From the Division of Clinical Immunology and
Transfusion Medicine at the Department of Laboratory Medicine
Karolinska Institutet, Stockholm, Sweden

NOVEL AUTOMATED PREPARATION AND COLD STORAGE OF BUFFY-COAT-DERIVED PLATELETS IN ADDITIVE SOLUTIONS: IN VITRO STUDIES

Per Sandgren

Karolinska Institutet

Stockholm 2008

Supervisor: Associate Professor Agneta Shanwell
Karolinska Institutet
Division of Clinical Immunology and Transfusion Medicine

Co-supervisor: Docent Hans Gulliksson
Karolinska Institutet
Division of Clinical Immunology and Transfusion Medicine

Opponent: Professor Gösta Berlin
Linköping University
Division of Clinical Immunology and Transfusion Medicine

Examination board:
Docent Lennart Rydberg
Sahlgrenska University
Department of Laboratory Medicine

Docent Nils Egberg
Karolinska Institutet
Department of Molecular Medicine and Surgery

Professor Lennart Eriksson
Karolinska Institutet
Department of Laboratory Medicine

Published by Karolinska Institutet. Printed by Universitets service US-AB

ISBN 978-91-7357-504-1

To Anna-Kari

ABSTRACT

This thesis focuses on (i) the in vitro quality of platelets (PLTs) prepared by novel automated techniques using the OrbiSac and the Atreus 2C+ systems; and (ii) evaluation of the ability to store PLTs for a prolonged time at 4ºC in an attempt to optimize the conditions under which PLTs are prepared and stored.

There has been an increase in the demand for supportive PLT transfusion therapy and in the search for a procedure for preparation of PLTs from pooled whole blood (WB)-derived buffy coats (BCs) for transfusion. Until now, this technique, which includes a sequence of manual steps, has been laborious and non-standardized, and has resulted in significant variance in PLT yield. This has led us to evaluate a novel automated system (OrbiSac) for the preparation of PLTs from BCs derived from WB and a novel automated system (Atreus 2C+) for preparation of BCs from WB, by studying PLT counts, and recovery and storage effects. The results of our in vitro studies suggest (i) that the OrbiSac technique is equivalent to the standard manual method regarding in vitro PLT characteristics during storage for 7 days, with uniform recovery of PLTs; and (ii) that PLTs derived from BCs produced using the novel automated Atreus 2C+ system to separate either fresh WB or WB stored overnight are equivalent to PLTs prepared using a semi-automated fresh WB separation process. Work detailed in this thesis (Papers I and IV) demonstrates that the Atreus 2C+ and the OrbiSac systems used in combination allow for automated production of PLTs with maintenance of in vitro PLT quality during 7 days of storage.

The development of the processing systems (Papers I and IV) was followed by an attempt to tackle the problem of how to refrigerate PLTs for transfusion (Papers II and III). Platelets are traditionally stored at 22ºC, which facilitates bacterial growth, and bacterial sepsis is regarded as the main risk of transfusion-transmitted diseases. For this reason, PLT storage is limited to 5 days. Storage at 4ºC would reduce not only the risk of bacterial growth, but may also delay the impairment of PLTs. Since the 1970s, considerable improvements have been made concerning processing and storage of PLTs, resulting in longer survival and better function, and providing an option for prolonged storage at 4ºC. Work detailed in this thesis demonstrates (i) that PLTs stored without agitation at 4ºC largely maintain their metabolic and cellular characteristics for 21 days of storage. We confirm that they lose their discoid shape and show that this loss of discoid shape during storage at 4ºC is associated with reductions in metabolic rate, and a decreased release of α-granule content. Furthermore (ii), we demonstrate that cold-induced activation, occurring at 4ºC, is not associated with increased expression of PLT membrane proteins and activation markers during long-term storage.

Our findings concerning the ability of cold temperatures to preserve PLT quality and prolong the storage period under modern blood bank conditions suggest that cold storage of PLTs may be possible in the future. However, this ability of PLTs to circulate and function in vivo remains to be demonstrated.

LIST OF PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals I-IV. The publications are reproduced in this thesis with kind permission of the publishers.

I. Larsson S, **Sandgren P**, Sjödin A, Vesterinen M, Gulliksson H. Automated preparation of platelet concentrates from pooled buffy coats: In vitro studies and experiences using the OrbiSac system. Transfusion 2005 May;45(5):743-51.

II. **Sandgren P**, Shanwell A, Gulliksson H. Storage of buffy-coat-derived platelets in additive solutions: in vitro effects of storage at 4^0C Transfusion 2006 May;46(5):824-34.

III. **Sandgren P**, Hansson M, Gulliksson H, Shanwell A. Storage of buffy-coat-derived platelets in additive solutions at 4^0C and 22^0C: flow cytometry analysis of platelet glycoprotein expression. Vox Sang. 2007 Jul;93(1):27-36.

IV. **Sandgren P,** Callaert M, Shanwell A, Gulliksson H. Storage of platelet concentrates from pooled buffy coats made of fresh and overnight-stored whole blood processed on the novel Atreus 2C+ system: in vitro study. Transfusion 2008. Published article online: 8-Jan-2008 doi: 10. 1111/j.1537-2995.2007.01593.x

OTHER PUBLICATIONS AND MANUSCRIPT BY THE SAME AUTHOR

I. Gulliksson H, Aubuchon JP, Vesterinen M, **Sandgren P,** Larsson S, Pickard CA, Herschel I, Roger J, Tracy JE, Langweiler M; Biomedical Excellence for Safer Transfusion Working Party of the International Society of Blood Transfusion. Storage of platelets in additive solutions: a pilot in vitro study of the effects of potassium and magnesium. Vox Sang. 2002 Apr;82(3):131-6.

II. Gulliksson H, Sjödin A, **Sandgren P**, Vesterinen M, Larsson S, Diedrich B. Automated preparation of platelet concentrates from pooled buffy coats using the Gambro OrbiSac system. Transfus Apheresis Sci. 2003 Aug;29(1):11-12.

III. Shanwell A, Diedrich B, Falker C, Jansson B, **Sandgren P**, Sundquist L, Svensson L, Vesterinen M, Gulliksson H. In vitro and in vivo comparison of paired apheresis platelet concentrates stored for 1 versus 7 days. Transfusion 2006 Jun;46(6):973-9.

IV. Diedrich B, **Sandgren P,** Jansson B, Gulliksson H, Svensson L, Shanwell A. In vitro and in vivo effects of potassium and magnesium on storage up to 7 days of apheresis platelet concentrates in platelet additive solution. Vox Sang. 2008 Feb;94(2):96-102.

V. **Sandgren P**, Hultenby K. Scanning and Transmission electron microscopy of platelets stored at 4^0C in additive solutions. (*Manuscript in preparation)*

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
BC	buffy coat
cAMP	cyclic adenosine monophosphate
CI	confidence interval
DAG	diacylglycerol
DEHP	2-diethylhexyl phthalate
DNA	deoxyribonucleic acid
DTS	dense tubular system
EGTA-AM	ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid-acetoxymethyl ester
ELISA	enzyme-linked immunosorbent assay
ESC	extent of shape change
FNHTR	febrile non-hemolytic transfusion reaction
G-protein	guanine nucleotide-binding protein
GDP	guanosine 5'-diphosphate
GP	glycoprotein
GT	glycosyltransferase
GTP	guanosine 5'-triphosphate
HLA	human leukocyte antigen
HSR	hypotonic shock response
IL	interleukin
IP3	inositol 1,4,5-triphosphate
LDH	lactate dehydrogenase
MFI	mean fluorescence intensity
MoAb	monoclonal antibody
MP	microparticle
MPV	mean platelet volume
NHTR	non-hemolytic transfusion reaction
OCS	open canalicular system
PAS	platelet-additive solution
PBS	phosphate-buffered saline
PF4	platelet factor 4
PIP	phosphatidylinositol phosphate
PKC	protein kinase C
PLT	platelet
PMP	platelet-derived microparticle
PVC	polyvinyl chloride
RANTES	regulated upon activation of normal T-cells expressed and secreted
RBC	red blood cell
RNA	ribonucleic acid
SD	standard deviation
SNARE	soluble N-ethylmaleimide-sensitive-factor attachment protein receptor

TCA	tricarboxylic acid cycle
TNF-α	tumor necrosis factor-α
TxA2	thromboxane A2
UDP-Gal	uridine 5[1]diphosphogalactose
vWF	von Willebrand factor
vWfR	von Willebrand factor receptor
WB	whole blood
WBC	white blood cell
β-TG	β-thromboglobulin

1 GENERAL INTRODUCTION

1.1 PLATELETS AND PLATELET TRANSFUSIONS – HISTORICAL BACKGROUND

At a session of the Academy of Science in Paris in 1842, Donne´ described platelets (PLTs), named “globulins”, as precursors or disintegration products of erythrocytes, leukocytes, fibrin, or bacteria (1). Interestingly, the first accurate PLT counts were reported by Hayem in 1878 (1). He referred to PLTs as hematoblasts since he thought they were precursors of erythrocytes. Bizzozero was the first to describe PLTs as distinct blood elements. In a paper published in 1882, he referred to them as “petites plaques” (1).

The value of PLT transfusions for thrombocytopenia was first reported in 1910, when Duke described three patients with bleeding due to thrombocytopenia, each of whom showed improvement with transfusions of PLT-containing fresh, whole blood (WB) (2) At that time PLTs were administrated by direct transfusion of WB without an anticoagulant. In 1916, Francis Rous and J. R. Turner introduced a citrate-glucose solution that permitted storage of blood for several days after collection. This allowed for blood to be stored in containers for later transfusion, and aided in the transition from the vein-to-vein method to transfusion of “stored blood”. This discovery also directly led to the establishment of the first blood 'depot' by the British during World War I. Oswald Robertson was credited as the creator of blood depots (3). After the war, Robertson went to China to work for the Rockefeller Foundation, and blood banking disappeared from Europe for almost 20 years.

In July 1936, the Spanish civil war broke out and the need for blood components in that country increased dramatically. As transfusion was not organized as a whole, the Republican army health services entrusted Dr. Duran-Jorda to develop a organization that could be self-sufficient in blood requirements. Duran-Jorda abandoned arm to arm transfusion and instead used citrated WB stored in sterile, 300 mL glass bottles (4). In the 1960´s breakable glass bottles for blood collection were replaced by plastic containers. The advent of plastic containers for blood collection made it feasible to centrifuge blood so that PLT- rich plasma could be prepared. Four to six fresh PLT-rich plasma units were considered a suitable PLT transfusion dose and PLT transfusions were now clearly established in several studies to substantially reduce the risk of death from hemorrhage during chemotherapy for leukemia (5-7).

During the 1970´s and 1980´s advances were made in the storage conditions for PLTs when S. Murphy and coworkers demonstrated the feasibility of storing PLTs at room temperature, revolutionizing PLT transfusion therapy (8-11). During the last few decades, considerable improvements have been made which have resulted in longer survival and better function of PLTs during storage, as shown by several in vivo and in vitro studies (12-14). The use of PLT additive solutions (PASs) has shown, that storage of PLTs in PASs permits better retention of properties during 5 days of storage, but may even be prolonged to 7 or more days (15-19).

Today, PLTs are used extensively to support patients receiving thrombocytopenia-inducing, intensive therapies for malignancies and solid tumors (20). The current reliance on PLT transfusions is reflected by the fact that in Sweden there has been roughly a tenfold increase in such transfusions since 1984. This brief historical overview is far from removes all the controversies that surround PLT transfusion practices today. Therefore, a review of the major problem areas that affect PLT quality will serve as background for understanding this large and complexed field.

Collection and preparation. Platelets are prepared by centrifuging WB obtained from a single blood donation. This process maximizes the yield of PLTs while limiting the number of red (RBCs) and white blood cells (WBCs). Optimal collection and preparation methods for PLTs require harvesting the largest possible number of PLTs without compromising their viability and function (21). A variety of methods for the preparation of PLTs for transfusion are available (16, 22, 23). Platelets have shown to be more or less activated after preparation (24-27), which may be due to different processing systems. Platelet units generally consist of PLTs and plasma or PLTs and PAS (16, 28)).

Storage. Platelets are stored at room temperature and constantly agitated to facilitate gas exchange (11, 21). Even so, numerous morphologic, biochemical and functional derangements occur during collection, processing and storage, which may affect post-transfusion function and survival (29). Room temperature storage of PLTs facilitates bacterial growth (30). For this reason, platelet storage is limited to five days (30), unless a bacterial contamination test has been performed, estimating the unit free from bacteria. In addition, PLTs release cytokines into the storage medium during storage (19), which may cause rigors and febrile reactions as well as allergic transfusion reactions (31-33).

Compatibility. Transfused PLTs can be destroyed by antibodies directed against class I HLA antigens presented on their membranes and, less commonly, by antibodies against ABO or PLT-specific antigens (34). Platelets have small amounts of ABO blood group antigens intrinsic to the membrane, as well as absorbed from plasma (35). Poor PLT recovery but a normal survival time has been reported when ABO-incompatible PLTs have been infused (36).

As ABO compatibility can influence the results of PLT transfusions and patients who experience poor increments from ABO-mismatched PLTs may therefore benefit from a trial of ABO-compatible PLTs before the initiation of HLA-matched PLT transfusion (36). A major cause of PLT refractoriness is alloimmunization against HLA class I antigens. However, no immunization against HLA will probably occur if leucocyte depleted PLTs are provided (37-39). Patients who are transfused on multiple occasions may develop PLT-reactive alloantibodies against HLA as well as to PLT-specific antigens. The PLT-specific alloantigen systems are located on glycoproteins (GPs) expressed at the surface of the PLT membrane (40). Platelets do not express Rh (D) antigen and Rh incompatibility does not influence PLT survival. However, Rh positive RBC contamination in the PLT unit may be sufficient to immunize an Rh negative recipient (41)

Indications for platelet transfusion and complications. Despite given guidelines with regard to indications for transfusion (42) the role of PLT transfusions, especially those given prophylactically to forestall bleeding, is not yet fully established and has been the subject of much discussion over the years. There are conflicting data regarding the optimal PLT dose for prophylactically transfusing patients with trombocytopenia and the most effective and efficient dose for prophylactic platelet transfusions are unknown (43). Febrile nonhemolytic transfusion reactions (FNHTRs) are a common complication of PLT transfusions, especially in adults (44, 45). Although prestorage of WBC reduction significantly reduce the rate of FNHTRs (45), FNHTRs still occur even after transfusion with PLTs that are leucocyte depleted before storage (45). One explanation for this may be the cytokines released from PLTs during storage (45). The incidence of FNHTRs appears to be less common among pediatric recipients of PLT transfusions than in adults (46). To date, few cases of confirmed bacterial contamination of PLT units have been reported (47). However, sepsis is still regarded as one of the main risks of transfusion-transmitted diseases (48, 49).

1.2 BLOOD PLATELETS

1.2.1 Platelet biology and structure

Understanding the biology of PLTs is important for the ability to identify and understand changes in PLTs that occur during processing and storage of PLT units intended for transfusion.

Platelets are small cells of great importance in maintaining hemostasis. They also play a significant role in many pathophysiologic processes, such as thrombosis, hemorrage, inflammation and tumor growth (50). Platelets are derived from the cytoplasm of megakaryocytes, arise in bone marrow and descend from pluripotent stem cells undergoing multiple deoxyribonucleic acid (DNA) replications without cell divisions, by the process of endomitosis. In endomitosis, early stages of mitosis occur within the nucleus without formation of the mitotic apparatus and metaphase plate and without division of the nucleus.

Megakaryocytes then begin a rapid cytoplasmic expansion phase characterized by the development of an elaborate demarcation membrane system and the accumulation of cytoplasmic proteins and granules essential for PLT function. During the following development, the megakaryocte cytoplasm undergoes a substantial reorganization into beaded cytoplasmic extensions called “pro-PLTs”, which released from the megakaryocyte and undergo further fragmentation into individual PLTs (50, 51).

Platelets, which are discoid in shape, circulate in a concentration of 150 to 400 x 10^9/L, and have a life span of 7 -10 days. The normal clearence of senile PLTs occurs primarily in the spleen and liver by macrophages that identify phagocytic signals expressed on the PLT surface (52). Not much is known about the PLT clearence mechanism, but one pathway involved in the clearence of damaged PLTs is suggested to be the macrophage scavenger receptor system (53).

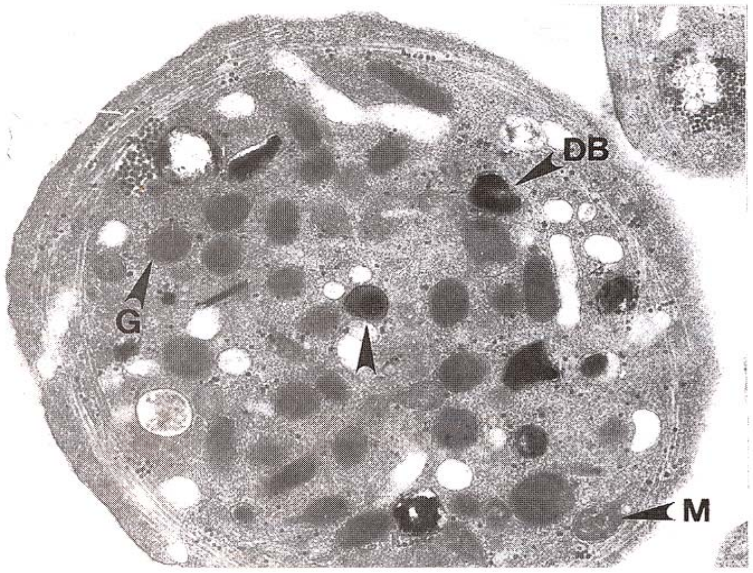
The only well-established mechanisms affecting PLT survival seems to be antibody-mediated PLT clearence, consumption of PLTs by coagulation processes, and loss due to massive bleeding. Antibody-mediated PLT destruction is a poorly understood process, although several lines of evidence suggest that splenic and hepatic macrophages may be involved, as well as destruction through phagocytosis (54, 55).

Platelets are the smallest of the many types of cells in circulating blood, averaging only 2.0 to 5.0 μm in diameter, 0.5 μm in thickness. They contain three major types of secretory organelles, namely, α-granules, dense bodies and lysosomes (51). (See Fig. 1) Alpha-granules are the most numerous of the PLT organelles, containing stored matrix adhesive proteins such as fibrinogen, fibronectin, thrombospondin, vitronectin and von Willenbrand factor (vWF).

Dense granule contains soluble activating agents such as adenosine diphosphate (ADP), serotonin and a small amount of P-selectin (51). Human PLTs contain few lysosomes. As PLTs do not isolate engulfed bacteria, which could be killed by lysosomal enzymes, the lysosomes in PLTs appear to be vestigal remnats with no known significant role in PLT function (56, 57).

Occasional multivesicular bodies are also present. Multivesicular bodies develop in the megakaryocyte by fusion of the small vesicles budding from the trans-Golgi zone of the Golgi complex, and may serve as sorting stations in the development of α-granules, dense bodies and lysosomes (58). Small numbers of relatively simple mitochondria are as well present in the PLT cytoplasm. They play an important role in energy metabolism (59). Other membrane-enclosed organelles or structures are also present in PLTs, such as glycosomes (60), electron-dense chains and clusters (61), and tubular inclusions (62).

Figure 1. A thin section of a discoid platelet (PLT) containing many α-granules (G), a few mitochondria (M), and several dense bodies (DB). Illustration taken from White, Platelets, second edition 2007 (Fig 3-41), Page 59, Copyright Elsevier (2007).



Platelets have an open canalicular system (OCS), a system of internal membranes formed into a network of tubules, which permeates the PLT. Not only is the OCS connected to the PLT surface membrane; it *is* surface membrane. When PLTs become activated, the OCS serves as a channel into which the PLT granules fuse and release their contents (63, 64). In addition, channels of the OCS provide a means for chemical and particulate substances to reach the deepest recesses of the cell (65). As a result, the OCS may be the major route for uptake and transfer of products from plasma to PLT α-granule (66, 67). Moreover, PLTs have two membrane systems: the OCS and the dense tubular system (DTS), which channels are distinguished from the canaliculi of the OCS (68, 69).

The lipid bilayer of the surface membrane and lining of the OCS are morphologically similar. Both are covered with GP receptors necesarry to facilitate PLT adhesion to a damage surface, and promote activation, aggregation and interaction with other cells (70). Some types of secretory organelles have GP receptors embedded in their membranes. P-selectin, which mediates binding of activated PLTs to granulocytes and monocytes (71), is stored in their membranes, as well as a portion of the major PLT adherence receptors, vWF and the integrin $\alpha_{IIb}\beta_3$.

The PLT cytoskeleton contains two actin filament-based components. One is the cytoplasmic actin filaments which fill the cytoplasm and mediate contractile events. The other is the membrane skeleton, which coats the plasma membrane and regulates properties of the membrane such as its contours and stability. In the unstimulated PLT, only 30-40% of the actin is polymerized into filaments; the rest is thought to be prevented from polymerizing by the association of thymosin β4 with monomeric actin,

and by the association of gelsolin with the barbed ends of preexisting actin filaments (72). When PLTs are activated, there is a rapid increase in actin polymerization; new filaments fill the extending filopodia and form a network at the periphery of the PLT. As a result of activation, myosin binds to cytoplasmic actin filaments, causing them to move towards the center of the PLT. As PLTs aggregate, additional cytoskeletal reorganizations occurs (72).

1.2.2. Platelet Function, short introduction

Platelets provide the first line of defence during the hemostatic process by formation of a hemostatic plug upon vessel injury that begins with apprehending circulating PLTs on exposed collagen and continues with the recruitment of additional PLTs into a growing PLT mass that will eventually be stabilized with cross-linked fibrinogen (73).

Formation of a PLT plug occurs at sites of vascular injury when circulating PLTs apprehend, and are activated by, exposed collagen and vWF that binds the Gp1bα subunit of the von Willebrand factor receptor (vWfR), allowing the accumulation of a PLT monolayer followed by binding collagen through GPVI and $\alpha_2\beta_1$ integrin. Ligand binding to vWF or GPVI initiates inside-out signals that activate PLT integrin $\alpha_{IIb}\beta_{3,}$ to bind fibrinogen to mediate firm adhesion and aggregation (73).

Additional PLTs then accumulate on the initial monolayer, a process for which $\alpha_{IIb}\beta_3$ activation is necessary but not sufficient. During this phase, the presence of receptors is necessary, which are able to rapidly respond to generated thrombin, secreted ADP or released thromboxane A2 (TxA2) to activate phospholipase C, increase the cytosolic Ca^{++} concentration and suppress synthesis of cyclic adenosine monophosphate (cAMP). Most of the receptors involved in these events are members of the superfamily of guanine nucleotide-binding protein (G-protein)-coupled receptors (73).

After PLT activation and aggregation have occurred, processes take place that consolidate the stability of the forming plug, as well as procoagulant events. In this thesis work, interactions between PLTs and the coagulation system have not been studied.

Figure 2. Scanning electron micrographs of different stages of PLT adhesion are shown in Figure 2. Resting platelet (left); Attached platelet showing shape change and pseudopia emission (center); Spread platelet (right)

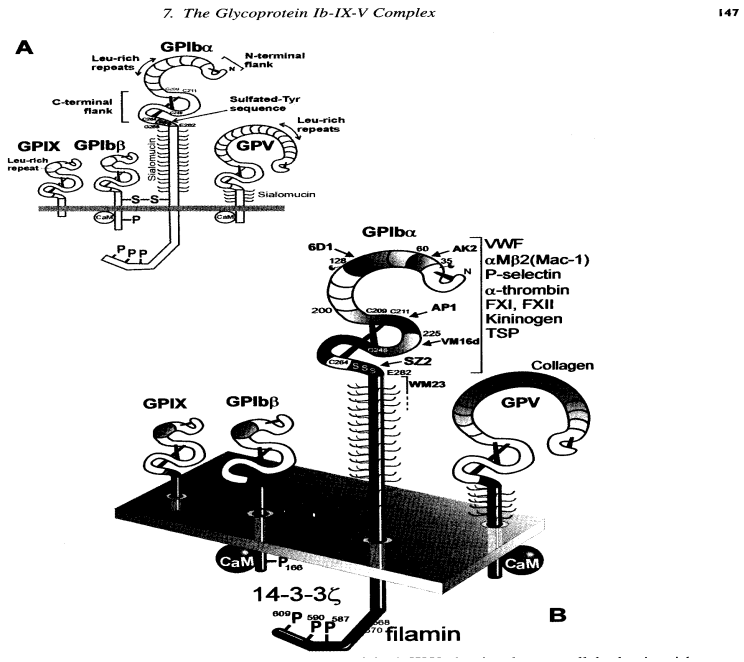
1.2.3 Platelet surface receptors

Because the main function of PLTs is hemostasis their major receptors have a direct role in this process, either in activating PLTs or as adhesive receptors interacting with damaged cell walls or with other PLTs to contribute to thrombos formation. The number of known receptors has expanded dramatically, and there have been breakthroughs in the identification and characterization of major receptors such as those for collagen, ADP, adenosine triphosphate (ATP), vWFR and G-protein-coupled receptors. Here, because of the focus of this thesis, I will discuss receptors which we studied to detect up-or downregulations, related to PLT quality during preparation and storage. Special attention has been given to the vWF-dependent adhesion receptor GPIbα due to this receptor´s importance in maintaining PLT viability in vivo, when PLTs have been exposed to a cold environment (74-78). In general, we studied up or down regulations of receptors associated with the main functions of PLTs, such as adhesion and aggregation

1.2.3.1 Platelet adhesion

The adhesion molecule vWF binds to subendothelium, which then allows PLTs to adhere to the injury site within 1-3 seconds of injury (79). Glycoprotein 1bα (GP1bα) is a subunit of the GPIb-IX-V complex, and is the receptor for vWF and a high affinity receptor for thrombin (80). (Fig. 3). The vWfR complex consists of four subunits, namely, GPIbα, GP1bβ, GPIX and GPV. Filamin binds to the cytoplasmic tail of the GPIbα subunit and links the vWfR complex to actin cytoskeleton. Glycoprotein Ibα´s extracellular domain can be divided into (i) the ligand binding domain, including the N-terminal flank, leucine rich repeats, the C-terminal flank and the sulfated region: and (ii) the C-terminal macroglycopeptide region.

Figure 3. Structure of the GPIb-IX-V complex as described by Robert K. Andrews published in "Platelets, second edition 2007, Fig 7-2, Page 147, Copyright Elsevier (2007).



1.2.3.2 Platelet aggregation

$\alpha_{IIb}\beta_{3,}$ (GPIIb-IIIa or CD41/CD61) is the major integrin on the PLT surface. Glycoprotein IIb, which is usually linked with GPIIIa, forms the GPIIb-IIIa complex. This complex serves as a receptor for soluble fibrinogen, fibrinonectin, thrombospondin, vWF and vitronectin (81*)* and plays a main role in the regulation of PLT aggregation and adhesion throughout haemostasis (82). During PLT activation, the expression level of GPIIb-IIIa on the PLT surface increases. A conformational change then occurs, which creates the active binding site for fibrinogen (83).

1.2.4. Intracellular signaling

A variety of PLT agonists, including thrombin, epinephrine, collagen from the subendothelium and ADP, cause intracellular signaling. In general, these events promote the activation of G-protein-coupled pathways. Platelets have at least five functionally different G-proteins. Once an agonist binds to its receptor, its associated G-protein releases guanosine 5'-diphosphate (GDP), binds guanosine 5'-triphosphate (GTP) and activates a signal-generating enzyme, such as phospholipase C. Phospholipase C hydrolyzes the precursor inositol phospholipid (PIP) PIP_2 into the second messengers inositol 1,4,5-triphosphate (IP_3) and 1,2 diacylglycerol (DAG). Inositol 1,4,5-triphosphate causes an increase in the cytosolic Ca^2+ concentration by releasing stored Ca^2+ from the DTS, and DAG activates proteinkinase C (PKC). These events promote TxA2 generation, shape change granule secretion, activation of the fibrinogen receptor integrin $\alpha_{IIb}\beta_{3,}$ (GPIIb-IIIa) and ultimately PLT aggregation (73, 84).

1.2.5 Granule secretion

Upon activation, PLTs secrete the contents of the dense granules and α-granules, as well as GPs with coagulating properties, which are "blebbed" from the plasma membrane. Annexin V is set free into the blood whenever cells are damaged. The Annexin V content of blood plasma or during storage can therefore be used as indicator for cell damage (85, 86). The process of membrane vesiculation and microparticle (MP) release in PLTs has been shown to require a calpain-dependent dissociation of membrane proteins from the submembrane cytoskeleton and most likely involves intracellular signaling by PLT protein kinases (87).

Alpha-granules contain adhesion molecules (e.g., P-selectin, vWf, thrombospondin, fibrinogen and fibronectin), fibrinolytic regulators, growth factors, chemokines (e.g., PLT factor 4 (PF4), the regulated upon activation of normal T-cells expressed and secreted (RANTES) and β-thromboglobulin (β-TG)), immunologic modulators and other proteins

(e.g., albumin). Some α-granule proteins are "PLT–specific", molecules that are synthesized only in megakaryocytes and unique to PLTs (e.g., PF4 and β-TG). Other molecules are "PLT– selective" (e.g., P-selectin, vWf and fibrinogen) as they are synthetisized or endocytosed by megakaryocytes or a small number of other cells (88-92).

Dense granules are less abundant than α-granules in human PLTs (93). They contain ions (e.g., Ca and Mg), nucleotides (ATP, GTP, ADP and GDP), a relatively small amount of membrane proteins (granulophysin and LAMP 2) and a transmitter (serotonin). In addition, P-selectin seems to be present in both α-granules and dense granules (94, 95). Platelet exocytosis involves reorganization of the actin structure, the movement of granules into close physical apposition with the plasma membrane, granule-plasma fusion and release of intracellular contents (96). Platelets secretion occurs through a soluble N-ethylmaleimide-sensitive-factor attachment protein receptor (SNARE) protein dependent mechanism (96). The formation of trans-SNARE complexes, bridging the gap between the granule and plasma membrane, is required for exocytosis (97-100). Increases in intracellular Ca^{2+}, acting through unidentified Ca^{2+} sensor molecules, leads to fusion of the granule with the plasma membrane, and granule secretion.

1.3 TECHNIQUES FOR PLATELET PREPARATION

A variety of methods for the preparation of PLTs for transfusion are available such as, PLT-rich plasma method (22, 101, 102), apheresis method (23) and buffy-coat (BC) method (16). In the following, I will focus on the preparation of PLT concentrates by pooling of BCs and addition of PASs.

1.3.1 Preparation of platelet concentrates by pooling of buffy coats and addition of platelet additive solutions.

Buffy-coat-derived PLTs has until now been prepared essentially by manual technique. The technique for the preparation of BC-PLTs, as described by Pietersz (12, 103) and several other authors (104, 105) is shown in Figures 4 and 5. The technique of removing the BCs from WB after centrifugation was initially introduced to reduce the number of WBCs, which are associated with FNHTRs (31, 106, 107), as well as to reduce the microaggregate formation by WBCs and PLTs during storage (108).

By use of an initial hard spin, most of the PLTs were concentrated in the BC layer. As an additional effect, BCs can be used to supply PLTs for the preparation of PLT concentrates by combining four to six BCs. To reduce the number of WBCs to $<1 \times 10^6$, the European standard for leukoreduced blood components, the preparation of PLTs from BCs can be combined with a leukofiltration step.

Platelet additive solution in combination with platelet concentrates prepared from pooled buffy coats (1).

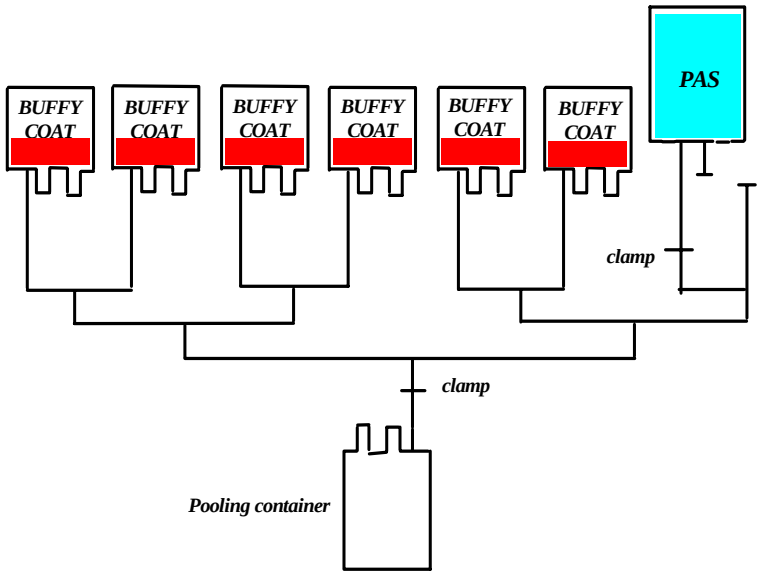


Fig 4. Preparation of platelet (PLT) concentrates by pooling of buffy-coats (BCs) and addition of synthetic PLT storage medium as follows: (1) each BC is prepared from whole blood (WB) by centrifugation at high speed to concentrate the PLTs in the BC layer (generally 3000 – 4000 g for 8-15 minutes), (2) each BC generally contains 20 to 30 mL of red cells, 25 to 35 mL of plasma and PLTs, (3) the BC containers (4-6) are connected to a pooling container by sterile connection equipment, (4) the BCs are transferred to pooling container and BC containers are washed with a PLT–storage medium to recover additional PLTs, and (5) the BC contents as well as the contents of the PLT-storage medium container are transferred to the pooling bag.

PAS in combination with platelet concentrates prepared from pooled buffy coats (2).

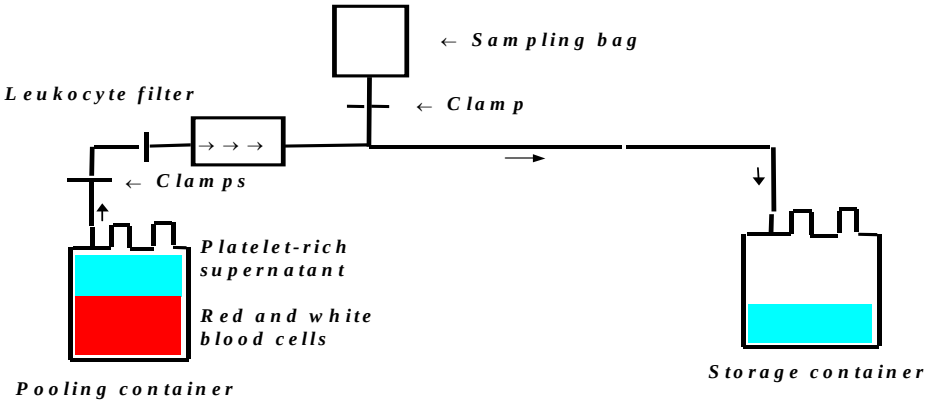


Fig 5. The preparation of platelet (PLT) concentrates using pooled buffy-coats (BCs) suspended in PLT storage medium is by preparation of PLT-rich supernatant and transferring the supernatant to a PLT storage container. After disconnecting the pooling bag and centrifugation at 500*g* for 7 – 9 minutes, a PLT-storage container is connected, often including a leukocyte depletion filter. The PLT-rich supernatant is transferred to through the filter to the storage container. The storage container is then disconnected and the PLT unit is ready for storage (Gulliksson 2003).

1.3.2 Novel automated preparation of platelets

The preparation of PLTs from BCs, however, is laborious and includes many manual steps, which makes automatization of the process attractive. In this thesis work, we evaluated the performance of a novel automated system for the preparation of PLTs from BCs derived from WB (OrbiSac), by studying the PLT counts, the recovery of PLTs, and the storage effects. In vitro studies and experiences with routine preparation of PLTs with the automated technique are further investigated and discussed in Paper I, as is also the further development of the preparation technique with introduction of an integrated leukoreduction filter, optimization of the preparation process, and standardization of the PLT content in the unit. Based on calculations from the original BC pool, the recovery of PLTs produced by the OrbiSac system was approximately 75 - 80%.

Several techniques for WB component separation into RBC concentrate, BCs and plasma have been developed to satisfy different requirements for preparation (109). However, these techniques all require a number of hand-over steps affecting staff time and production flow in the blood centre. Novel automated equipment, the Atreus 2C+ system, eliminates several of these hand-over steps by combining them into one integrated process, which has several potential benefits. In synergy with the OrbiSac system, the Atreus 2C+ system, allows for an almost fully automated production of PLTs. The in vitro quality of PLTs prepared with the automated technique and derived from BCs, obtained from either fresh WB or overnight stored WB and processed on the Atreus 2C+ system, has been investigated and discussed in Paper IV in this thesis.

1.4 STORAGE OF PLATELETS

Several factors influence the quality of the PLTs during storage. Including the preparation method, the composition of the storage bag, additive solutions, the temperature, the PLT count and agitation of the concentrates. Since it is of vital importance, we will start by discussing the PLT metabolism.

1.4.1 Platelet metabolism

In order to maintain viability, PLTs must continously generate new ATP to meet their energy needs (110). There are two major metabolic pathways through which PLTs can generate ATP, one that requires oxygen, the tricarboxylic acid cycle (TCA) and the respiratory chain, and one which does not require oxygen, glycolysis. Generally in cell metabolism, glucose has a dual role as a substrate, on the one hand for glycolysis, resulting in two molecules of lactic acid being formed from one molecule of glucose, and on the other for the TCA and oxidative processes, with CO_2 and water as end products (110). Some data suggest that the major part (85 %) of the energy generation is via the TCA cycle (110). One possibility is that glucose may primarily be used as substrate for glycolysis, but may not be the primary substrate for oxidative metabolism. It is also known that fatty acids and amino acids may be used as substrates for oxidative metabolism of stored PLT cells. (111). Consequently, approximately 15 % of the energy generation may be generated by glucose metabolism (110). However, in Papers II and III, we reported that when PLTs are suspended in storage media, and stored at 22^0C, their metabolism depends on the presence of glucose during the entire storage period. This finding has also been described in previous reports (16).

In glycolysis, glucose is converted to lactate and a free hydrogen ion, which cannot leave the storage bag (18). The hydrogen ion, converted from glucose during glycolysis, can be buffered by the bicarbonate in the plasma and converted to H_2O and CO_2 (8). The bicarbonate in the plasma is limited and will not tolerate extended lactate concentrations above certain levels. Above these levels, the pH will fall rapidly, which might result in lost PLT viability (8). However, in some studies, addition of acetate and/or pyruvate to PLTs stored in plasma slowed the rate of bicarbonate consumption by approximately 50% as compared with reference PLTs, and thereby inhibited the fall in pH (112). Acetate cannot act directly as a buffer, but can be oxidized by PLTs, leading to production of bicarbonate (113).

A decrease in pH has been shown to be associated with increased formation of lactate (9, 114) and reduced glucose levels (114). In one study, these changes were linearly related to pH (114). Various other factors, apart from the oxygen permeability affect the pH such as the acetate metabolism (115, 116). The buffer capacity of the medium paticularly bicarbonate, will also affect the pH (112, 114, 116) and an earlier report has shown that low pH levels is related to deterioration of PLT adenine nucleotid levels (117). To facilitate gas exchange, mixing has been found to greatly affect the viability of PLTs stored at 22^0C (118-120). The temperature has also been shown to influence the glucose consumption and lactate accumulation (121, 122)

1.4.2 Platelet storage containers

One of the most significant advances in PLT transfusion therapy was the development of plastic, gas- permeable storage containers (9). These storage bags are specifically designed to allow adequate gas (O_2 and CO_2) exchange. The earliest storage bags made of polyvinyl chloride (PVC) and a 2-diethylhexyl phthalate (DEHP) plasticizer did not allow PLT storage beyond 3 days. Aerobic metabolism was not maintained, which resulted in lactic acid production and a rapidly falling pH, booth of which affect in vivo recovery and survival (9, 110). The next generation of storage containers, composed of either PVC and non-DEHP plasticizers such as butyryl-tri-hexyl citrate, or other non-PVC plastic containers, was more gas permeable, allowing the storage of PLTs for 5 days at 20 to 24^0C, while maintaining acceptable degrees of in vitro function and in vivo survival (10, 123, 124, 125).

1.4.3 Agitation of platelets

Platelets are stored with continous gentle agitation because most measures of in vitro PLT function and structure deterioate more rapidly when PLTs are stored without agitation (126). A more recent study suggests that pH will decrease more rapidly when the PLTs are stored without agitation (120). In addition, agitation seems to prolong PLT survival following transfusion (127). Horizontal agitation on a flat-bed agitator is generally preferred over circular rolling "Ferris wheel"–type agitation (14, 128). Agitation is thought to enhance the transport of gases such as O_2 and CO_2 through the storage container. However, surface interactions between the agitated PLTs and the plastic container may also result in shear stresses capable of activating PLTs. Although, considerable improvements have been made concerning processing and storage of PLTs, resulting in longer survival and better function (16, 28, 129) and although research suggests that PLTs can sustain 4 days without agitation (120), most evidence supports the standard that PLTs be routinely stored with agitation (118). It is important to remove air bubbles from the storage bag preceding agitation, as the foam occurring after preparation may interact with PLTs, causing activation and release reactions (130).

1.4.4 Storage in synthetic media

The storage of PLTs in PAS might have several advantages. Optimized synthetic storage media might help counteract the PLT storage lesion (see section 1.4.5), thereby facilitating extended storage. Numerous PASs have been formulated with this idea in mind. Because additive solutions replace 60–80% of the plasma in the original PLT unit, these formulations are predicted to have additional benefits: reduced allergic and febrile transfusion reactions (131), decreased transfusion of unwanted antibodies (anti-A, anti-B and antibodies against HLA antigen) and increased plasma made available for fractionation (132).

A recent multicenter randomized study of the efficasy of transfusions with PLTs stored in PAS II versus plasma showed no difference regarding bleeding complications or transfusion intervals, and support the view that adverse transfusion reactions occur significantly less after transfusion with PAS II (133). The composition of several PASs as described by Eriksson is shown in Table 2.

Table 2. Composition in mmol/L of the different platelet additive solutions used (Eriksson)

	PlasmaLyte A	**PAS-II**	**PAS-III**	**PAS-IIIM**	**Composol**
NaCl	90	115.5	77.3	69.3	90
KCl	5	-	-	5	5
$MgCl_2$	3	-	-	1.5	1.5
Na_3 citrate	-	10	10.8	10.8	11
Na phosphate	-	-	28.2	28.2	-
Na acetate	27	30	32.5	32.5	27
Na gluconate	23	-	-	-	23

In an additive solution unit, the final medium contains 20–40% donor plasma. This carried-over plasma provides glucose for PLT metabolism (134). The electrolytes contained in the PAS are in such amounts that when added to the PLTs the final composition contains the electrolytes in the approximate concentrations found in normal blood plasma (135). There are good reasons for believe that addition of Mg and K ions may also increase the ability of PLTs to withstand storage (19, 129, 136).

The buffering capacity of PASs without acetate is generally very limited compared with that of plasma (113, 137). Therefore, several additive solutions also contain acetate, which serves as a second metabolic fuel and has the added benefit of providing a buffer effect by generating bicarbonate (138) as well as phosphate, having two possible roles in PLT metabolism: acting as a buffer to prevent a fall in pH, and stimulating PLT glycolysis (134).

Citrate included in the storage medium affects the rate of glucose consumption and lactate production and is useful as an anticoagulant for the PLTs (28, 138). In addition, L-carnitine may be a useful additive in PLT preservation by limiting lactate accumulation (139). The components described illustrate the complexity of the effects of additives and their interdependence and interaction. The optimal storage solution probably still has to be found.

1.4.5 The platelet storage lesion

It has long been recognized that PLT viability and function decline over time, a phenomenon known as the "platelet storage lesion" (140-143). Numerous morphologic, biochemical and functional derangements occur during PLT collection, processing and storage. These changes are important because they are associated with decreased posttransfusion survival (29). However, a recent report showed that in vivo recovery and survival of PLTs stored for 7 days decreased (144), but met suggested criteria (145). Therefore, randomized patient transfusion studies will provide additional verification of the suitability of 7-day storage of PLTs (144).

Platelets continue to be metabolically active at room temperature. Products of metabolism, such as lactate accumulate, and a fall in pH has been reported. It has also been shown that if the pH drops below 6.0–6.2, survival in vivo is severely diminished (140). Platelets also tend to become activated during storage. Over time, an increasing fraction of PLTs in a concentrate will change from a discoid (resting) shape to a spherocytic shape. Platelet specific cytokines, such as ß-TG, RANTES and PF4 accumulate in the storage medium, reflecting granule release (19). P-selectin (CD62), a constituent of α-granules in resting PLTs which can be detected on the activated PLT surface after α-granule secretion (71, 146), as well as CD63, suggested as a marker for dense granule release (147, 148), has been shown to increase during storage (149).

Some of the morphological and biochemical changes that characterize this storage lesion are reminiscent of cell death by apoptosis (150). Platelets do contain all proteins required for apoptosis, including cytochrome C, procaspase 9, procaspase 8 and procaspase 3. Addition of cytochrome C to PLT lysates results in formation of active caspase 3, illustrating that at least the so-called “intrinsic pathway” of apoptosis is functional. So far, studies of the possible contribution of apoptotic-like processes to the formation of PLT-derived microparticles (PMPs) and other responses during activation, as well as during storage, have yielded conflicting data (151, 152). However, earlier work suggests that proteins involved in cell apoptosis are unlikely to account for the PLT storage lesion, as caspase activation is a late event during PLT storage (153).

While a variety of methods have been applied to evaluate PLT quality during storage (154), there appears to be no in vitro test that clearly predicts the in vivo recovery, survival and function of transfused PLTs. However, a battery of in vitro tests reflecting the different aspects of PLT function is so far the best tool and is widely used in an attempt to identify the PLT storage lesion by detecting changes that occur during processing and storage of PLTs.

Some selected in vitro tests are thought to be crucial. Swirling should be present, because its absence is highly predictive of poor posttransfusion PLT increments (155, 156). The swirling phenomenon is observed when a light beam through a PLT concentrate is broken by discoid PLTs, representing the shape of discoid PLTs. The absence of the swirling phenomenon has been reported to correlate well with PLT disc-to-sphere transformation (157). Throughout storage, pH should be in the range of 6.8−7.4, and glucose should be present in the PLT unit as energy fuel (105). Moreover, it is suggested that PLT ATP levels should be >4.0 μmol/10^{11} PLTs since lower concentrations may be associated with loss of in vivo viability. (158).

Hypotonic shock response (HSR) as well as extent of shape change (ESC), has been shown to be suitable in vitro tests to predict post transfusion efficacy (140, 159, 160).

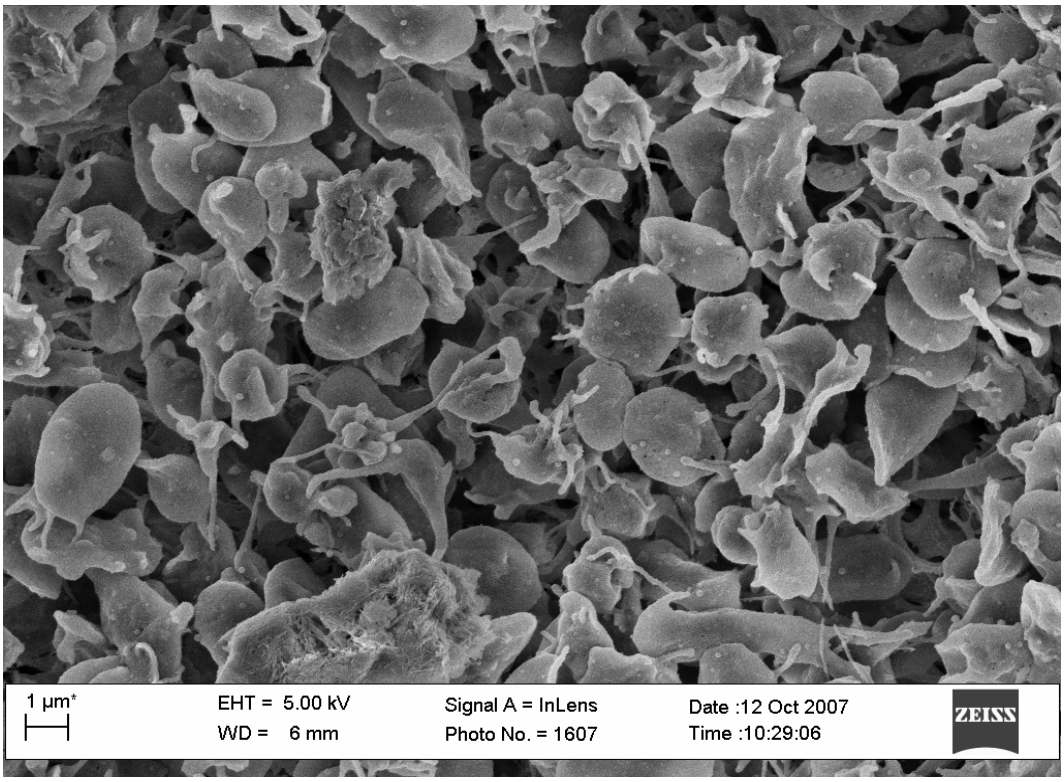
Three approaches (16) seem to be of specific importance to optimizing the long-term storage of PLTs. They are (i) to reduce the activation of PLTs during the collection of blood and during the preparation and storage of PLTs; (ii) to reduce the metabolic rate of glucose consumption and lactate production; and (iii) to ensure that glucose is available in the storage medium during the entire storage period.

1.4.6 Bacterial contamination

Storage of PLTs at room temperature facilitates bacterial growth, and bacterial sepsis is now regarded as one of the main risks of transfusion-transmitted diseases (161, 162). For this reason, PLT storage is limited to five days, unless a bacterial contamination test has not been performed, estimating the unit free from bacteria. Two strategies to dealing with the bacterial contamination issue seem to be current practice in transfusion medicine. One of the most widely used strategies for decreasing post transfusion bacterial sepsis risk is bacterial detection. Some investigators have shown that bacterial screening technology is useful for eliminating the transfusion of PLTs that contain high levels of contaminating bacteria though there may be limitations to detecting lower bacterial levels (163). Consequently, these data suggest that bacterial screening does not prevent all transfusion-transmitted bacterial infections.

Pathogen inactivation is the other strategy, This is based on one of two methods, the first of which involves psoralen-based compounds intercalating into helical regions of pathogens to form “cross-links”, which inhibits replication of any bacterial DNA or ribonucleic acid (RNA) when exposed to photochemical treatment (164, 165). However, such treatment may affect the PLTs and it has been reported that mitochondrial DNA in such PLTs is substansially modified (166). The other method involves technology based on the use of riboflavin and ultraviolet light to generate chemical reactions in the nucleic acids of pathogens, which prevents replication and leads to inactivation (167, 168). Figure 6 shows scanning electron micrographs (day 2) of PLTs prepared by the OrbiSac system, stored in PAS II (T-Sol) and estimated free from bacteria.

Figure 6. Scanning electron micrographs (day 2) of platelets (PLTs) prepared by the OrbiSac system and stored in PAS II (T-Sol). (Sandgren, Hultenby)



1.5 COLD STORAGE OF PLATELETS

Storage at 4^0C is another approach to dealing with bacterial contamination that occurs at 22^0C storage. Storage at 4^0C would reduce not only the risk of bacterial growth, but may also delay the impairment of PLTs and prolong the storage period. It has been known for over 30 years that PLTs stored at 4^0C have a shorter circulation time than PLTs stored at 22^0C, after transfusion into human volonteers (169). When refrigerated murine PLTs are injected into mice, they showed dramatically reduced circulation times as well (77). Storage of PLTs at temperatures below 15^0C increases cytosolic Ca, actin polymerization and shape change (170, 171). Such PLTs also change from discoid to spherocytic shape (170).

The discoid shape of the PLTs was thought for long to be the best predictor of normal PLT survival time in the circulation (140, 143, 172, 173). An attempt to maintain refrigerated PLTs in a discoid shape using cytochalasin B, an actin assembly inhibitor and ethylene glycol-bis (β-aminoethylether) -N,N,N',N'-tetraacetic acid-acetoxymethyl ester (EGTA-AM), an intracellular calcium chelator (174), did, however, not increase the circulation time of transfused murine PLTs (77) nor of baboon PLTs (175). Some authors even claim that non-discoid PLTs may survive after transfusion and even revert to a discoid shape (176).

Recently the mechanism responsible for the reduced circulation times of transfused refrigirated PLTs, clearence, was described (77). In refrigerated PLTs, the vWfR (Gp1bα or CD42b) is irreversibly rearranged to form a cluster on the surface of the PLTs. The integrin receptor of hepatic macrophages recognizes the clusters with their exposed incomlete GPIbα N-linked glycans on the surface of the PLTs and removes them from the circulation. Over 30 years ago, Jamison and Barber (177) proposed that an externally disposed glycosyltransferase (GT) activity mediates PLT adhesion and other functions. Subsequent work ruled out such activity in nucleated cells and established the Golgi apparatus as the primary site of such enzymes, although no further studies examined their role vis-a`-vis PLTs. Recently the existence of GT activity in PLTs was reported (78) and it was described how megakaryocytes package and deliver Golgi-associated GTs into PLTs and their surfaces using dense granules (178).

These findings suggest possible new roles of PLT GTs and carbohydrates. As a consequense of these findings, some studies suggest that enzymatic galactosylation with uridine 5^1diphosphogalactose (UDP-gal) can inhibit lectin binding between the integrin receptor of hepatic macrophages and the clusters on the surface of the PLTs and prevent phagocytosis. Galactosyltransferase enzymes transfer galactose into the exposed incomplete GPIbα N-linked glycans residues using UDP-Gal as the substrate. Addition of UDP-Gal is suitable before or after cooling and the glycan modification is stable during storage (78).

Uridine 5^1diphosphogalactose, a normal constituent of human cells is detectable in body fluids (179). Recent work shows that spherocytic murine PLTs, when protected against clearence (78), function well and that spherocytosis does not damage many aspects of PLT function (180). Moreover, chilled and rewarmed circulating murine PLTs protected against clearence are not activated and they function normally after an in vivo injury (78). In addition, recent work also shows that human WB PLTs exposed to 4^0C adhere and aggregate under close to physiological conditions (181)

On the basis of these results, other groups including ourselves are now reinvestigating PLT storage at 4^0C (74-76). To date, there is some evidence that Hoffmeister et al.'s approach, which suggests that galactosylation with UDP-Gal of PLTs can inhibit lectin binding before or after cooling (78), allows cold storage of human PLTs and provides the protective effect for human PLTs stored cold, as well as prolonging the survival time of refrigerated human PLTs in a rabbit model (182). A recent study also observed that galactosylation prevents in vitro recognition by macrophages of short term-stored refrigirated PLTs. In contrast to PLTs stored at 4^0C for a short period, the phagocytic ratio of galactosylated PLTs increased by approximately twofold after 14 days of storage (183). These results indicate a possibility that a different clearence mechanism might operate after long-term storage in plasma.

An attempt to tackle the practical problem of how to refrigerate PLTs for transfusion can therefore be divided into two separate problems, the "clearance problem" and the "storage problem". Both the clearance problem, which involves a shorter circulation time after transfusion, and the storage problem have been known about since the 1970s, especially the detrimental changes in terms of in vivo behavior when the storage time at 4^0C exceeded 24 hours (169, 184).

Since the 1970s, considerable improvements have been made concerning processing and storage of PLTs, resulting in longer survival and better function (16-18, 28, 105, 140, 185) and providing an option for prolonged storage at 4^0C. As UDP-Gal may provide a solution to the clearance problem, the ability to store PLTs over a prolonged period of time at 4^0C under modern human blood banking conditions is being further investigated and discussed in Papers II and III in this thesis.

2 AIMS OF THE STUDY

The overall aim of the present thesis work has been to optimize platelet transfusion therapy by -

1) introducing a novel automated technique, the OrbiSac system for preparation of PLTs from pooled WB-derived BCs (Paper I);

2) investigating the effects on in vitro PLT quality of storing either WB or BCs overnight before preparation of PLTs, by using a novel automated equipment, the Atreus 2C+ system for BC preparation (Paper IV);

3) studying the in vitro quality of PLTs stored at 4^0C (Papers II and III);

4) investigating the PLT storage lesion by studying changes that occur during processing and storage of PLTs; and

5) implementing a new method for studying GPs, which play an important role in the main function of PLTs.

3 MATERIAL AND METHODS

Materials and methods have been described in detail in each paper. Selected methods are discussed here.

3.1 Preparation and storage of platelets

Platelets were prepared by novel automated technique, using the OrbiSac system, and stored in PAS II (T-Sol). This technique was introduced prior to the study described in Paper I and was further used as production system for PLTs during the following studies.

3.2 Preparation of buffy coats from whole blood

In all studies, BC were produced using a quadruple-blood bag system, the Top-and-Top system (Imuflex-CRC; Terumo, Tokyo, Japan), made of PVC with DEHP as plasticizer, A total of 450 mL WB was collected from healthy blood donors who met standard donation criteria. The WB containers were kept at room temperature (20-24^{o}C) by cooling plates (Sebra, Tucson, AZ, USA) and centrifuged at 2700 g for 10 minutes at 22^{o}C within 8 hours of collection. Centrifugation was immediately followed by separation into RBCs, plasma and BCs using automated equipment, the Terumo automatic component extractor (T-ace; Terumo, Tokyo, Japan). Buffy coats were kept at room temperature (20-24^{o}C) over night without agitation. This preparation procedure of BCs from WB, however, is laborious and includes many manual steps, which makes automatization of the process attractive (Paper IV).

3.2.1 Buffy coats obtained from fresh and overnight stored whole blood and processed on the novel Atreus 2C+ system

A novel automated equipment, the Atreus 2C+ system (Gambro BCT, Zaventem, Belgium) was used which eliminates several hand-over steps that are necessary during the process described in section 3.2, by combining them into one integrated process. This has several potential benefits. The Atreus equipment consists of an automatic centrifuge, hydraulic expressor, valve/sealer, and barcode reader all packaged into one machine which automatically separates WB into a BC, RBC and plasma unit (see Fig. 7). The “2C” in the name “Atreus 2C+” stands for “two components + a BC unit”.

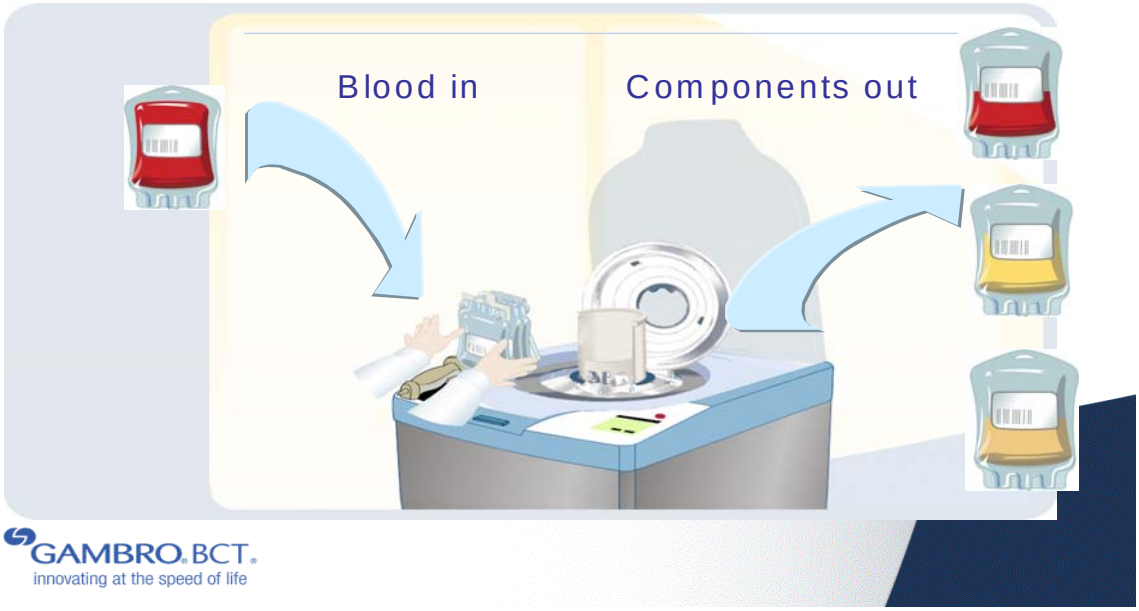


Figure 7. The Atreus process: the Atreus system automatically separates WB into plasma, a BC and an RBC unit.

There are some possible advantages to storing the WB overnight preceding preparation of blood components. They include the following:

1) All WB units will be available the following morning, allowing very effective routine production
2) The staff for blood component preparation will primarily be needed during business hours
3) The number of transports of WB from collection sites will be significantly reduced
4) Platelet yield from BC for the preparation of pooled BC-derived PLTs may be improved.

On the other hand, the quality of plasma (186, 187), RBCs (188, 189) and PLTs (190-195) may be affected by overnight storage of WB. Prolonged incubation of PLTs with metabolically active granulocytes, as in WB stored at room temperature, may result in higher levels of PLTt activation. Polymorphonuclear granulocytes are numerous in WB, but absence in the PLT unit. Granulocytes may become activated in citrated blood by complement fractions, lipids or contact with foreign surfaces (196, 197).

If the practice of storing WB overnight, preceding preparation of PLTs, contributes to initially higher levels of PLT activation, this may increase during the storage period and thereby reduce the ability of the PLTs to withstand storage. Therefore, WB was processed either within 8 hours of collection or stored overnight before processing (See Paper IV). Either the BC or WB units rested overnight without agitation at 22 ± 2 °C preciding further preparations.

3.3 Automated preparation of platelets with the OrbiSac system

The OrbiSac system (Gambro BCT, Karlskoga, Sweden) is designed for automated preparation of PLTs from pooled BCs (Paper I). In a first step, ABO-matched BCs and PASs are pooled in a ring-shaped container; this step includes washing of the BC containers with PAS. In a second step, the contents of the ring-shaped pooling container are mixed and centrifuged, followed by transfer of the PLT-rich supernatant into a container placed in the center of the centrifuge. This process is monitored by a system of pressure sensors and photocells. In the first design, the number of WBCs in the PLT-rich supernatant was reduced by filtration after removal of the disposable parts from the equipment. In the next design, a WBC reduction filter and PLT storage container were integrated in the system. Different steps of the preparation process are shown in Paper I. The Atreus 2C+ system, used together with the OrbiSac system (Fig. 8) allows for almost fully automated production of PLTs.

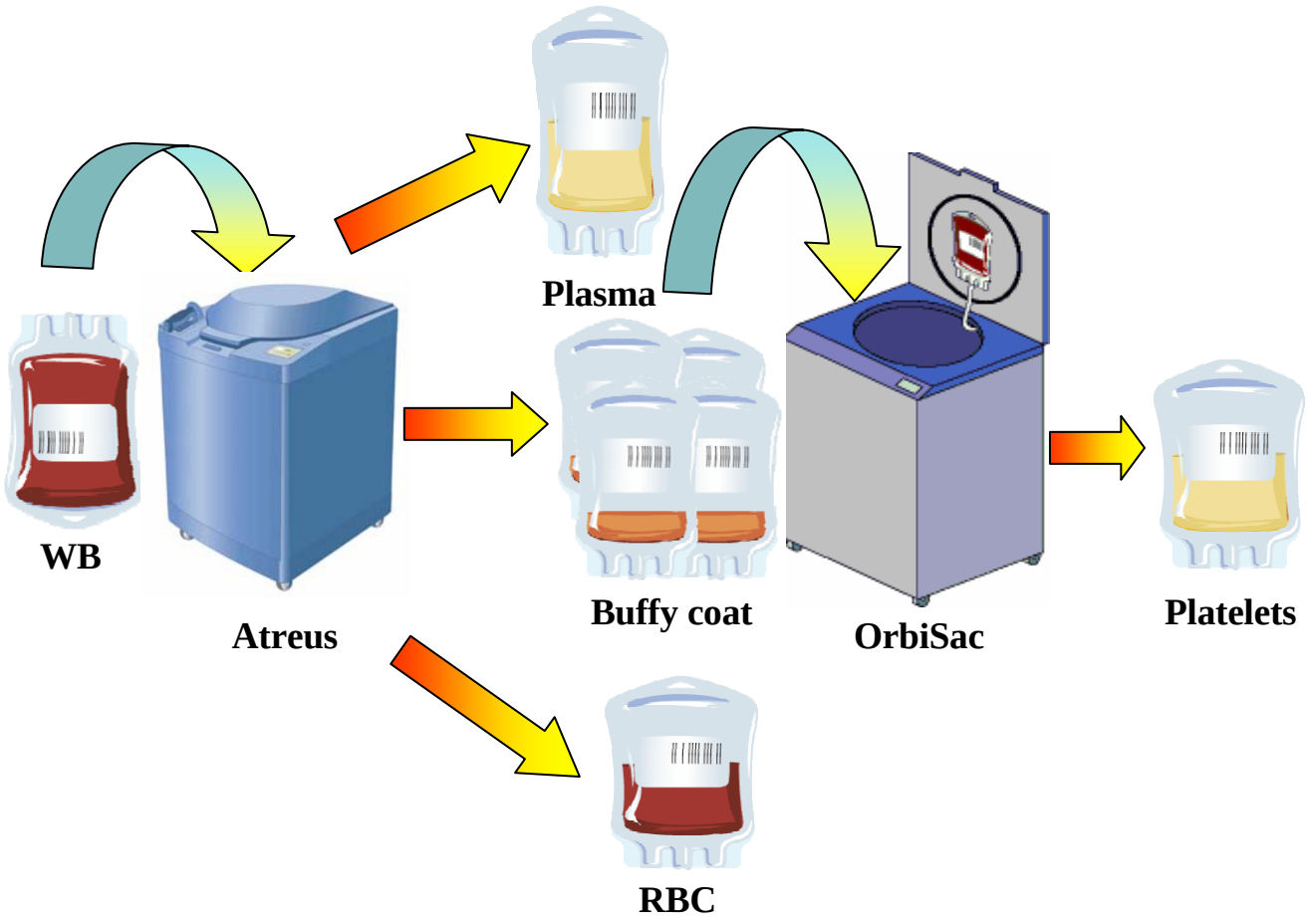


Figure 8. A synergy between the Atreus and the OrbiSac systems allows for automated production of platelets (PLTs). The Atreus system automatically separates whole blood (WB) into plasma, a buffy coat (BC) and a red cell unit (RBC). After Atreus processing, 4 to 6 BCs and 300 mL of platelet additive solution (PAS) can be pooled and processed into a leukoreduced platelet concentrate (LR-PLT) by using the OrbiSac system.

3.4 IN VITRO STUDIES

The overall PLT function is difficult to measure. Unfortunately, there is no test that identifies all problems with PLT function. While a variety of methods have been applied to evaluate PLT quality during storage (154)**,** there appears to be no in vitro test that clearly predicts the in vivo recovery, survival and function of transfused PLTs. Therefore, a battery of in vitro tests reflecting the different aspects of PLT function is so far the best tool we have to identify the PLT storage lesion by detecting changes that occur during processing and storage of PLTs. Several of these changes are important because they are associated with decreased posttransfusion survival (29).

3.4.1 Metabolic and cellular parameters

Some selected metabolic and cellular parameters are crucial. As previously mentioned, swirling should be present, because its absence is highly predictive of poor posttransfusion PLT count increments (155, 156); pH should be in the range of 6.8−7.4, and glucose should be present in the PLT unit as energy fuel throughout storage (105, 155). Platelet ATP levels should be >4.0 μmol/10^{11} PLTs since lower concentrations may be associated with loss of in vivo viability (158). In addition to pH and the other selected metabolic and cellular parameters (PLT counts, mean PLT volume (MPV), pO_2, pCO_2, lactate, bicarbonate, the WBC count and lactate dehydrogenase (LDH) activity, a marker for disintegration of PLTs), the in vitro evaluation of PLT storage lesions also requires a panel of assays able to measure PLT activation and function (123, 143). Hypotonic shock response as well as ESC (Paper II-IV) has been shown to be a suitable in vitro test to predicting posttransfusion efficacy (159, 160, 198).

Measurements of PLT counts and MPV were performed using CA 620 Cellguard (Boule Medical, Stockholm, Sweden). The volume (mL) was calculated by weighing the contents of the storage bag, in grams, on a scale (Mettler PB 2000 and 3000, Mettler-Toledo, Albstadt, Switzerland) and the result, in grams, was divided by 1.01 (1.01 g/mL is the density of the storage medium composed of approximatly 70 % PAS and 30% plasma). Using a blood gas analyzer (ABL 705; Radiometer, Copenhagen, Denmark), we measured the pH, pO_2, pCO_2, glucose and lactate levels. Bicarbonate was automatically given by the blood gas analysator, based on other measured variables. The assessment of swirling was done by inspection and grading with Bertolini´s method (155, 156). The WBC count on day 1 was determined with a Nageotte chamber and a microscope (Zeiss, standard, Chester, VA) (199).

Hypotonic shock response reactivity reactivity and the ESC measurements were performed using a dedicated microprocessor based instrument (SPA 2000, Chronolog, Havertown, PA, USA) with the modifications of these tests described by VandenBroeke et al (160). The total ATP concentration, (μmol/10^{11}) was determined with a Luminometer (Orion Microplate Luminometer, Berthold Detection Systems GmbH, Pforzheim, Germany) on the basis of principles described by Lundin (200). Lactate dehydrogenase activity as a marker of disintegration of PLTs, was measured using a spectrophotometric method (Spectrophotometer DMS 100, Varian Techtron, Springvale, Australia) (201).

3.4.2 Cytokine analysis

The basis for many FNHTRs associated with PLT transfusion therapy is cytokine elaboration and accumulation in the storage bag.

Febrile non-haemolytic transfusion reactions may occur after PLT transfusions (44, 45). Anti-leucocyte alloantibodies in the recipient, and leucocytes in the blood component transfused, have been considered as being responsible for most of the FNHTRs (202). In many patients, however, no leucocyte antibodies can be found (203). A correlation between the increased levels of interleukin (IL)-1, IL-6 and tumour necrosis factor-α (TNF-α), derived from WBCs in the PLT unit, and FNHTRs, has been shown (107, 204).

Prestorage filtration is effective in preventing the generation of these cytokines during the storage of PLTs (32, 205), but FNHTRs still occur, even after transfusion with PLTs that are leucocyte depleted before storage (45). One explanation for this may be cytokines released from PLTs during storage, because these cytokines also occur in filtered PLTs (45, 206, 207). The RANTES, β-TG, PF4 and probably IL-7, are stored in the α-granule and released into the PLT suspension media when PLTs are activated or destroyed (19, 208).

The effect of passive transfusion of RANTES into recipients of PLTs is not clear. In recent work, no correlation was seen between the concentration of RANTES and FNHTRs (209**)**. Higher concentrations (668 ± 223 ng/ml) of RANTES were associated with allergic reactions in another study (45). Therefore, in the present attempt to improve preparation and storage conditions, we estimated whether new conditions (Paper II and III) as well as novel preparation of BCs (Paper IV) may reduce the levels of PLT-derived cytokine plasma concentrations by use of commercial enzyme-linked immuno-sorbent assay (ELISA) kits (Quantikine ELISA kit; R&D Systems, Abingdon, UK).

3.4.3 Flow cytometry analysis

In Paper II, we evaluated the metabolic and cellular in vitro characteristics during 21 days of PLTs storage in additive solution (T-Sol) at 4^0C without agitation. However, several vital questions need to be assessed about PLT storage at 4^0C, especially whether the estimated changes in morphology (i.e., the loss of discoid shape) at 4^0C, reported in Paper II, are related to increased expression of PLT membrane proteins and activation markers during long-term storage. Previous work has shown that refrigeration of PLTs increases cytosolic Ca, actin polymerization and shape change (170, 171). As inside-out activation is Ca^{2+}-dependent and involves changes in the conformations of both the ligand-binding extracellular region and the cytoplasmic tails, we suggest that inside-out signaling and cytoskeletal rearrangements eventually result in increased expression of receptors and activation markers during prolonged storage at 4^0C.

Therefore, a flow cytometry analysis method FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) was set up (Paper III) to study PLT GPs that play an important role in adhesion, aggregation and to evaluate activation of stored PLTs (154).

We fixed the PLT samples by adding an equal volume of 1% paraformalaldehyde in phosphate-buffered saline (PBS), (PFA-PBS, pH 7.2-7.4) before staining with the fluorochrome-labelled monoclonal antibodies (MoAbs) P-selectin (CD62p), CD63, CD41, CD61 and CD42b. The first, CD62p, a constituent of alpha granules in resting PLTs, can be detected on the activated PLT surface after alpha granule secretion (146). The second, CD63 reacts with lysosomal membrane linked GP3, subsequently identified as granulophysin, a PLT dense granule GP suggested being a marker for granule release (147, 148). Human PLTs contain few lysosomes. As mentioned, they contain granulophysin (CD63) which can be released when PLTs are exposed to maximum stimulation in vitro.

However, acid hydrolyses from the lysosomes may not be secreted at sites of vascular injury in vivo (70), which leads us to the conclusion that the lysosomal role in PLT function is still unknown. Therefore, we suggested that CD63 may reflect dense granule release. The third, CD41 (GPIIb), which is usually linked with CD61 (GPIIIa), forms the GPIIb/-IIIa complex. This complex seems to be involved in PLT aggregation, serves as a receptor for soluble fibrinogen, fibrinonectin, thrombospondin, vWF and vitronectin (81) and plays a main role in the regulation of PLT aggregation and adhesion throughout haemostasis (82).

During PLT activation, the expression level of CD61/CD41 increases on the PLT surface, a conformational change then occurs, which creates the active binding site for fibrinogen (83). Recently the importance of vWF-dependent adhesion receptor (GPIbα/CD42b) in maintaining PLT viability in vivo was clearly shown in animal murine models (77, 78). This was therefore of specific interest, and it was further investigated and discussed in Paper III in this thesis. All MoAbs were purchased from Immunotech (Beckman Coulter, Marseilles, France). The implemented flow cytometric analysis method for studying PLT GP expression and activation markers was used in Paper IV as well, to estimate whether novel automated technique for BC preparation and WB stored overnight preciding preparation of PLTs contributes to higher levels of PLT activation.

3.5 Statistical analyses

Mean values and standard deviations (SDs) are usually given. In the in vitro studies (Experiments 1 and 4, Paper I), the two-tailed t-statistic evaluation for the two sample test was used to compare the population means. Statistical comparisons were carried out on a one-to-one basis, with a paired t-test statistic and a two-sided probability of 0.05 to reject the null hypothesis.

In Papers II–IV, repeated measurement analysis of variance (ANOVA) was performed, as well as post hoc multiple comparisons (Paper IV), in an attempt to identify differences between specific groups. Different groups were studied over time (days). Days was the repeated factor and group a between factor. The p-value for the interaction term between days and groups is reported, as well as the 95% confidence interval (CI) as a measure of dispersion of the results (Paper III). The analyses were carried out in the Statistica software, version 14.0 (SPSS, Chicago, IL, USA).

4 RESULTS AND DISCUSSION

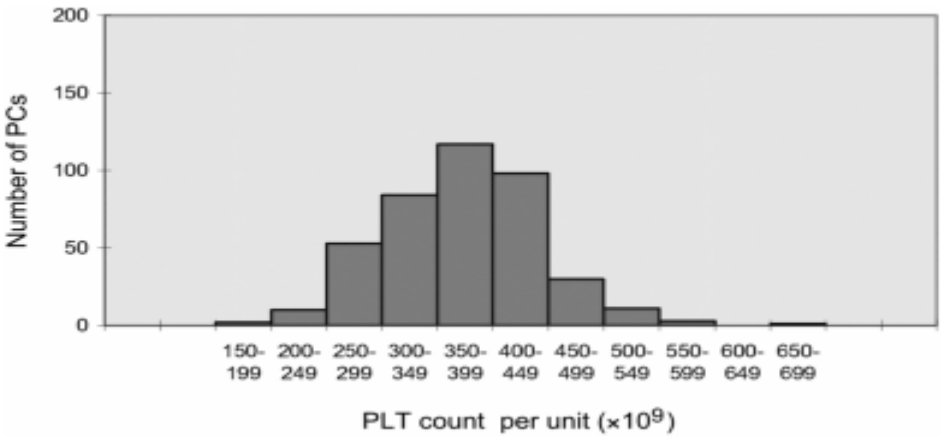
4.1 PAPER I

The aim of Study I (Paper I) was to evaluate PLTs prepared by the novel automated OrbiSac system, from pooled BCs stored in an PAS. Experiment 1 was a paired in vitro study of PLTs (from six BCs), prepared by automated and manual procedures. Experiments 2 and 3 evaluated PLTs prepared (from six BCs) using the OrbiSac system; Experiment 3 included selection of BCs based on donor data. Experiment 4 was a paired in vitro study of PLTs (from six BCs) with an integrated WBC filter and two different storage containers. Experiment 5 evaluated PLTs (from six BCs) using the OrbiSac system with an integrated WBC filter. Experiment 6 was similar to Experiment 5, with computer-selected pools of five BCs. The in vitro studies evaluated the effects of 7-day storage of PLTs regarding PLT metabolism and disintegration.

In *Experiment 1*, we found similar in vitro results for PLTs prepared by the manual technique as for PLTs prepared using the automated technique and stored for 7 days. The PLT content of PLTs prepared manually was significantly higher than in units prepared using the OrbiSac system. This difference was probably related to rinsing of the BCs into the pooling bags, which at the time could be done more effectively with the manual system. This situation probably explains the lower values of lactate, pCO_2, and bicarbonate on days 3, 5, and 7 with the OrbiSac system, because fewer PLTs produce less lactate and CO_2. A similar difference in the glucose level was observed on day 7. Differences in pH and pCO_2 on day 1 may be associated with sampling and measurement before obtaining chemical equilibrium in the PLT storage medium. No other differences in PLT metabolism were observed, except for an occasional slight difference in ATP on day 5. The results of Experiment 1, in which we compared the PLTs prepared by the novel OrbiSac system, and reference PLTs prepared by the standard manual procedures and stored for 7 days in T-Sol, suggest that the two methods are similar regarding PLT in vitro characteristics.

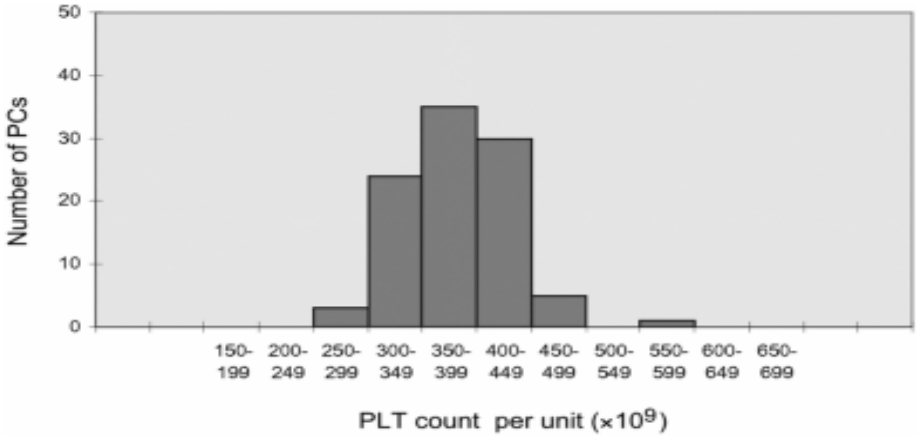
In *Experiment 2*, the automated technique was used for routine preparation of PLTs. The PLT content ($370 \times 10^9 \pm 70 \times 10^9$/unit; mean ± SD) in our experiment remained essentially the same as over the years when the PLTs were prepared manually (Fig. 9). Also the distrubution of PLT content in the the range of $150 - 700 \times 10^9$/unit remained essentially the same as over the years. These results suggest that the variations in PLT content are present already in the initial pool of six BCs, because the recovery is very uniform.

Figure 9. Experiment 2: automated routine preparation of platelets (PLT) units (n = 409) from random pools of six buffy coats (BCs). Distribution of PLT contents (number of units in the range 150×10^9-700×10^9 PLTs/unit).



In *Experiment 3,* we studied the effects of combining BCs in the pools on the basis of PLT concentration in doonor blood (Fig. 10). We found that the variations in PLT content were reduced and less concentrates were out of the optimal range 300 - 450 $\times 10^9$/unit.

Figure 10. Experiment 3: automated preparation of platelets (PLTs) (n = 98) from pools of six buffy coats (BCs) selected on the basis of blood donor PLT concentration in whole blood (WB). Distribution of PLT contents (number of units in the range 150×10^9-700×10^9 PLTs/unit).

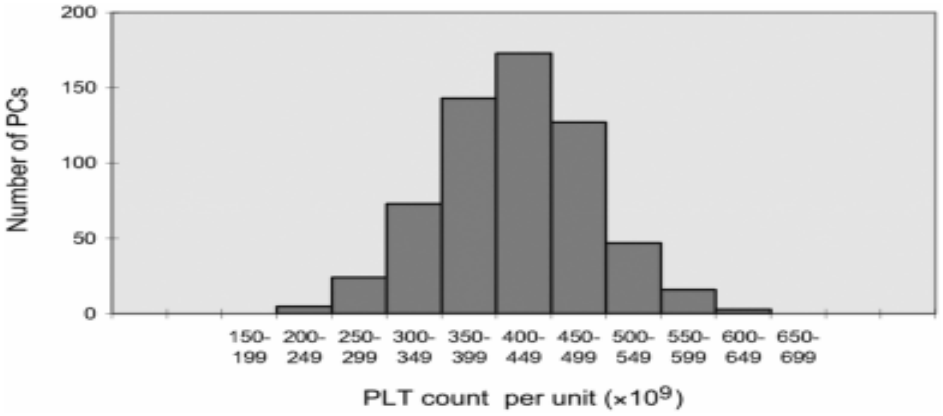


Experiment 4. Different plastic, gas- permeable storage containers are available. These storage bags are specifically designed to allow adequate gas (O_2 and CO_2) exchange (9, 110, 114). An attempt to evaluate the most optimal storage bag contributes to advances in PLT transfusion therapy. Therefore, in Experiment 4, a paired in vitro study of PLTs from six BCs with the OrbiSac system with an novel integrated WBC depletion filter (evaluated in another study) was comparing two different storage containers. Our findings suggest that the new kit for in-process WBC depletion with the ELP (Gambro BCT, Zaventem, Belgium) PLT storage container barelt affected the PLT in vitro data during storage for 7 days, compared to the reference technique with the UPX–80 (Terumo, Tokyo, Japan) PLT storage container. The differences include slightly lower consumption of glucose and production of lactate in test units and consequently, a higher pH at the end of the storage period. In addition, we also found that introduction of the integrated system involved an improvement in the preparation process to reduce

the loss of PLTs, which may be due to less manual involvement during the preparation process.

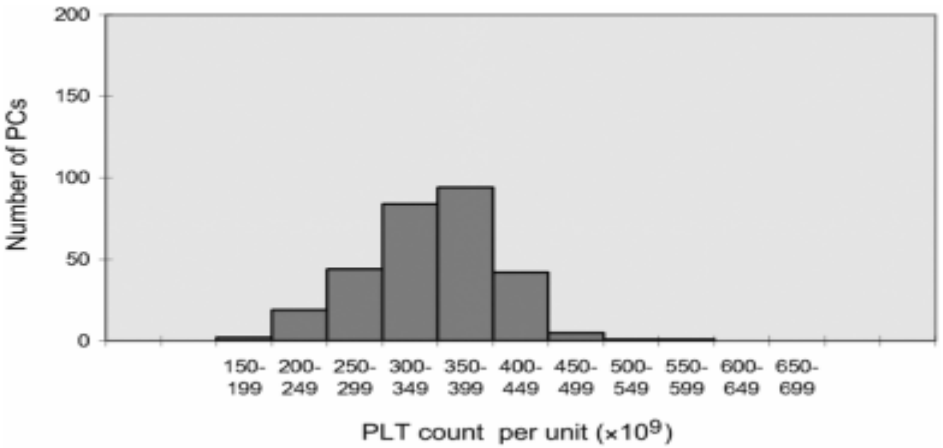
Experiment 5 involved automated routine preparation of PLTs (n=611) from random pools of six BCs, using the novel integrated filter and storage container (Fig. 11). As the recovery of PLTs produced by the OrbiSac system was very uniformed, the results confirm that the variations in PLT content are present already in the initial pool of six BCs, which may be due to the variations in the PLT concentration in doonor blood. Moreover, we found that the integrated system improves PLT content to over 400 $\times 10^9$/unit concerning mean value.

Figure 11. Experiment 5: Automated routine preparation of platelet (PLT) units (n = 611) from random pools of six buffy coats (BCs), with an integrated filter and storage container. Distribution of PLT contents (number of units in the range 150×10^9-700×10^9 PLTs/unit).



As a consequense of improving the process in Experiment 5, the number of BCs could be reduced in Experiment 6 (Fig. 12). Based on the results in Experiment 3, a novel computerized selection of BCs based on blood doonor concentration was used. We found that a higher percentage of PLT units than in Experiment 3 had PLT contents outside the range of 300 - 450 $\times 10^9$/unit.

Figure 12. Experiment 6: Automated routine preparation of platelet (PLT) units (n = 292) from pools of five buffy coats (BCs, with an integrated filter and storage container and computerized selection of BCs), Orbiselect. Distribution of PLT contents (number of PLT units in the range 150×10^9-700×10^9 PLTs/unit).



In summary, in Paper I we have shown that the novel automated OrbiSac technique for the preparation of PLTs from pooled BCs is equivalent to the standard manual technique regarding PLT in vitro charecteristics during 7 days of storage. We achieved a uniform recovery of PLTs, which resulted in satisfactory PLT and WBC contents according to current Eurpeen standards. We found that our optimism was not unfounded regarding the introduction on a large scale of this more effective technique for the preparation of PLTs in a near future. The OrbiSac system in is now in clinical use and provides all of Karolinska University Hospitals PLTs. The system is also in use in several other blood centres in Sweden, as well as in several other Nordic and Europeen countries. At the Karolinska University Hospital, two persons can produce approximately 40 PLT units/day. In the region of 5000 units/year are produced by the OrbiSac system.

4.2 Paper II

Platelets are traditionally stored at 22°C, but this needs to be reconsidered. Storage of PLTs at room temperature accommodates bacterial growth, and bacterial sepsis is currently regarded as the main risk of transfusion-transmitted diseases and the reason for the present limit of PLT storage to 5 days (161-163). This short shelf life severely reduces the availability of PLTs and can contribute to chronic PLT shortages. Storage at 4°C, however, would not only reduce the risk of bacterial growth, but may also delay PLT ageing.

In this study, PLTs stored at 4°C without agitation were compared to those stored at 22°C with agitation. To determine the in vitro effects of incubation at 37°C, we incubated the aliquots from PLTs stored at 4°C and at 22°C for 1 hour at 37°C before the analysis. Due to the paired in vitro study design, The PLT content on day 1 of the four groups of PLT units were uniformed within the range of 290 – 298 x 10^9/unit.

Cellular assays

During storage, the PLT content declined more in units stored at 22°C than units stored at 4°C (measuring 180 × 10^9 and 265 × 10^9/unit, respectively, on Day 21). Therefore, LDH activity increased more when PLTs were stored at 22°C than when they were stored at 4°C, indicating discharge of PLTs at 22°C storage. Consequently, LDH was reduced during the entire storage period of 21 days when PLTs were stored at 4°C. Loss of the discoid shape visibly increased the size of the PLT (MPV). The MPV was significantly lower in preincubated PLTs stored at 4°C than in those stored at 4°C. These findings suggest that the preincubation of PLTs at 37°C, which have been stored at 4°C significantly influences PLT size (i.e., MPV), but causes only minor changes in LDH activity.

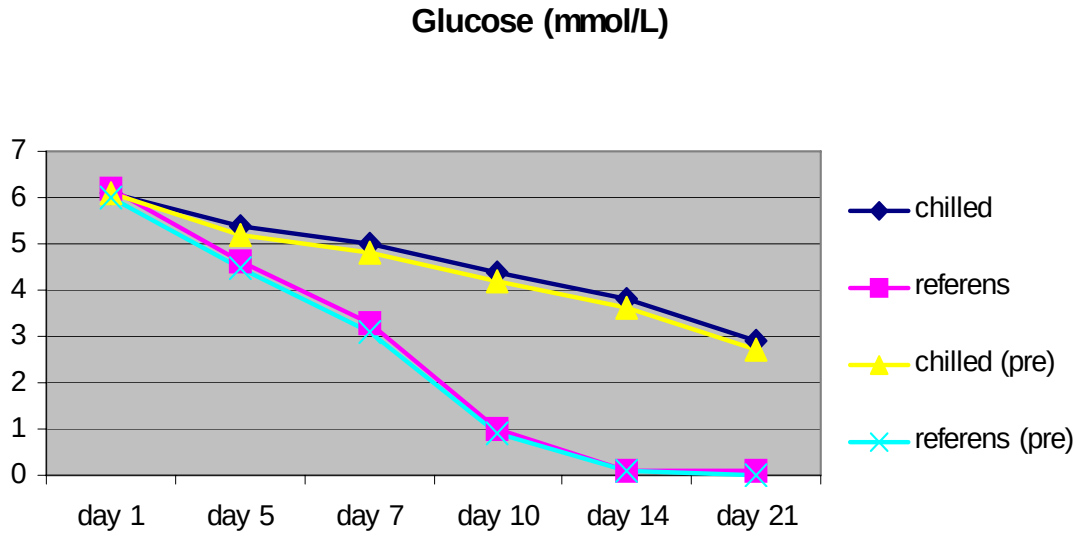
The loss of the discoid shape in several PLTs in units stored at 22°C, visualized by the increased MPV, was most certainly related to the PLT storage lesion (140-143). On the other hand, an increase in MPV at 4°C probably visualizes cytoskeleton rearrangements (170, 171).

Metabolic assays

We found that glucose declined less and lactate increased less in PLTs stored at 4°C than in PLTs stored at 22°C. Not only pH, but also ATP, and pCO_2 were better maintained after Day 10 in PLTs stored at 4°C compared with PLTs stored at 22°C. Bicarbonate was better maintained from Day 14 in PLTs stored at 4°C compared with 22°C. The oxygen tension (pO_2) was higher in PLT units stored at 4 and 22°C than in preincubated PLTs stored at 4 and 22°C. However, incubation of PLTs for 1 hour at 37°C before the analysis caused only minor changes in glucose consumption, lactate and CO_2 production, oxygen consumption, pH, bicarbonate and ATP concentration.

The analysis of the metabolic assays suggests that the storage of PLTs at the latter temperature significantly affects PLT metabolism compared with storage at 22°C. The storage at 4°C reduces the metabolic rate of glucose consumption and lactate production. Figure 13 illustrates that due to the reduced rate of glycolysis, glucose is available in the storage medium during the entire storage period of 21 days when PLTs are stored at 4°C.

Figure 13. (chilled = 4°C, reference = 22°C, pre = preincubated).



We found that PLT metabolism depends on the presence of glucose during the entire storage period, when PLTs are suspended in storage medium and stored at 22°C. The total consumption of glucose on Day 10 is associated with immediate cessation in the production of lactate and CO_2, a decrease in ATP levels, an increase in pH levels, a change in PLT size (MPV), and delayed disintegration of PLTs (LDH), followed by a decrease in the PLT count and an increase in the release of α-granule content on Day 14, when glucose is completely consumed on Day 10.

Hypotonic shock response, extent of shape change, and swirling

We found that HSR and ESC were higher in PLTs stored at 22°C than in those stored at 4°C, indicating change of refrigirated PLTs from a discoid to spherocytic shape (170, 174). Hypotonic shock response and ESC were higher in preincubated PLTs stored at 22°C than in reference PLTs stored at the same temperature. Slight differences in HSR and ESC were detected between PLTs stored at 4°C with or without preincubation, indicating irreversible cold-induced activation. Swirling was fully maintained for

7 days in PLTs stored at 22°C. No swirling during the storage period was observed in PLTs stored at 4°C. Consequently, preincubated PLTs stored at 22°C responded better to HSR reactivity and ESC compared with PLTs stored at 22°C whithout preincubation. Therefore, these interesting in vitro results support the view that non-discoid PLTs may even revert to a discoid shape when exposed to 37^0C (176). Moreover, we found close agreement between these findings and previous studies, which suggests that preincubation of PLTs for 1 hour at 37°C improves recovery after transfusion (210, 211).

Cytokine analysis

The concentrations of RANTES, β-TG, and PF4 increased during storage in all units, but were higher in PLT units stored at 22°C than in units stored at 4°C. In PLT units stored at 22 and 4°C and incubated for 1 hour at 37°C before the analysis, the cytokine concentration was slightly higher than in PLT units that had not been preincubated. These results show that the storage of PLTs at 4°C reduces the release of cytokines from the PLTs into the PLT unit during storage, and that preincubation causes only minor changes, here illustrated by the release of RANTES during 21 days of storage (see Fig 14**).**

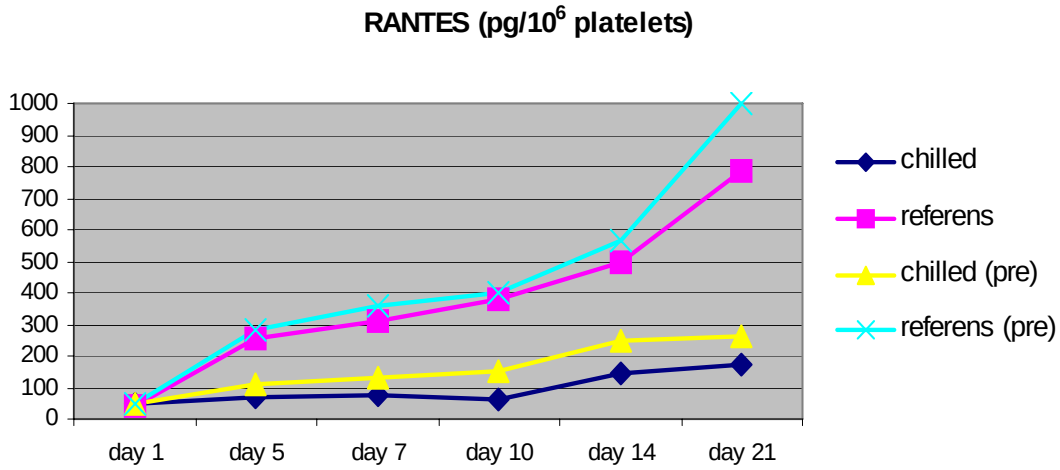


Figure 14 (chilled = 4°C, reference = 22°C, pre = preincubated).

The RANTES chemokine, β-TG, and PF4 are stored in the PLT α-granule and higher levels of these cytokines indicate PLT activation or disintegration (208) as the basis for many FNHTRs associated with PLT transfusion is cytokine elaboration and accumulation in the storage bag (45). Platelet storage at 4°C will probably reduce the risk of FNHTRs.

To summarize, PLTs stored at 4°C without agitation maintain their metabolic and cellular characteristics to a great extent during 21 days of storage. We confirm that they lose their discoid shape (with changes in ESC, MPV and swirling) and have shown that this loss of discoid shape during storage at 4°C is associated with reductions in metabolic rate and a decreased release of α-granule content. Therefore, our findings concerning storage at 4°C do not suggest cold-induced activation at a level associated

with the release of PLT-derived cytokines (RANTES, β-TG, and PF4). These results indicate that the cytoskeleton rearrangements, caused by cold induced activation when PLTs are stored at 4^0C, contribute less to in vitro effects associated with decreased PLT quality than does activation caused by what is known as "the PLT storage lesion" at 22^0C storage. We found that preincubated PLTs stored at 22°C responded better to HSR reactivity and ESC than those stored at 22°C whithout preincubation, which supports the view that shape change may to some extent be reversible.

4.3 Paper III

In Paper II, we found that PLTs stored in additive solution (T-Sol) at 4ºC without agitation maintain acceptable metabolic and cellular in vitro characteristics for 21 days of storage. However, several vital questions needed to be assessed about PLT storage at 4ºC, especially whether changes in morphology (i.e., the loss of discoid shape) at 4ºC are related to increased expression of PLT membrane proteins and activation markers during long-term storage. Previous work has shown that refrigeration increases cytosolic Ca, actin polymerization, and shape change (170, 171). As inside-out activation is Ca^{2+}-dependent and involves changes in the conformations of both the ligand-binding extracellular region and the cytoplasmic tails, we suggest that inside-out signaling and cytoskeletal rearrangements will eventually result in increased expression of receptors and activation markers during prolonged storage at 4ºC.

The results of metabolic and cellular assays

This study resembles the results in Paper II with regard to various aspects of PLT metabolism and cellular quality after prolonged storage at 4ºC.

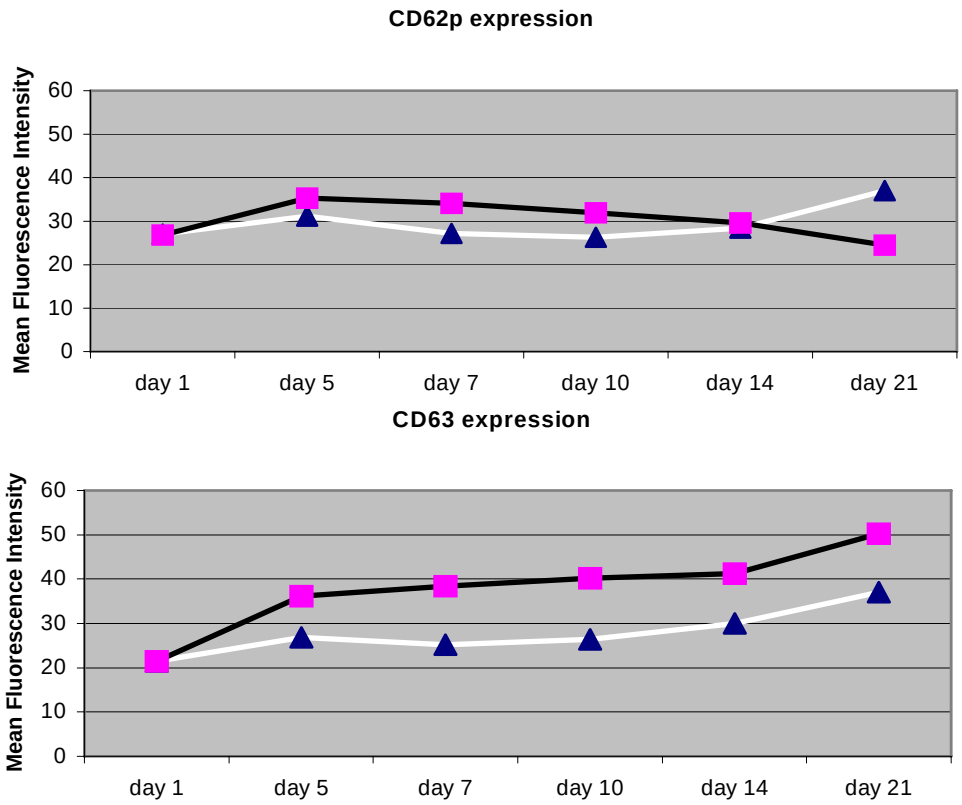
As in Paper II, we found that PLTs stored at 4ºC show characteristic morphological changes related to activation from a discoid to a spherocytic shape, a phenomenon that is referred to as "cold-induced activation" (77, 170). In this study, we asked ourselves whether this change in shape after storage at 4 ºC is really as important as in PLTs stored at 22ºC. For PLTs stored at room temperature, the ESC assay and the HSR assay are regarded as fairly good predictors of PLT recovery and survival (140). However, no single in vitro test can predict the recovery and survival in vivo (154).

In this paper (Paper III) and in Paper II, we have shown that PLTs stored at 4ºC perform poorly in the HSR as well as in the ESC assay, as compared with PLTs stored at room temperature. However, Hoffmeister et al. (2003), studying animal models, found that a spherocytic shape alone is insufficient to cause rapid clearance of PLTs and some authors claim that non-discoid PLTs may survive after transfusion and even revert to a discoid shape (176). As PLTs stored at 4ºC perform excellently with regard to most of the in vitro parameters, with the exception of parameters that reflect changes in shape, we suggest that the ESC and HSR assays may be of no value in predicting the quality of PLTs stored at 4ºC.

The results of the flow cytometric analysis

We found that the activation marker CD62p remained almost unchanged during storage in all units. By contrast, the expression of CD63 was higher in PLTs stored at 22ºC than in PLTs stored at 4ºC (Fig. 15).

Figure 15. CD62p expression as well as CD63 expression during 21 days of storage. Squares = 22ºC; triangles = 4ºC.



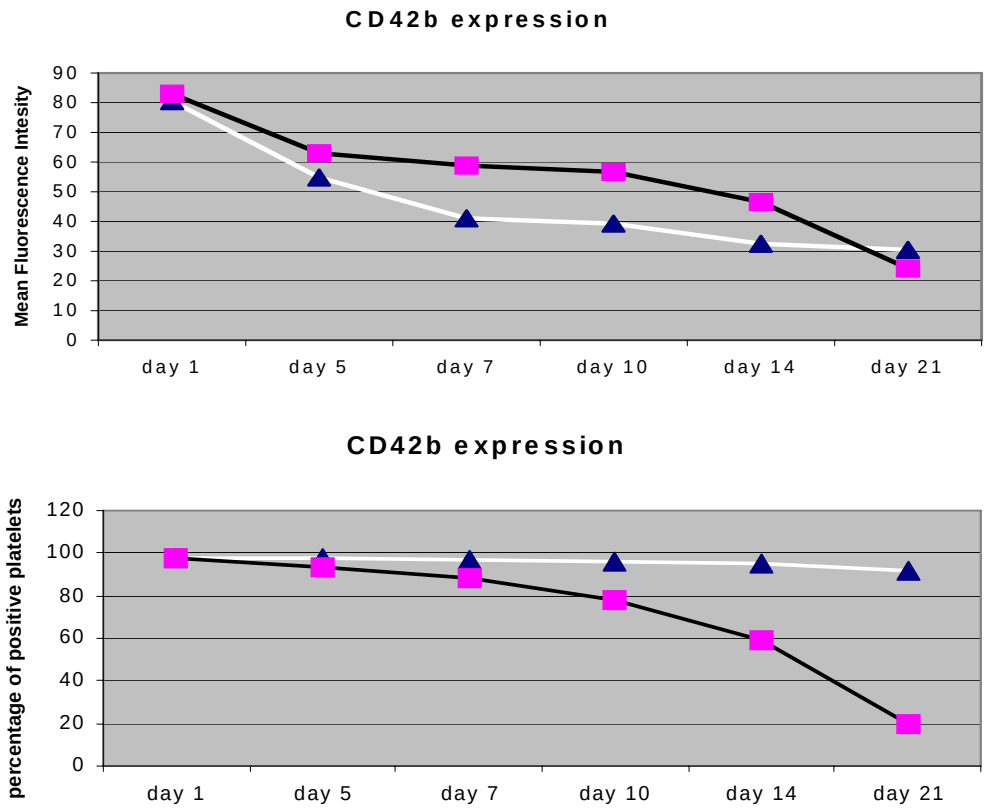
These data indicate that CD63 has a closer relation to modifications in PLT morphology, as judged by a reduction in ESC values, an increase in the release of RANTES, and a loss of swirling, than CD62p. We suggested that the lower expression of CD62p may be due to the fact that cell-surface expression of CD62p can be cleaved from the surface by various proteases (212).

Therefore, our results imply that cell surface expression of CD63 may remain unchanged during the entire storage period and that CD63 may therefore be a better marker pf PLT activation than CD62p. In contrast to results reported by other investigators (213, 214), the CD63 antigen in our study was expressed along a continuum with no distinct positive or negative cells (since mean fluorescence intensity (MFI) values reflect the density of the antigen on the surface). Since we wanted to follow possible up- or downregulations of surface antigens caused by the preparation and storage itself, it would have been informative to establish the percentage of PLTs expressing CD63 as well. However, due to the fact that the CD63 antigen was expressed along a continuum with no distinct positive or negative cells we realized that only MFI should be presented, and that a percentage may be misleading.

After day 14, the increase in the expression of CD62p and CD63 in PLTs stored at 4ºC was associated with an increase in the release of RANTES, reflecting increased PLT activation. No significant difference in CD41 expression was detected. The surface expression of CD61 was higher in PLTs stored at 22ºC than in PLTs stored at 4ºC. Our findings in cold-stored PLTs are in line with those in cold-treated mouse PLTs which suggests that such treatment does not cause up-regulation of GPIIb/-IIIa (83). Since we observed >98% CD41/CD61 in all our experiments, we suggest that MFI, which reflects the density of the antigen on the PLT surface, is a more informative measure.

The expression of CD42b (Fig. 16) decreased during storage in all units (MFI), but was higher in PLT units stored at 22ºC compared with PLTs stored at 4ºC, with the exception of day 21. The percentage of PLTs expressing CD42b remained high in PLTs stored at 4ºC, but gradually decreased in PLTs stored at 22ºC. These data indicate that GPIbα remains on the surface of refrigerated PLTs during long-term storage even if the density of the antigen is reduced/rearranged.

Figure 16. CD42b expression during 21 days of storage. Squares = 22ºC; triangles = 4ºC.



As GP1bα plays a major role in the clearence of transfused refrigerated PLTs (77), further studies of the vWFR complex subunits GPIbα, GPIX, and GPV are needed, investigating the relationship between vWFR clustering/conformational changes and long-term storage at 4ºC.

In summary, the problem of how to refrigerate PLTs for transfusion can be divided into two separate problems, the storage problem and the clearance problem.

In this study, we have shown that storage at 4ºC, which leads to "cold-induced activation", is not associated with increased expression of human PLT membrane proteins and activation markers during long-term storage. Moreover, a current report suggests that if the reduction in CD42b expression during 4ºC storage reflects the irreversible rearrangement in clustering of the vWFR (GP1bα or CD42b), as shown in animal models, glycan modification (78) may prolong the circulation in humans of PLTs stored at 4ºC and thus the clearance problem may be solved.

Consequently, these effects of a cold temperature on human PLT GP expression during long-term storage may prove to be of practical value with regard to the storage problem. These data confirm the metabolic and cellular findings of Paper II. We have shown that the cytoskeleton rearrangements caused by cold-induced activation when PLTs are stored at 4ºC contribute less to upregulations of receptors associated with PLT activation. Such upregulations are normally observed when PLTs are stored at room temperature and are affected by the PLT storage lesion, and therefore make it tempting to speculate that shape change induced by cold storage does not contribute to decreased PLT quality to the same extent as shape change induced by the PLT storage lesion that occurs at 22ºC storage.

4.4 Paper IV

In Paper IV, we continued developing the automated preparation of blood component processing started in Paper I, by investigating the effects of storing either WB or BCs overnight before preparation of PLT by using a novel automated equipment, the Atreus 2C+ system for BC preparation, in combination with the OrbiSac technique. Different aspects and effects on other blood components, associated with WB overnight storage preceding preparation of PLTs, are mentioned in section 3.2.1. Results are discussed here.

We studied PLTs, prepared using the OrbiSac system, which were derived either from BCs obtained from fresh WB (Atreus F, (n = 10)) or WB stored overnight (Atreus S, (n = 10)) and prepared using the Atreus 2C+ system. Platelets (n = 10) obtained from BCs prepared from fresh WB using conventional blood bags (TaT-F, control) were studied in parallel and used as a reference.

Cellular assays

The PLT counts on day 1 are given in Table 3. From day 1 and during the following storage period, the PLT counts and contents were significantly higher in Atreus S than in Atreus F and TaT-F PLTs. In contrast to a previously investigator report (193), these results are in agreement with those of others (215) who have shown that the PLT yield significantly improves when the WB is stored overnight before processing. We found no significant differences in PLT counts and contents between Atreus F and TaT-F

PLTs throughout storage. No significant difference in MPV and LDH activity between groups was detected.

Table 3. Comparison of platelets (PLTs) derived from buffy coats (BCs) obtained from fresh blood and whole blood (WB) stored overnight and processed using the Atreus 2C+ system (Gambro BCT, Zaventem, Belgium) or fresh WB obtained using the Top-and-Top system (Imuflex-CRC; Terumo, Tokyo, Japan) on day 1. Results are expressed as means ± standard deviations (SDs) (n = 10).

WB processing system	**PLT concentrates**		
	Volume (mL)	**Platelets (10^9/L)**	**Leukocytes (10^6/units)**
Atreus, fresh WB	357±27	896±136	<0.2
Atreus, stored WB	365±10	988±122	<0.2
Top-and-Top system	381±14	854±62	<0.2

Metabolic assays

We found no significant differences in glucose consumption, lactate production, bicarbonate, and ATP. Other indicators of PLT metabolism associated with preparation using the Atreus 2C+ system were similar to those reported for PLTs using the routine procedure, except for occasional small differences in pH, PCO_2, and PO_2 values between the groups. As no evident differences in PLT metabolism were observed, we suggest that these differences may be associated with effects of sampling and measurements on the chemical equilibrium, as well as differences in PLT yield when the WB was stored overnight.

Hypotonic shock response, extent of shape change, and swirling

We found that HSR and ESC values decreased during storage in all units. Hypotonic shock response was slightly higher in Atreus S PLTs, but no significant difference between Atreus F and TaT-F PLTs was detected. By contrast, ESC was significantly higher in TaT-F than in the other two groups. No significant difference with regard to ESC between Atreus F and Atreus S was detected. Based on these results, we suggest that the small though statistically significant fall in HSR in PLTs obtained from fresh WB, compared with PLTs from stored WB, may be explained by the fact that a larger amount of PAS was used due to a lower PLT content in fresh blood.

To clarify: to achive the dilution performed with plasma, required for the HSR and ESC test (160), more PAS needs to be present when the PLT content is lower. Consequently, the lower response may have been caused by the impact of the PAS rather than by differences in PLT quality. Compared with a previous investigator, we noted that all HSR and ESC levels on day 7 were below the level in a plasma storage environment, for which poor in vivo viability has previously been predicted to occur (198) in PLTs stored for 7 days. Although we modified our ESC and HSR tests based

on the technical implications described by VandenBroeke et al., we still observed decreased response to HSR and in particular ESC when PLTs had been stored in PAS. As previously mentioned this phenomenon is likely to be associated with the measurement technique rather than with effects associated with PLT quality and PLT storage environment. Since HSR levels were fairly similar in all three groups during storage, the decreased levels of ESC on day 7 will probably be of no consequence, especially when swirling remained at the highest levels (score = 2) for all units at all times. Therefore, HSR and ESC tests that are affected by the PAS may be considered to be more a reflection of the PLT storage lesion over the storage period than be associated with certain quality levels based on performance in 100% plasma.

Cytokine analysis

The concentrations of RANTES increased during storage in all units. No significant difference in the concentration of released RANTES between groups was detected. A previous investigator has shown that higher concentrations of released RANTES into the PLT unit were associated with allergic reactions (45). In our study, the concentrations of RANTES, in pg/10^6 PLTs, on day 7 were below the value noted in the latter study in all units.

Flow cytometric analysis

In general, we found that the percentage of PLTs expressing the activation markers CD62p and CD63, as well as the MFI increased during storage. We found hardly any difference between the different WB processing systems that may have affected the PLTs before preparation. The increase in surface expression has a close relation to modifications in PLT morphology, as judged by a reduction in ESC values, followed by an increase in the release of RANTES which clearly reflects effects of the PLT storage lesion (140, 142, 143).

The surface expression (MFI) of CD41 and CD61, as well as the percentage of PLTs remained almost unchanged during storage in all units. These results are in agreement with previous data (214, 216), showing no change during storage in the expression of CD41 (GPIIb) and CD61 (GPIIIa). Significant differences (MFI) between all groups were found for CD41 ($p<0.01$), and a significant difference (MFI) between Atreus S and TaT-F PLTs was detected for CD61 ($p = 0.026$). As the expression levels of CD41/CD61 remained almost unchanged during storage, we suggest that it seems most unlikely that the differences in density of the antigen on the surface between groups will be of any clinical relevance.

We found that the percentage of PLTs expressing CD42b decreased during storage in all units. A strong correlation between the percentage of PLTs able to bind antibodies that recognize the N-terminal of CD42b and in vivo recovery has been reported (217). In our study, the percentage of PLTs expressing CD42b was higher (>91% on day 7) than that associated with poor in vivo recovery in the mentioned study. We found no statistical difference in the percentage of PLTs expressing CD42b between the groups, and our findings are in accordance with those of others who have shown that storage of PLTs reduces the expression of GPIbα on the surface of the PLTs (218-220).

To summarize, the results presented in Paper IV suggest that PLTs derived from pooled BCs obtained either from fresh WB or from WB stored overnight and processed using the Atreus 2C+ system are equivalent to PLTs derived from the standard routine procedure with regard both to PLT in vitro characteristics during 7 days of storage and to satisfactory PLT recovery, according to current standards. In addition, we found that overnight storage of WB improved PLT recovery and content. The mechanisms behind this phenomenon are still unknown, but it is tempting to speculate that the suggested higher levels of PLT activation that may occur after collection may to some extent be reversible when the WB is stored overnight, as compared with PLT production from fresh WB. Furthermore, the possibility of processing PLTs from fresh WB gives possitive side effects with regard to higher plasma (186, 189) and RBC (188, 189) quality.

The Atreus 2C+ system, which in synergy with the OrbiSac system (Fig 8) allows for an almost fully automated production of PLTs, and brings several distinct advantages such as (i) standardized preparation resulting in a more uniformed PLT component (ii) elimination of several hand-over steps and thus reducing labour effort and (iii) the fact that PLTs can be derived from BCs, obtained from either fresh or overnight stored WB, whatever preferred, potentially increasing efficiency in the production flow of PLTs at the blood centres. Therefore, there is good reason for optimism that this more effective technique for the preparation of BCs from WB can be introduced on a large scale in the near future, meeting the demand for improvements in the preparation-process of PLTs.

5 GENERAL CONCLUSIONS

This thesis work has focused on (i) investigation of the in vitro quality of PLTs prepared by novel automated techniques (the OrbiSac and Atreus 2C+ systems); and (ii) evaluation of the ability to store PLTs for a prolonged time at 4ºC in an attempt to optimize the conditions under which PLTs are prepared and stored.

The major findings of this thesis are:

1) We showed that the technique for the preparation of PLTs from pooled BCs using the novel automated OrbiSac system is equivalent to the standard manual technique with regard to in vitro PLT characteristics during 7 days of storage.

2) We achieved uniform recovery of PLTs, which resulted in satisfactory PLT and WBC contents, according to current European standards.

3) We confirmed that PLTs derived from pooled BCs obtained either from fresh WB or from WB stored overnight, and processed using the novel Atreus 2C+ system, is equivalent to PLTs obtained using standard routine procedures with regard to in vitro PLT characteristics over 7 days of storage. The novel system of BC preparation represents satisfactory PLT recovery and content, according to current European standards.

4) We found that overnight storage of WB improved PLT recovery and content.

5) We demonstrated that PLTs stored at 4ºC without agitation largely maintain their metabolic and cellular characteristics during 21 days of storage.

6) We found that using preincubated PLTs stored at 22ºC causes only minor changes. However, better response to HSR reactivity and ESC was noted compared with PLTs stored at 22ºC without preincubation, which indicates that non-discoid PLTs stored at 22ºC may to some extent revert to a discoid shape when exposed to 37ºC.

7) We clarified that shape change occurring when PLTs are stored at 4ºC is not associated with increased expression of membrane proteins and activation markers during long-term storage. Since increased expression is associated with PLT activation and decreased PLT quality, these findings suggest that PLTs are better preserved at 4ºC. However, such PLTs must be demonstrated to circulate and function in vivo.

6 FUTURE PERSPECTIVES

The problem of how to refrigerate PLTs for transfusion can be divided into two separate problems, the clearance problem and the storage problem. The clearance mechanism of refrigerated PLTs, due to their shortened in vivo survival after transfusion, has recently been clarified (77-78). These results, in combination with our findings concerning the capability of a cold temperature to preserve PLT quality and prolong the storage period, make it therefore tempting to speculate that cold storage of PLTs may be possible in the future. However, several vital questions need to be addressed in a future study of PLT storage at 4ºC. They are listed below.

As GPIbα seems to play a major role in the clearance of transfused, refrigerated PLTs, further studies of the vWFR complex subunits GPIbα, GPIX, and GPV are needed, investigating the relationship between vWfR clustering/conformational changes during long-term storage under modern blood bank conditions at 4ºC. Studies with electron microscopy may provide a better understanding of the influence of a cold environment on morphology as well as on receptors in human PLTs processed and stored under modern blood bank conditions.

Future research would need to include an investigation of glycosylation of PLT surface proteins in vitro, as an approach to protecting PLTs stored at 4ºC from clearance.

Previous work has shown that refrigeration of PLTs increases cytosolic Ca, actin polymerization, and shape change (170, 171). Inside-out activation is Ca^{2+}-dependent and involves changes in the conformations of both the ligand-binding extracellular region and the cytoplasmic tails of receptors. Therefore, inside-out signaling and cytoskeletal rearrangements may be the underlying factor, causing irreversible rearrangements in PLT surface receptors.

Consequently, one approach to storing refrigerated PLTs in a discoid shape, of using addition of protecting substances such as cytochalasin B, an actin assembly inhibitor, and EGTA-AM, an intracellular calcium chelator, may clarify whether cytoskeletal rearrangements have any influence on the “cluster phenomenon”.

Additional hopes for the future would be that progress in answering the abovementioned vital questions would lead us to the ability to perform in vivo studies of PLTs stored at 4ºC, probably with addition of a protecting substance against clearance.

7 SVENSK SAMMANFATTNING

Trombocyttransfusioner används idag rutinmässigt inom sjukvården och har förbättrat överlevnaden för många olika patientkategorier. Vid olika blodsjukdomar, efter transplantation med hematopoetiska stamceller och vid cytostatikabehandling förstörs patientens egna trombocyter och trombocyter från blodgivare behöver tillföras. Syftet med denna avhandling är att bidra till en optimering av de betingelser som påverkar trombocyternas kvalité genom en utveckling av processystemen för trombocytframställning samt studier av möjligheten för trombocyter att kunna förvaras i kyla. Framställning och förvaring av trombocytkoncentrat är komplicerat. Vanligaste metoden i Europa är den s.k ”buffy-coat” metoden. För framställning av en transfusionsdos ($300\text{-}400x10^9$) så behövs trombocyter från 4-6 blodgivningar. Trombocyterna selekteras sedan ut med hjälp av fraktionerad centrifugeringsteknik och trombocytförvaringsmedium tillsätts. Trombocytkoncentrat framställda ur buffy-coats från normala blodgivare har fram till nyligen framställts via manuell teknik. Manuell teknik är tids- och personalkrävande samt uppvisar en brist på standardisering av antal trombocyter ämnade för transfusion.

Delarbete I och IV i denna avhandling visar att trombocyter framställda via ny automatiserad teknik (Atreus och OrbiSac, Gambro BCT) bibehåller en god cellulär och metabolisk kvalité under 7 dagars förvaring. Resultaten i delarbete I visar att detta industriprojekt har inneburit att trombocytkoncentrat som framställts via automatiserad teknik, även leukocytfiltrerats i samma process. För att standardisera trombocytkoncentrationen i de via automatiserad teknik framställda trombocytkoncentraten, har ett specifikt dataprogram tagits fram, vilket selekterar ut 5 st. buffy-coats för trombocytframställning med utgångspunkt baserat på blodgivarnas perifert cirkulatoriska trombocytinnehåll. I delarbete IV vidare utvecklas process-systemet för trombocytframställning. Resultaten visar att automatisering med ny teknik för framställning av buffy-coats som utgångsmaterial för trombocyter med god metabolisk och cellulär kvalité, där helblodet förvarats antingen över natt eller processats inom 8 timmar, är möjlig.

Forskning inom trombocytområdet har under det senaste decenniet bland annat varit inriktad på att förbättra överlevnad och funktion av trombocyter under en rumstempererad förvaringsperiod. Flera grupper har demonstrerat att förvaring av trombocyter i tillsatslösning (PAS = platelet additive solution) ger en förbättrad överlevnad och funktion i upp till 7 dagar. Men metaboliska och funktionella förändringar sker liksom risk för bakterietillväxt under förvaring i rumstemperatur. Delarbete II och III presenterade i denna avhandling indikerar på möjligheten att kylförvara trombocyter, styrkt av resultat som visar att trombocyter förvarade vid 4^0C minimerar många av de negativa förändringar som sker när trombocyter förvaras vid 22^0C. Förvaring vid 4^0C skulle kunna innebära att man avlägsnar bakterieproblemet, förlänger förvaringstiden samt bättre bibehåller trombocyternas metaboliska och cellulära kvalité innan transfusion. Dock måste kylförvarade trombocyters förmåga att cirkulera och fungera efter transfusion påvisas i vidare forskning.

8 ACKNOWLEDGEMENTS

This work has been carried out at the Division of Clinical Immunology and Transfusion Medicine, Department of Laboratory Medicine at Karolinska Institutet, Stockholm, Sweden. I would like to express my sincere gratitude to my colleagues, friends and family, who have supported me throughout these years. Particulary, I would like to acknowledge:

Associate professor **Agneta Shanwell**, my main supervisor and Dr. **Hans Gulliksson**, my co-supervisor, for providing me with great opportunity to work in this disipline of platelet research, for accepting me as PhD student, for your trust, encouragement, support and patience, for sharing your great depth of scientific knowledge and for your great efforts in revising my manuscripts and thesis.

My co-authors, MD. **Stella Larsson,** first author in Paper I. Pharm D**. Martine Callaert** and Dr. **Mona Hansson** for your great efforts in revising and contribution to my manuscripts. **Gertrud Naess** for cooperation preciding our presentation in Cape Town, South Africa. MD. **Peter Pihlstedt**, Dr. **Geert van Waeg,** Dr. **Niclas Högberg** and MM**. Philippe Jacquet** Gambro BCT for kindly supplying equipment, technical advice and assistance.

Dr. **Kjell Hultenby** for helping me with excellent scanning electron microscopy pictures of stored platelets. Ph.D stud. collegues**, Nahreen Tynngård, Håkan Samuelsson**, **Ola Bjärbo, Gunilla Björling** and **Sofia Nordman** for interesting discussions, cooperation and social support. MD**. Beatrice Diedrich** for providing me the great opportunity to participate in her work with the isotope analysis of transfused platelets. **Bo Nilsson** for expert assistance with statistical analysis of data. **Elisabeth Ishizaki** for teaching me flow cytometry. MD**. Margareta Genberg-Andren** and Prof**. Mats Sjöquist** for your efforts. Dr. **Zoe Walsh** for linguistic revision. **Agneta Sjödin** for your help, interest and support. **Mervi Vesterinen** and **Ingeborg Ohlsson** for valuable practical instructions as well as **Birgitta Hammarborg** always friendly and heplful. **Anita Eriksson** and **Jaenette Hallberg** for cooperation at ISBT in Athens. Ph.D **Alain Segers** and Dr. **Joan Sid** for cooperation during ISBT Madrid. **Gunilla Rosendahl** for your help. **Camilla Söderlund**, **Narin Baderkhan,** and **Tomas Eriksson** at Clinical Immunology and Transfusion Medicine, for being good lab friends.

Family and friends:
My wife, my everything, **Anna-Kari** for your love and support throughout our life together, for sharing all the happiness and sadness with me, for always being there with me. **Börje Malm** for your overall support, your fighting spirit, for sharing your depth of society knowledge and for the stimulating discussions about society and life. **Johan Grill and Johanna Malm** for their integrity and independence, for everything throughout all the years, especially our Christmas celebrations. My parents, **Karin** and **Sven Sandgren** for your loyal support through all the years.

Maria Bäck, for your love and her family: **Anna-Karin Bäck**, **Sebastian** and **Kurt**. **Ulla-Britt Hellberg** for your interest and support. **Johan Stenberg** for sharing memorable moments in the K2 as well as in other sports activities, for all your help, inspiration and good friendship. **Mats Eriksson** for sharing sports and good friendship. **Mats** and **Kerstin Wänglund,** for beeing good neighbours and friends. My old friends throughout the years: **John Kieraldo, Per Bergstedt**, **Robert Hammar, Magnus Karlsson** and **Anna Granström**. **Björn Lindberg, Moa** and **Marcus** and all the friends around them for unforgettable moments. Finally, without songs from **Ulf Lundell** and **Neil Young** and words from **Carl-Magnus Stolt, Hermann Hesse** and **Arthur Schopenauer** this work has never been completed.

9 REFERENCES

1. Tocantins. Historical notes on blood platelets. Blood. 1948;3:1073-82.
2. Duke. The relation of blood platelets to hemorrhagic disease. JAMA. 1910;55:1185-92.
3. Hess JR, Schmidt PJ. The first blood banker: Oswald Hope Robertson. Transfusion. 2000 Jan;40(1):110-3.
4. Grifols. The contribution of Dr. Duran-Jorda to the advance and development of the european blood transfusion. Vox Sanguinis. 2007 July 2007;93(Abstract of the XVII Regional Congress of the ISBT, Europe):24-.
5. Alvarado J, Djerassi I, Farber S. Transfusion of Fresh Concentrated Platelets to Children with Acute Leukemia. J Pediatr. 1965 Jul;67:13-22.
6. Freireich EJ, Kliman A, Gaydos LA, Mantel N, Frei E, 3rd. Response to Repeated Platelet Transfusion from the Same Donor. Ann Intern Med. 1963 Sep;59:277-87.
7. Hersh EM, Bodey GP, Nies BA, Freireich EJ. Causes of Death in Acute Leukemia: a Ten-Year Study of 414 Patients from 1954-1963. Jama. 1965 Jul 12;193:105-9.
8. Murphy S. Platelet storage for transfusion. Semin Hematol. 1985 Jul;22(3):165-77.
9. Murphy S, Gardner FH. Platelet storage at 22 degrees C: role of gas transport across plastic containers in maintenance of viability. Blood. 1975 Aug;46(2):209-18.
10. Murphy S, Kahn RA, Holme S, Phillips GL, Sherwood W, Davisson W, et al. Improved storage of platelets for transfusion in a new container. Blood. 1982 Jul;60(1):194-200.
11. Murphy S, Sayar SN, Gardner FH. Storage of platelet concentrates at 22 degrees C. Blood. 1970 Apr;35(4):549-57.
12. Pietersz RN, de Korte D, Reesink HW, van den Ende A, Dekker WJ, Roos D. Preparation of leukocyte-poor platelet concentrates from buffy coats. III. Effect of leukocyte contamination on storage conditions. Vox Sang. 1988;55(1):14-20.
13. Shanwell A, Gulliksson H, Berg BK, Jansson BA, Svensson LA. Evaluation of platelets prepared by apheresis and stored for 5 days. In vitro and in vivo studies. Transfusion. 1989 Nov-Dec;29(9):783-8.
14. Snyder EL, Pope C, Ferri PM, Smith EO, Walter SD, Ezekowitz MD. The effect of mode of agitation and type of plastic bag on storage characteristics and in vivo kinetics of platelet concentrates. Transfusion. 1986 Mar-Apr;26(2):125-30.
15. Adams GA, Rock G. Storage of human platelet concentrates in an artificial medium without dextrose. Transfusion. 1988 May-Jun;28(3):217-20.
16. Gulliksson H. Defining the optimal storage conditions for the long-term storage of platelets. Transfus Med Rev. 2003 Jul;17(3):209-15.
17. Hogman CF. Aspects of platelet storage. Transfus Sci. 1994 Dec;15(4):351-5.
18. Murphy S. The efficacy of synthetic media in the storage of human platelets for transfusion. Transfus Med Rev. 1999 Jul;13(3):153-63.

19. Shanwell A, Falker C, Gulliksson H. Storage of platelets in additive solutions: the effects of magnesium and potassium on the release of RANTES, beta-thromboglobulin, platelet factor 4 and interleukin-7, during storage. Vox Sang. 2003 Oct;85(3):206-12.
20. Wallace EL, Churchill WH, Surgenor DM, Cho GS, McGurk S. Collection and transfusion of blood and blood components in the United States, 1994. Transfusion. 1998 Jul;38(7):625-36.
21. Slichter SJ. Preservation of platelet viability and function during storage of concentrates. Prog Clin Biol Res. 1978;28:83-100.
22. Boomgaard MN, Gouwerok CW, Palfenier CH, Pankalla-Blandeau IE, Veldman HA, de Korte D, et al. Pooled platelet concentrates prepared by the platelet-rich-plasma method and filtered with three different filters and stored for 8 days. Vox Sang. 1995;68(2):82-9.
23. Schooneman F. [Concentration of platelets from apheresis: methods of preparation]. Transfus Clin Biol. 1994;1(6):489-99.
24. Holme S, Sweeney JD, Sawyer S, Elfath MD. The expression of p-selectin during collection, processing, and storage of platelet concentrates: relationship to loss of in vivo viability. Transfusion. 1997 Jan;37(1):12-7.
25. Rivera J, Sanchez-Roig MJ, Rosillo MC, Moraleda JM, Vicente V. Stability of glycoproteins Ib/IX and IIb/IIIa during preparation and storage of platelet concentrates: detection by binding assays with epitope-defined monoclonal antibodies and physiological ligands. Vox Sang. 1994;67(2):166-71.
26. Triulzi DJ, Ness PM. Intraoperative hemodilution and autologous platelet rich plasma collection: two techniques for collecting fresh autologous blood. Transfus Sci. 1995 Mar;16(1):33-44.
27. Wang C, Mody M, Herst R, Sher G, Freedman J. Flow cytometric analysis of platelet function in stored platelet concentrates. Transfus Sci. 1999 Apr;20(2):129-39.
28. Gulliksson H. Platelet storage media. Transfus Apher Sci. 2001 Jun;24(3):241-4.
29. Chernoff A, Snyder EL. The cellular and molecular basis of the platelet storage lesion: a symposium summary. Transfusion. 1992 May;32(4):386-90.
30. Sazama K. Reports of 355 transfusion-associated deaths: 1976 through 1985. Transfusion. 1990 Sep;30(7):583-90.
31. Heddle NM. Febrile nonhemolytic transfusion reactions to platelets. Curr Opin Hematol. 1995 Nov;2(6):478-83.
32. Muylle L, Peetermans ME. Effect of prestorage leukocyte removal on the cytokine levels in stored platelet concentrates. Vox Sang. 1994;66(1):14-7.
33. Muylle L, Wouters E, Peetermans ME. Febrile reactions to platelet transfusion: the effect of increased interleukin 6 levels in concentrates prepared by the platelet-rich plasma method. Transfusion. 1996 Oct;36(10):886-90.
34. Novotny VM, van Doorn R, Witvliet MD, Claas FH, Brand A. Occurrence of allogeneic HLA and non-HLA antibodies after transfusion of prestorage filtered platelets and red blood cells: a prospective study. Blood. 1995 Apr 1;85(7):1736-41.
35. Dunstan RA, Simpson MB, Knowles RW, Rosse WF. The origin of ABH antigens on human platelets. Blood. 1985 Mar;65(3):615-9.

36. Lee EJ, Schiffer CA. ABO compatibility can influence the results of platelet transfusion. Results of a randomized trial. Transfusion. 1989 Jun;29(5):384-9.
37. Fisher M, Chapman JR, Ting A, Morris PJ. Alloimmunisation to HLA antigens following transfusion with leucocyte-poor and purified platelet suspensions. Vox Sang. 1985;49(5):331-5.
38. Oksanen K, Kekomaki R, Ruutu T, Koskimies S, Myllyla G. Prevention of alloimmunization in patients with acute leukemia by use of white cell-reduced blood components--a randomized trial. Transfusion. 1991 Sep;31(7):588-94.
39. Saarinen UM, Kekomaki R, Siimes MA, Myllyla G. Effective prophylaxis against platelet refractoriness in multitransfused patients by use of leukocyte-free blood components. Blood. 1990 Jan 15;75(2):512-7.
40. Halle L. [Platelet alloantigenic systems]. Transfus Clin Biol. 1998 Oct;5(5):362-5.
41. Jakobowicz R, Williams L, Silberman F. Immunization of Rh-negative volunteers by repeated injections of very small amounts of Rh-positive blood. Vox Sang. 1972;23(4):376-81.
42. Platelet transfusion therapy. National Institutes of Health Consensus Conference. Transfus Med Rev. 1987 Dec;1(3):195-200.
43. Cid J, Lozano M. Lower or higher doses for prophylactic platelet transfusions: results of a meta-analysis of randomized controlled trials. Transfusion. 2007 Mar;47(3):464-70.
44. Heddle NM, Klama LN, Griffith L, Roberts R, Shukla G, Kelton JG. A prospective study to identify the risk factors associated with acute reactions to platelet and red cell transfusions. Transfusion. 1993 Oct;33(10):794-7.
45. Kluter H, Bubel S, Kirchner H, Wilhelm D. Febrile and allergic transfusion reactions after the transfusion of white cell-poor platelet preparations. Transfusion. 1999 Nov-Dec;39(11-12):1179-84.
46. Couban S, Carruthers J, Andreou P, Klama LN, Barr R, Kelton JG, et al. Platelet transfusions in children: results of a randomized, prospective, crossover trial of plasma removal and a prospective audit of WBC reduction. Transfusion. 2002 Jun;42(6):753-8.
47. Schrezenmeier H, Walther-Wenke G, Muller TH, Weinauer F, Younis A, Holland-Letz T, et al. Bacterial contamination of platelet concentrates: results of a prospective multicenter study comparing pooled whole blood-derived platelets and apheresis platelets. Transfusion. 2007 Apr;47(4):644-52.
48. Blajchman MA, Goldman M. Bacterial contamination of platelet concentrates: incidence, significance, and prevention. Semin Hematol. 2001 Oct;38(4 Suppl 11):20-6.
49. Goldman M. Bacterial contamination of platelet concentrates: where are we today? Vox Sang. 2004 Jul;87 Suppl 2:90-2.
50. Levin. The evolution of mammalian platelets. In: Michelson AD ed Platelets Second edition: Academic Press/Elsevier Science. 2007:3-18.
51. Italiano J. Megakaryocyte development and platelet formation. In: Michelson AD ed Platelets Second edition: Academic Press/Elsevier Science. 2007:23-39.

52. Yamao T, Noguchi T, Takeuchi O, Nishiyama U, Morita H, Hagiwara T, et al. Negative regulation of platelet clearance and of the macrophage phagocytic response by the transmembrane glycoprotein SHPS-1. J Biol Chem. 2002 Oct 18;277(42):39833-9.
53. Brown SB, Clarke MC, Magowan L, Sanderson H, Savill J. Constitutive death of platelets leading to scavenger receptor-mediated phagocytosis. A caspase-independent cell clearance program. J Biol Chem. 2000 Feb 25;275(8):5987-96.
54. Turner CP, Hadley AG. The role of P-selectin in the immune destruction of platelets. Br J Haematol. 2003 May;121(4):623-31.
55. Wiener E, Abeyakoon O, Benchetrit G, Lyall M, Keler T, Rodeck CH. Anti-HPA-1a-mediated platelet phagocytosis by monocytes in vitro and its inhibition by Fc gamma receptor (FcgammaR) reactive reagents. Eur J Haematol. 2003 Feb;70(2):67-74.
56. Lewis JC, Maldonado JE, Mann KG. Phagocytosis in human platelets: localization of acid phosphatase-positive phagosomes following latex uptake. Blood. 1976 May;47(5):833-40.
57. White JG. Platelets are covercytes, not phagocytes: uptake of bacteria involves channels of the open canalicular system. Platelets. 2005 Mar;16(2):121-31.
58. Heijnen HF, Debili N, Vainchencker W, Breton-Gorius J, Geuze HJ, Sixma JJ. Multivesicular bodies are an intermediate stage in the formation of platelet alpha-granules. Blood. 1998 Apr 1;91(7):2313-25.
59. Salganicoff L, Fukami MH. Energy metabolism of blood platelets. I. Isolation and properties of platelet mitochondria. Arch Biochem Biophys. 1972 Dec;153(2):726-35.
60. White JG. Platelet glycosomes. Platelets. 1999;10:242-6.
61. White JG. Electron dense chains and clusters in human platelets. Platelets. 2002 Aug-Sep;13(5-6):317-25.
62. White JG. Medich giant platelet disorder: a unique alpha granule deficiency I. Structural abnormalities. Platelets. 2004 Sep;15(6):345-53.
63. Behnke O. Electron microscopic observations on the membrane systems of the rat blood platelet. Anat Rec. 1967 Jun;158(2):121-37.
64. Breton-Gorius J. Development of two distinct membrane systems associated in giant complexes in pathological megakaryocytes. Ser Haematol. 1975;8(1):49-67.
65. White JG. Uptake of latex particles by blood platelets: phagocytosis or sequestration? Am J Pathol. 1972 Dec;69(3):439-58.
66. Escolar G, White JG. The platelet open canalicular system: a final common pathway. Blood Cells. 1991;17(3):467-85; discussion 86-95.
67. White JG, Escolar G. The blood platelet open canalicular system: a two-way street. Eur J Cell Biol. 1991 Dec;56(2):233-42.
68. Gerrard JM, White JG, Rao GH, Townsend D. Localization of platelet prostaglandin production in the platelet dense tubular system. Am J Pathol. 1976 May;83(2):283-98.
69. White JG, Gerrard JM. Ultrastructural features of abnormal blood platelets. A review. Am J Pathol. 1976 Jun;83(3):589-632.
70. White JG. Platelet structure. In: Michelson AD ed Platelets Second edition: Academic Press/Elsevier Science. 2007;2:45-73.

71. Barnard MR, MacGregor H, Mercier R, Ragno G, Pivacek LE, Hechtman HB, et al. Platelet surface p-selectin, platelet-granulocyte heterotypic aggregates, and plasma-soluble p-selectin during plateletpheresis. Transfusion. 1999 Jul;39(7):735-41.
72. Fox JE. The platelet cytoskeleton. Thromb Haemost. 1993 Dec 20;70(6):884-93.
73. Brass LS, TJ. Zhu L. Woulfe DS. Signal transduction during platelet plug formation. In: Michelson AD ed Platelets Second edition: Academic Press/Elsevier Science. 2007:319-39.
74. Badlou BA, Spierenburg G, Ulrichts H, Deckmyn H, Smid WM, Akkerman JW. Role of glycoprotein Ibalpha in phagocytosis of platelets by macrophages. Transfusion. 2006 Dec;46(12):2090-9.
75. Badlou BA, van der Meer PF, Akkerman JW, Smid WM, Pietersz RN. Metabolic energy reduction by glucose deprivation and low gas exchange preserves platelet function after 48 h storage at 4 degrees C. Vox Sang. 2007 May;92(4):311-8.
76. Badlou BA, Wu YP, Smid WM, Akkerman JW. Platelet binding and phagocytosis by macrophages. Transfusion. 2006 Aug;46(8):1432-43.
77. Hoffmeister KM, Felbinger TW, Falet H, Denis CV, Bergmeier W, Mayadas TN, et al. The clearance mechanism of chilled blood platelets. Cell. 2003 Jan 10;112(1):87-97.
78. Hoffmeister KM, Josefsson EC, Isaac NA, Clausen H, Hartwig JH, Stossel TP. Glycosylation restores survival of chilled blood platelets. Science. 2003 Sep 12;301(5639):1531-4.
79. Nurden AT, Caen JP. Specific roles for platelet surface glycoproteins in platelet function. Nature. 1975 Jun 26;255(5511):720-2.
80. Berndt MC, Shen Y, Dopheide SM, Gardiner EE, Andrews RK. The vascular biology of the glycoprotein Ib-IX-V complex. Thromb Haemost. 2001 Jul;86(1):178-88.
81. McGregor JL, Brochier J, Wild F, Follea G, Trzeciak MC, James E, et al. Monoclonal antibodies against platelet membrane glycoproteins. Characterization and effect on platelet function. Eur J Biochem. 1983 Mar 15;131(2):427-36.
82. Fullard JF. The role of the platelet glycoprotein IIb/IIIa in thrombosis and haemostasis. Curr Pharm Des. 2004;10(14):1567-76.
83. Shattil SJ. Function and regulation of the beta 3 integrins in hemostasis and vascular biology. Thromb Haemost. 1995 Jul;74(1):149-55.
84. Brass LF, Manning DR, Cichowski K, Abrams CS. Signaling through G proteins in platelets: to the integrins and beyond. Thromb Haemost. 1997 Jul;78(1):581-9.
85. Krailadsiri P, Seghatchian J. Annexin V and platelet antigen expression is not altered during storage of platelet concentrates obtained with the AMICUS cell separator. Transfus Sci. 1999 Aug;21(1):101-2.
86. Krailadsiri P, Seghatchian J. Effect of processing and storage on platelet activation, cellular injury and microvesiculation. Transfus Apher Sci. 2001 Jun;24(3):237-8.
87. Fox JE, Austin CD, Boyles JK, Steffen PK. Role of the membrane skeleton in preventing the shedding of procoagulant-rich microvesicles from the platelet plasma membrane. J Cell Biol. 1990 Aug;111(2):483-93.

88. Beckstead JH, Stenberg PE, McEver RP, Shuman MA, Bainton DF. Immunohistochemical localization of membrane and alpha-granule proteins in human megakaryocytes: application to plastic-embedded bone marrow biopsy specimens. Blood. 1986 Feb;67(2):285-93.
89. Gear AR, Camerini D. Platelet chemokines and chemokine receptors: linking hemostasis, inflammation, and host defense. Microcirculation. 2003 Jun;10(3-4):335-50.
90. Handagama P, Rappolee DA, Werb Z, Levin J, Bainton DF. Platelet alpha-granule fibrinogen, albumin, and immunoglobulin G are not synthesized by rat and mouse megakaryocytes. J Clin Invest. 1990 Oct;86(4):1364-8.
91. Niewiarowski S. Proteins secreted by the platelet. Thromb Haemost. 1977 Dec 15;38(4):924-38.
92. Schmaier AH. Platelet forms of plasma proteins: plasma cofactors/substrates and inhibitors contained within platelets. Semin Hematol. 1985 Jul;22(3):187-202.
93. White JG. The dense bodies of human platelets: inherent electron opacity of the serotonin storage particles. Blood. 1969 Apr;33(4):598-606.
94. Israels SJ, Gerrard JM, Jacques YV, McNicol A, Cham B, Nishibori M, et al. Platelet dense granule membranes contain both granulophysin and P-selectin (GMP-140). Blood. 1992 Jul 1;80(1):143-52.
95. Norcott JP, Solari R, Cutler DF. Targeting of P-selectin to two regulated secretory organelles in PC12 cells. J Cell Biol. 1996 Sep;134(5):1229-40.
96. Reed G. Platelet secretion. In: Michelson AD ed Platelets Second edition: Academic Press/Elsevier Science. 2007:309-18.
97. Chen D, Bernstein AM, Lemons PP, Whiteheart SW. Molecular mechanisms of platelet exocytosis: role of SNAP-23 and syntaxin 2 in dense core granule release. Blood. 2000 Feb 1;95(3):921-9.
98. Lemons PP, Chen D, Whiteheart SW. Molecular mechanisms of platelet exocytosis: requirements for alpha-granule release. Biochem Biophys Res Commun. 2000 Jan 27;267(3):875-80.
99. Polgar J, Chung SH, Reed GL. Vesicle-associated membrane protein 3 (VAMP-3) and VAMP-8 are present in human platelets and are required for granule secretion. Blood. 2002 Aug 1;100(3):1081-3.
100. Polgar J, Lane WS, Chung SH, Houng AK, Reed GL. Phosphorylation of SNAP-23 in activated human platelets. J Biol Chem. 2003 Nov 7;278(45):44369-76.
101. Efeoglu C, Akcay YD, Erturk S. A modified method for preparing platelet-rich plasma: an experimental study. J Oral Maxillofac Surg. 2004 Nov;62(11):1403-7.
102. Landesberg R, Burke A, Pinsky D, Katz R, Vo J, Eisig SB, et al. Activation of platelet-rich plasma using thrombin receptor agonist peptide. J Oral Maxillofac Surg. 2005 Apr;63(4):529-35.
103. Pietersz RN, Reesink HW, Huijgens PC, van Oers MH. Preparation of leucocyte-poor platelet concentrates from buffy coats. IV. Clinical evaluation. Vox Sang. 1988;55(3):129-32.
104. Eriksson L, Hogman CF. Platelet concentrates in an additive solution prepared from pooled buffy coats. 1. In vitro studies. Vox Sang. 1990;59(3):140-5.

105. Gulliksson H. Additive solutions for the storage of platelets for transfusion. Transfus Med. 2000 Dec;10(4):257-64.
106. Ezidiegwu CN, Lauenstein KJ, Rosales LG, Kelly KC, Henry JB. Febrile nonhemolytic transfusion reactions. Management by premedication and cost implications in adult patients. Arch Pathol Lab Med. 2004 Sep;128(9):991-5.
107. Heddle NM, Klama L, Singer J, Richards C, Fedak P, Walker I, et al. The role of the plasma from platelet concentrates in transfusion reactions. N Engl J Med. 1994 Sep 8;331(10):625-8.
108. McCullough J, Dodd R, Gilcher R, Murphy S, Sayers M. White particulate matter: report of the ad hoc industry review group. Transfusion. 2004 Jul;44(7):1112-8.
109. Heaton WA, Rebulla P, Pappalettera M, Dzik WH. A comparative analysis of different methods for routine blood component preparation. Transfus Med Rev. 1997 Apr;11(2):116-29.
110. Kilkson H, Holme S, Murphy S. Platelet metabolism during storage of platelet concentrates at 22 degrees C. Blood. 1984 Aug;64(2):406-14.
111. Cesar J, DiMinno G, Alam I, Silver M, Murphy S. Plasma free fatty acid metabolism during storage of platelet concentrates for transfusion. Transfusion. 1987 Sep-Oct;27(5):434-7.
112. Murphy S, Shimizu T, Miripol J. Platelet storage for transfusion in synthetic media: further optimization of ingredients and definition of their roles. Blood. 1995 Nov 15;86(10):3951-60.
113. Bertolini F, Murphy S, Rebulla P, Sirchia G. Role of acetate during platelet storage in a synthetic medium. Transfusion. 1992 Feb;32(2):152-6.
114. Moroff G, Friedman A, Robkin-Kline L. Factors influencing changes in pH during storage of platelet concentrates at 20-24 degree C. Vox Sang. 1982 Jan;42(1):33-45.
115. Guppy M, Whisson ME, Sabaratnam R, Withers P, Brand K. Alternative fuels for platelet storage: a metabolic study. Vox Sang. 1990;59(3):146-52.
116. Holme S. Effect of additive solutions on platelet biochemistry. Blood Cells. 1992;18(3):421-30; discussion 31-4.
117. Filip DJ, Eckstein JD, Sibley CA. The effect of platelet concentrate storage temperature on adenine nucleotide metabolism. Blood. 1975 Jun;45(6):749-56.
118. Hunter S, Nixon J, Murphy S. The effect of the interruption of agitation on platelet quality during storage for transfusion. Transfusion. 2001 Jun;41(6):809-14.
119. Slichter SJ, Harker LA. Preparation and storage of platelet concentrates. I. Factors influencing the harvest of viable platelets from whole blood. Br J Haematol. 1976 Nov;34(3):395-402.
120. van der Meer PF, Gulliksson H, Aubuchon JP, Prowse C, Richter E, de Wildt-Eggen J. Interruption of agitation of platelet concentrates: effects on in vitro parameters. Vox Sang. 2005 May;88(4):227-34.
121. Rock G, Figueredo A. Metabolic changes during platelet storage. Transfusion. 1976 Nov-Dec;16(6):571-9.
122. Sandgren P, Shanwell A, Gulliksson H. Storage of buffy coat-derived platelets in additive solutions: in vitro effects of storage at 4 degrees C. Transfusion. 2006 May;46(5):828-34.

123. Snyder EL. Activation during preparation and storage of platelet concentrates. Transfusion. 1992 Jul-Aug;32(6):500-2.
124. Snyder EL, Ezekowitz M, Aster R, Murphy S, Ferri P, Smith E, et al. Extended storage of platelets in a new plastic container. II. In vivo response to infusion of platelets stored for 5 days. Transfusion. 1985 May-Jun;25(3):209-14.
125. AuBuchon JP, Taylor H, Holme S, Nelson E. In vitro and in vivo evaluation of leukoreduced platelets stored for 7 days in CLX containers. Transfusion. 2005 Aug;45(8):1356-61.
126. Vainer H, Prost-Dvojakovic RJ, Jaisson F, Letohic F. Studies on human platelets stored at 20-22 degrees C without agitation. Acta Haematol. 1976;56(3):160-73.
127. Kunicki TJ, Tuccelli M, Becker GA, Aster RH. A study of variables affecting the quality of platelets stored at "room temperature". Transfusion. 1975 Sep-Oct;15(5):414-21.
128. Holme S, Vaidja K, Murphy S. Platelet storage at 22 degrees C: effect of type of agitation on morphology, viability, and function in vitro. Blood. 1978 Aug;52(2):425-35.
129. Gulliksson H, AuBuchon JP, Vesterinen M, Sandgren P, Larsson S, Pickard CA, et al. Storage of platelets in additive solutions: a pilot in vitro study of the effects of potassium and magnesium. Vox Sang. 2002 Apr;82(3):131-6.
130. Tanoue K, Mano Y, Kuroiwa K, Suzuki H, Shibayama M, Yamazaki H. Consumption of platelets in decompression sickness of rabbits. J Appl Physiol. 1987 May;62(5):1772-9.
131. de Wildt-Eggen J, Nauta S, Schrijver JG, van Marwijk Kooy M, Bins M, van Prooijen HC. Reactions and platelet increments after transfusion of platelet concentrates in plasma or an additive solution: a prospective, randomized study. Transfusion. 2000 Apr;40(4):398-403.
132. Ringwald J, Zimmermann R, Eckstein R. The new generation of platelet additive solution for storage at 22 degrees C: development and current experience. Transfus Med Rev. 2006 Apr;20(2):158-64.
133. Kerkhoffs JL, Eikenboom JC, Schipperus MS, van Wordragen-Vlaswinkel RJ, Brand R, Harvey MS, et al. A multicenter randomized study of the efficacy of transfusions with platelets stored in platelet additive solution II versus plasma. Blood. 2006 Nov 1;108(9):3210-5.
134. Gulliksson H, Larsson S, Kumlien G, Shanwell A. Storage of platelets in additive solutions: effects of phosphate. Vox Sang. 2000;78(3):176-84.
135. Holme S, Heaton WA, Courtright M. Improved in vivo and in vitro viability of platelet concentrates stored for seven days in a platelet additive solution. Br J Haematol. 1987 Jun;66(2):233-8.
136. B. Diedrich PS, B. Jansson, H. Gulliksson, L. Svensson, A. Shanwell In vitro and in vivo effects of potassium and magnesium on storage up to 7 days of apheresis platelet concentrates in platelet additive solution. Vox Sang. 2007;(OnlineEarly Articles). doi:10.1111/j.1423-0410.2007.01002.x
137. Shimizu T, Murphy S. Roles of acetate and phosphate in the successful storage of platelet concentrates prepared with an acetate-containing additive solution. Transfusion. 1993 Apr;33(4):304-10.

138. Gulliksson H. Storage of platelets in additive solutions: the effect of citrate and acetate in in vitro studies. Transfusion. 1993 Apr;33(4):301-3.
139. Sweeney JD, Blair AJ, Cheves TA, Dottori S, Arduini A. L-carnitine decreases glycolysis in liquid-stored platelets. Transfusion. 2000 Nov;40(11):1313-9.
140. Holme S. Storage and quality assessment of platelets. Vox Sang. 1998;74 Suppl 2:207-16.
141. Rudderow D, Soslau G. Permanent lesions of stored platelets correlate to pH and cell count while reversible lesions do not. Proc Soc Exp Biol Med. 1998 Feb;217(2):219-27.
142. Seghatchian J. Platelet storage lesion: an update on the impact of various leukoreduction processes on the biological response modifiers. Transfus Apher Sci. 2006 Feb;34(1):125-30.
143. Seghatchian J, Krailadsiri P. The platelet storage lesion. Transfus Med Rev. 1997 Apr;11(2):130-44.
144. Shanwell A, Diedrich B, Falker C, Jansson B, Sandgren P, Sundkvist L, et al. Paired in vitro and in vivo comparison of apheresis platelet concentrates stored in platelet additive solution for 1 versus 7 days. Transfusion. 2006 Jun;46(6):973-9.
145. Murphy S. Radiolabeling of PLTs to assess viability: a proposal for a standard. Transfusion. 2004 Jan;44(1):131-3.
146. de Bruijne-Admiraal LG, Modderman PW, Von dem Borne AE, Sonnenberg A. P-selectin mediates Ca(2+)-dependent adhesion of activated platelets to many different types of leukocytes: detection by flow cytometry. Blood. 1992 Jul 1;80(1):134-42.
147. Metzelaar MJ, Korteweg J, Sixma JJ, Nieuwenhuis HK. Comparison of platelet membrane markers for the detection of platelet activation in vitro and during platelet storage and cardiopulmonary bypass surgery. J Lab Clin Med. 1993 Apr;121(4):579-87.
148. Nishibori M, Cham B, McNicol A, Shalev A, Jain N, Gerrard JM. The protein CD63 is in platelet dense granules, is deficient in a patient with Hermansky-Pudlak syndrome, and appears identical to granulophysin. J Clin Invest. 1993 Apr;91(4):1775-82.
149. Matsubayashi H, Weidner J, Miraglia CC, McIntyre JA. Platelet membrane early activation markers during prolonged storage. Thromb Res. 1999 Feb 15;93(4):151-60.
150. Seghatchian J, Krailadsiri P. Platelet storage lesion and apoptosis: are they related? Transfus Apher Sci. 2001 Feb;24(1):103-5.
151. Shcherbina A, Remold-O'Donnell E. Role of caspase in a subset of human platelet activation responses. Blood. 1999 Jun 15;93(12):4222-31.
152. Wolf BB, Goldstein JC, Stennicke HR, Beere H, Amarante-Mendes GP, Salvesen GS, et al. Calpain functions in a caspase-independent manner to promote apoptosis-like events during platelet activation. Blood. 1999 Sep 1;94(5):1683-92.
153. Plenchette S, Moutet M, Benguella M, N'Gondara JP, Guigner F, Coffe C, et al. Early increase in DcR2 expression and late activation of caspases in the platelet storage lesion. Leukemia. 2001 Oct;15(10):1572-81.

154. Cardigan R, Turner C, Harrison P. Current methods of assessing platelet function: relevance to transfusion medicine. Vox Sang. 2005 Apr;88(3):153-63.
155. Bertolini F, Agazzi A, Peccatori F, Martinelli G, Sandri MT. The absence of swirling in platelet concentrates is highly predictive of poor posttransfusion platelet count increments and increased risk of a transfusion reaction. Transfusion. 2000 Jan;40(1):121-2.
156. Bertolini F, Murphy S. A multicenter evaluation of reproducibility of swirling in platelet concentrates. Biomedical Excellence for Safer Transfusion (BEST) Working Party of the International Society of Blood Transfusion. Transfusion. 1994 Sep;34(9):796-801.
157. Bertolini F, Murphy S. A multicenter inspection of the swirling phenomenon in platelet concentrates prepared in routine practice. Biomedical Excellence for Safer Transfusion (BEST) Working Party of the International Society of Blood Transfusion. Transfusion. 1996 Feb;36(2):128-32.
158. Holme S, Heaton WA, Courtright M. Platelet storage lesion in second-generation containers: correlation with platelet ATP levels. Vox Sang. 1987;53(4):214-20.
159. Valeri CR, Feingold H, Marchionni LD. The relation between response to hypotonic stress and the 51Cr recovery in vivo of preserved platelets. Transfusion. 1974 Jul-Aug;14(4):331-7.
160. VandenBroeke T, Dumont LJ, Hunter S, Nixon J, Murphy S, Roger J, et al. Platelet storage solution affects on the accuracy of laboratory tests for platelet function: a multi-laboratory study. Vox Sang. 2004 Apr;86(3):183-8.
161. Blajchman MA. Bacterial contamination of blood products and the value of pre-transfusion testing. Immunol Invest. 1995 Jan-Feb;24(1-2):163-70.
162. Blajchman MA, Ali AM, Richardson HL. Bacterial contamination of cellular blood components. Vox Sang. 1994;67 Suppl 3:25-33.
163. Blajchman MA, Beckers EA, Dickmeiss E, Lin L, Moore G, Muylle L. Bacterial detection of platelets: current problems and possible resolutions. Transfus Med Rev. 2005 Oct;19(4):259-72.
164. McCullough J, Vesole DH, Benjamin RJ, Slichter SJ, Pineda A, Snyder E, et al. Therapeutic efficacy and safety of platelets treated with a photochemical process for pathogen inactivation: the SPRINT Trial. Blood. 2004 Sep 1;104(5):1534-41.
165. Mohammadi T, Pietersz RN, Vandenbroucke-Grauls CM, Savelkoul PH, Reesink HW. Detection of bacteria in platelet concentrates: comparison of broad-range real-time 16S rDNA polymerase chain reaction and automated culturing. Transfusion. 2005 May;45(5):731-6.
166. Bruchmuller I, Losel R, Bugert P, Corash L, Lin L, Kluter H, et al. Effect of the psoralen-based photochemical pathogen inactivation on mitochondrial DNA in platelets. Platelets. 2005 Dec;16(8):441-5.
167. Cardo LJ, Rentas FJ, Ketchum L, Salata J, Harman R, Melvin W, et al. Pathogen inactivation of Leishmania donovani infantum in plasma and platelet concentrates using riboflavin and ultraviolet light. Vox Sang. 2006 Feb;90(2):85-91.
168. Cardo LJ, Salata J, Mendez J, Reddy H, Goodrich R. Pathogen inactivation of Trypanosoma cruzi in plasma and platelet concentrates using riboflavin and ultraviolet light. Transfus Apher Sci. 2007 Oct 18.

169. Becker GA, Tuccelli M, Kunicki T, Chalos MK, Aster RH. Studies of platelet concentrates stored at 22 C nad 4 C. Transfusion. 1973 Mar;13(2):61-8.
170. Hoffmeister KM, Falet H, Toker A, Barkalow KL, Stossel TP, Hartwig JH. Mechanisms of cold-induced platelet actin assembly. J Biol Chem. 2001 Jul 6;276(27):24751-9.
171. Oliver AE, Tablin F, Walker NJ, Crowe JH. The internal calcium concentration of human platelets increases during chilling. Biochim Biophys Acta. 1999 Jan 12;1416(1-2):349-60.
172. Dumont LJ, AuBuchon JP, Whitley P, Herschel LH, Johnson A, McNeil D, et al. Seven-day storage of single-donor platelets: recovery and survival in an autologous transfusion study. Transfusion. 2002 Jul;42(7):847-54.
173. Murphy S. What's so bad about old platelets? Transfusion. 2002 Jul;42(7):809-11.
174. Winokur R, Hartwig JH. Mechanism of shape change in chilled human platelets. Blood. 1995 Apr 1;85(7):1796-804.
175. Valeri CR, Ragno G, Marks PW, Kuter DJ, Rosenberg RD, Stossel TP. Effect of thrombopoietin alone and a combination of cytochalasin B and ethylene glycol bis(beta-aminoethyl ether) N,N'-tetraacetic acid-AM on the survival and function of autologous baboon platelets stored at 4 degrees C for as long as 5 days. Transfusion. 2004 Jun;44(6):865-70.
176. Mintz PD, Anderson G, Avery N, Clark P, Bonner RF. Assessment of the correlation of platelet morphology with in vivo recovery and survival. Transfusion. 2005 Aug;45(2 Suppl):72S-80S.
177. Barber AJ, Jamieson GA. Platelet collagen adhesion characterization of collagen glucosyltransferase of plasma membranes of human blood platelets. Biochim Biophys Acta. 1971 Dec 21;252(3):533-45.
178. Wandall. Megakaryocytes package and deliver golgiassociated glycosyltransferases into platelets and to platelet surfaces using dense granules. Blood. 2005;ASH Annual meeting and exposition, Atlanta.
179. Arthur PG, Kent JC, Potter JM, Hartmann PE. Lactose in blood in nonpregnant, pregnant, and lactating women. J Pediatr Gastroenterol Nutr. 1991 Oct;13(3):254-9.
180. Italiano JE, Jr., Bergmeier W, Tiwari S, Falet H, Hartwig JH, Hoffmeister KM, et al. Mechanisms and implications of platelet discoid shape. Blood. 2003 Jun 15;101(12):4789-96.
181. Sandgren P. Blood samples exposed to 4 degrees C: Effects on a platelet adhesion and aggregation test the cone and platelet analyzer (CPA) Impact R. Vox Sang. 2006;91 suppl. 3:265.
182. Tang RC, Huang CY, Pei XP, Chen BA, Li CP, Shi GY. [Evaluation of the effects of glycosylation on in vivo survival of cold-storage human platelets by using rabbit model]. Zhongguo Shi Yan Xue Ye Xue Za Zhi. 2005 Dec;13(6):1113-6.
183. Babic AM, Josefsson EC, Bergmeier W, Wagner DD, Kaufman RM, Silberstein LE, et al. In vitro function and phagocytosis of galactosylated platelet concentrates after long-term refrigeration. Transfusion. 2007 Mar;47(3):442-51.
184. Murphy S, Gardner FH. The effect of temperature on platelet viability. Vox Sang. 1969 Jul;17(1):22.
185. Holme S. Platelet storage in a liquid environment. Transfus Sci. 1994 Jun;15(2):117-30.

186. Nilsson L, Hedner U, Nilsson IM, Robertson B. Shelf-life of bank blood and stored plasma with special reference to coagulation factors. Transfusion. 1983 Sep-Oct;23(5):377-81.
187. Vermeer C, Soute BA, Ates G, Hellings JA, Brummelhuis HG. Contributions to the optimal use of human blood. VIII. Stability of blood coagulation factor VII during collection and storage of whole blood and plasma. Vox Sang. 1976;31(1 SUPPL):55-67.
188. Knutson F, Loof H, Hogman CF. Pre-separation storage of whole blood: the effect of temperature on red cell 2,3-diphosphoglycerate and myeloperoxidase in plasma. Transfus Sci. 2000 Aug 1;23(1):83.
189. Thibault L, Beausejour A, de Grandmont MJ, Lemieux R, Leblanc JF. Characterization of blood components prepared from whole-blood donations after a 24-hour hold with the platelet-rich plasma method. Transfusion. 2006 Aug;46(8):1292-9.
190. Boeri N, Saleun S, Pelissier E, Saleun JP, Aiach M, Rendu F. Influence of a 12-hour, 22 degrees C holding period for buffy coats on the preparation of platelet concentrates stored in plasma. Transfusion. 1994 Oct;34(10):881-6.
191. Ledent E, Wasteson A, Berlin G. Growth factor release during preparation and storage of platelet concentrates. Vox Sang. 1995;68(4):205-9.
192. Perez-Pujol S, Lozano M, Perea D, Mazzara R, Ordinas A, Escolar G. Effect of holding buffy coats 4 or 18 hours before preparing pooled filtered PLT concentrates in plasma. Transfusion. 2004 Feb;44(2):202-9.
193. Racz Z, Baroti C. Storage of platelet concentrates from overnight-stored blood and overnight-stored buffy coat: in vitro studies. Vox Sang. 1995;68(3):160-3.
194. Sanz C, Pereira A, Faundez AI, Ordinas A. Prolonged holding of whole blood at 22 degrees C does not increase activation in platelet concentrates. Vox Sang. 1997;72(4):225-8.
195. Sanz C, Pereira A, Vila J, Faundez AI, Gomez J, Ordinas A. Growth of bacteria in platelet concentrates obtained from whole blood stored for 16 hours at 22 degrees C before component preparation. Transfusion. 1997 Mar;37(3):251-4.
196. Schleuning M, Bock M, Mempel W. Complement activation during storage of single-donor platelet concentrates. Vox Sang. 1994;67(2):144-8.
197. Silliman CC, Clay KL, Thurman GW, Johnson CA, Ambruso DR. Partial characterization of lipids that develop during the routine storage of blood and prime the neutrophil NADPH oxidase. J Lab Clin Med. 1994 Nov;124(5):684-94.
198. Holme S, Moroff G, Murphy S. A multi-laboratory evaluation of in vitro platelet assays: the tests for extent of shape change and response to hypotonic shock. Biomedical Excellence for Safer Transfusion Working Party of the International Society of Blood Transfusion. Transfusion. 1998 Jan;38(1):31-40.
199. Moroff G, Eich J, Dabay M. Validation of use of the Nageotte hemocytometer to count low levels of white cells in white cell-reduced platelet components. Transfusion. 1994 Jan;34(1):35-8.
200. Lundin A. Use of firefly luciferase in ATP-related assays of biomass, enzymes, and metabolites. Methods Enzymol. 2000;305:346-70.

201. King J. A routine method for the estimation of lactic dehydrogenase activity. J Med Lab Technol. 1959 Oct;16:265-72.
202. Brubaker DB. Clinical significance of white cell antibodies in febrile nonhemolytic transfusion reactions. Transfusion. 1990 Oct;30(8):733-7.
203. Decary F, Ferner P, Giavedoni L, Hartman A, Howie R, Kalovsky E, et al. An investigation of nonhemolytic transfusion reactions. Vox Sang. 1984;46(5):277-85.
204. Muylle L, Joos M, Wouters E, De Bock R, Peetermans ME. Increased tumor necrosis factor alpha (TNF alpha), interleukin 1, and interleukin 6 (IL-6) levels in the plasma of stored platelet concentrates: relationship between TNF alpha and IL-6 levels and febrile transfusion reactions. Transfusion. 1993 Mar;33(3):195-9.
205. Wadhwa M, Seghatchian MJ, Dilger P, Sands D, Krailadisiri P, Contreras M, et al. Cytokines in WBC-reduced apheresis PCs during storage: a comparison of two WBC-reduction methods. Transfusion. 2000 Sep;40(9):1118-26.
206. Bubel S, Wilhelm D, Entelmann M, Kirchner H, Kluter H. Chemokines in stored platelet concentrates. Transfusion. 1996 May;36(5):445-9.
207. Fujihara M, Ikebuchi K, Wakamoto S, Sekiguchi S. Effects of filtration and gamma radiation on the accumulation of RANTES and transforming growth factor-beta1 in apheresis platelet concentrates during storage. Transfusion. 1999 May;39(5):498-505.
208. Boehlen F, Clemetson KJ. Platelet chemokines and their receptors: what is their relevance to platelet storage and transfusion practice? Transfus Med. 2001 Dec;11(6):403-17.
209. Wakamoto S, Fujihara M, Kuzuma K, Sato S, Kato T, Naohara T, et al. Biologic activity of RANTES in apheresis PLT concentrates and its involvement in nonhemolytic transfusion reactions. Transfusion. 2003 Aug;43(8):1038-46.
210. Hutchinson RE, Kunkel KD, Schell MJ, Jackson CW, Nelson EJ, Wang WC, et al. Beneficial effect of brief pre-transfusion incubation of platelets at 37 degrees C. Lancet. 1989 May 6;1(8645):986-8.
211. Shanwell A, Wikman A, Ringden O. Pretransfusion incubation of apheresis platelets at 37 degrees C improves posttransfusion recovery. Transfusion. 1992 Oct;32(8):715-8.
212. Kostelijk EH, Fijnheer R, Nieuwenhuis HK, Gouwerok CW, de Korte D. Soluble P-selectin as parameter for platelet activation during storage. Thromb Haemost. 1996 Dec;76(6):1086-9.
213. Dijkstra-Tiekstra MJ, Pietersz RN, Huijgens PC. Correlation between the extent of platelet activation in platelet concentrates and in vitro and in vivo parameters. Vox Sang. 2004 Nov;87(4):257-63.
214. George JN, Pickett EB, Heinz R. Platelet membrane glycoprotein changes during the preparation and storage of platelet concentrates. Transfusion. 1988 Mar-Apr;28(2):123-6.
215. Pietersz RN, de Korte D, Reesink HW, Dekker WJ, van den Ende A, Loos JA. Storage of whole blood for up to 24 hours at ambient temperature prior to component preparation. Vox Sang. 1989;56(3):145-50.
216. Rinder HM, Snyder EL, Bonan JL, Napychank PA, Malkus H, Smith BR. Activation in stored platelet concentrates: correlation between membrane expression of P-selectin, glycoprotein IIb/IIIa, and beta-thromboglobulin release. Transfusion. 1993 Jan;33(1):25-9.

217. Holme S, Bode A, Heaton WA, Sawyer S. Improved maintenance of platelet in vivo viability during storage when using a synthetic medium with inhibitors. J Lab Clin Med. 1992 Feb;119(2):144-50.
218. Bolin RB, Medina F, Cheney BA. Glycoprotein changes in fresh vs. room temperature-stored platelets and their buoyant density cohorts. J Lab Clin Med. 1981 Oct;98(4):500-10.
219. Lozano ML, Rivera J, Gonzalez-Conejero R, Moraleda JM, Vicente V. Loss of high-affinity thrombin receptors during platelet concentrate storage impairs the reactivity of platelets to thrombin. Transfusion. 1997 Apr;37(4):368-75.
220. Turner CP, Sutherland J, Wadhwa M, Dilger P, Cardigan R. In vitro function of platelet concentrates prepared after filtration of whole blood or buffy coat pools. Vox Sang. 2005 Apr;88(3):164-71.