complementing sanitary practices currently in use. Several efforts have been made in this direction: as an example, the International Society on Thrombosis and Haemostasis has published standards to match current proteomic information on platelets with all other available scientific data, in order to realize the first platelet protein database[59].

Moreover, a proteomic platform, termed PlateletWeb, has been recently developed by Dittrich and colleagues, framing the pool of platelet "omic" data so far harvested. This database, available at http://plateletweb.bioapps.biozentrum.uni-wuerzburg.de, also contains information about protein structure and intra/intercellular interactions[60]. Indeed, as results from protein-protein interaction-oriented studies will be growingly available, it would become fundamental to characterize protein networks and pathways, rather than single molecules alone, which are likely to be involved in modulating biological functioning of platelets, as well as of other transfusion relevant blood components (red blood cells[61], for example). Undoubtedly, the rapid build-up of proteomic, transcriptomic and metabolomic information in medical sciences, due to the rapid technical progress in "omic" sciences, could be potentially translated into dramatic improvements of current clinical practice. However, this link requires a wise discernment of the connections between accumulated data, not to get lost in this vastness.

**Key words:** platelets, platelet concentrates, storage, cold activation.

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