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Author: Kerkhoffs, Jean-Louis Henri
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Efficacy of platelet transfusions

Jean-Louis H. Kerkhoffs

Promotiereeks HagaZiekenhuis

Het HagaZiekenhuis van Den Haag is trots op medewerkers die fundamentele bijdragen leveren aan de wetenschap en stimuleert hen daartoe. Om die reden biedt het HagaZiekenhuis promovendi de mogelijkheid hun dissertatie te publiceren in een speciale Haga uitgave, die onderdeel is van de promotiereeks van het HagaZiekenhuis. Daarnaast kunnen promovendi in het wetenschapsmagazine HagaScoop van het ziekenhuis aan het woord komen over hun promotieonderzoek.

Efficacy of platelet transfusions

Efficacy of platelet transfusions

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van Doctor

aan de Universiteit Leiden, op gezag van

Rector Magnificus prof. mr. P.F. van der Heijden,

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Co-promotor:	Dr. P.W. Wijermans	HagaZiekenhuis, Den Haag
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	Prof. Dr. S.J. Slichter	University of Washington
		School of Medicine

# Contents

Chapter 1

Introduction

1

1 Introduction

PLATELET TRANSFUSIONS: PRODUCTS AND ENDPOINTS IN RANDOMIZED CONTROLLED TRIALS

Under the assumption that thrombocytopenia correlates with bleeding complications, guidelines advise to institute a platelet transfusion policy to prevent and treat bleeding complications in haemato-oncology patients with thrombocytopenia due to myelosuppressive diseases and/or treatment.[1-4] At the start of the research for this thesis platelet transfusion therapy appeared to be a fact of life and a point of no return had been achieved, underlining the statement by Schiffer in 1992 "unfortunately, it will be scientifically impossible to perform studies 20 years after the horse is out of the barn to prove the value of this approach".[5] Meanwhile clinicians paid only limited attention to the quality of the platelet products as long as they were available on demand. The product we tested in our first randomized controlled trial (RCT), platelets stored in an additive solution, was just one of the last of several modifications since the systematic preventive use of platelets. Studies concerning platelet transfusions conducted between the early eighties until the late nineties mainly investigated product modifications to reduce adverse reactions, such as (non)-immunological refractoriness and febrile transfusion reactions. It was only in the last decade that clinical efficacy became an issue, even more to blood bankers than to clinicians.[6] The modifications which have been performed to improve the safety profile are typically characterised by removing one or more components after blood donation (figure 1). This introduction aims to recapitulate platelet transfusions from a historical perspective and the development of policy guidelines, mainly focussing on the clinical evidence for the several product modifications throughout time.

Figure 1: *Figure 1 shows the several general modifications in the production of platelet products. Not shown is the proces of pooling (possible since the introduction of sterile docking devices in the eighties) leading to multidonor Platelet Rich Plasma v.s. Buffy-coat platelet products.*
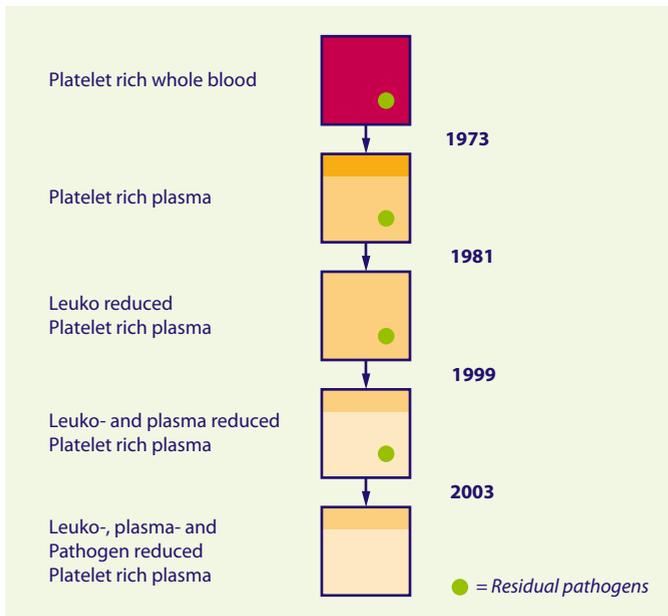
One of the first physicians investigating the fate of transfused platelets and a potential method to treat bleeding was Duke in 1911.[7] Using blood from a polycythemic donor Duke et al determined that donor platelets survived five to six days following transfusion in a young patient probably suffering from congenital thrombocytopenia. In contrast to the transfusion of blood of a normal donor, blood of the polycythemic donor resulted in a longer lasting rise of the platelet count and a reduction in bleeding time (figure 2).[8] A platelet survival study in 22 patients using a direct transfusion of polycythemic blood after a first transfusion using a normal donor to prevent "platelet hunger" suggested a survival of 48 – 72 hours, potentially influenced by active bleeding and an enlarged spleen. Bleeding time was shortened promptly after transfusion, a beneficial effect which lasted approximately 24 hours longer than the platelet survival.[9]

Figure 2: *Figure 2 shows one of the first in vivo experiments studying the survival of transfused platelets in a thrombocytopenic patient. PLT= Platelets*
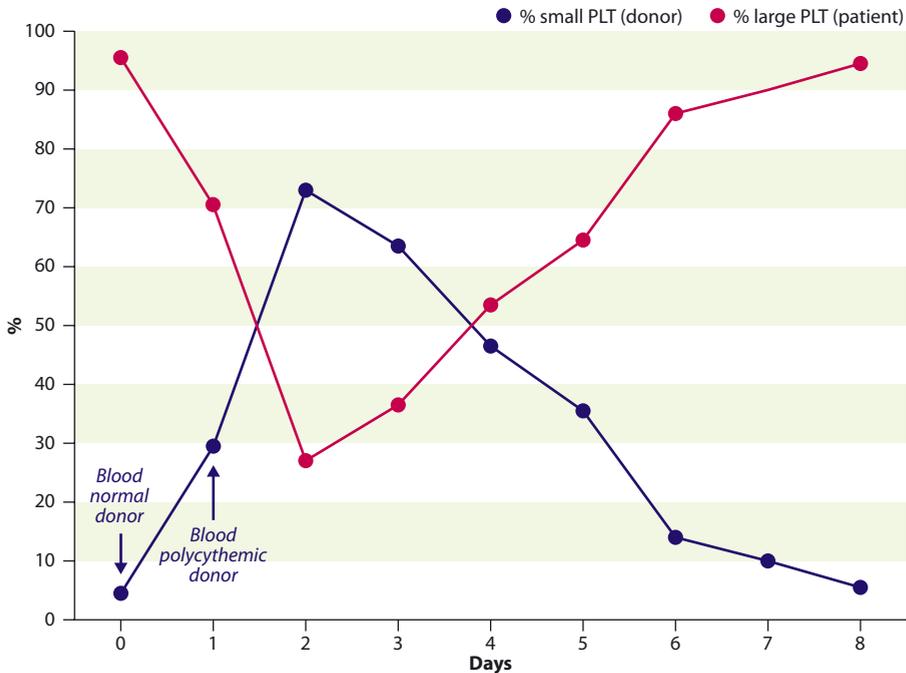
Klein and Freireich et al developed a technique for platelet separation by differential centrifugation to obtain platelets for clinical use and a first study comparing fresh versus banked blood in the management of bleeding was performed.[10, 11] In one of the first reviews platelet transfusions were already considered as a part of the "total care" for patients with malignancies, however the difficulty of an objective evaluation of the "self-apparent" effects of platelet transfusions and the need for controlled studies was also emphasized.[12] A state-of-the-art review concerning the development and quality of platelet products as well as guidance for clinical use stated that the indication for platelet transfusions was haemorrhage associated with severe thrombocytopenia, defined as a platelet count less than 10×10^9/l.[13] Two autopsy studies showed that fatal haemorrhages are frequently encountered in patients with acute leukaemia and two of these studies

reported a dramatic reduction of fatal haemorrhages after the implementation of platelet transfusions.[14, 15] The first prospective landmark study in 92 patients with acute leukaemia by Gaydos et al showed a relationship between the platelet count as well as a decrease in platelet count and the risk of bleeding, although no threshold level could be observed. However, it was after this publication that the concept of prophylactic rather than therapeutic platelet transfusions emerged (figure 3).[16]

Figure 3: *Figure 3 shows the number of patients included in randomised trials studying platelet transfusions. Policy driven RCTs are trials investigating indication (prophylactic versus therapeutic), thresholds and doses. Product related RCTs classically compare an alternative platelet product, for instance using additive solutions, with conventional platelet products, usually platelets stored in plasma*
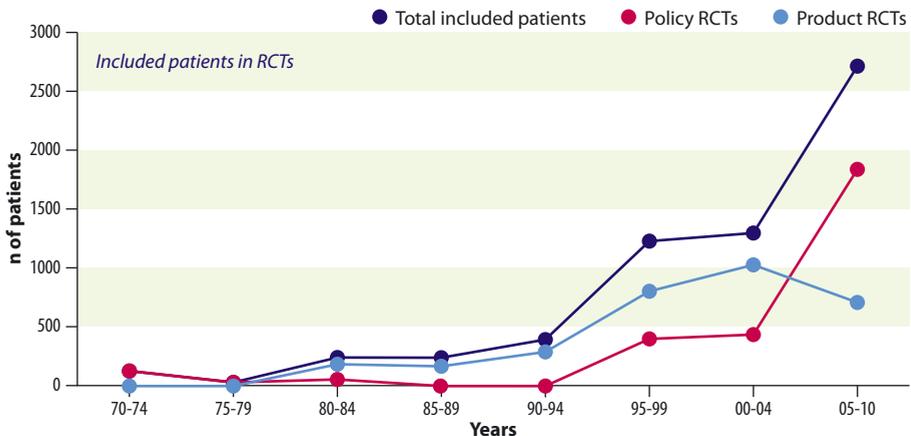
Since this publication, several strategies have been studied in clinical trials investigating the use of prophylactic transfusions as opposed to therapeutic platelet transfusions, platelet transfusion thresholds and platelet transfusion dose. In parallel with these trials the manufacturing of platelet products has constantly evolved with the development of several ways to harvest platelets, sterile docking device, storage bags and the introduction of additive solutions as well as pathogen reduction techniques, driven by emerging blood-borne infections and bacterial growth associated with prolonged storage of platelets at ambient temperature. Thus far 5 RCTs have studied prophylactic platelet transfusions as opposed to therapeutic transfusions.[17-21] Based on the three oldest studies, the combined relative risk for major or more severe bleeding complications was 0.49 (95%CI 0.28 – 0.87) favouring prophylactic platelet transfusions.[17, 19, 23, 22] More recently, a prophylactic versus therapeutic trial in patients with acute myeloid leukaemia reported also that bleeding complications were significantly increased in the therapeutic arm. There were 5 minor cerebral haemorrhagic complications and 2 lethal cerebral haemorrhages in the therapeutic arm as opposed to zero in the prophylactic arm.[21] Four RCTs and six observational studies compared different platelet transfusion thresholds for prophylactic platelet transfusions from as low as 5 x 10^9/l to 30 x 10^9/l.[27-32] Despite the heterogeneity of the studies, none of these studies has shown a difference with regard to bleeding complications, although lowering the platelet transfusion trigger led to a significant reduction in the mean number of platelet transfusions.[22] Before the recent publication of the PLADO and the STOP trial, 4 RCTs and 1 observational study

investigated platelet dose.[33-38] These trials were initiated to investigate the "optimal" platelet dose. Both economical as well as safety issues, i.e. minimizing donor exposure, are the main driving factors to reduce the transfusion threshold as well as the platelet dose. Both the PLADO as well as the STOP have made two important points. Firstly and most importantly, haemorrhagic complications are very frequent despite the use of prophylactic platelet transfusions (the % of patients with WHO ≥ 2 bleeding: PLADO 70%, STOP 50% without differences between the study arms with platelet doses reaching from ± 0.8 x 10^{11}/m^2 (lowest dose in the STOP trial) to 4.4 x 10^{11}/m^2 (highest dose in the PLADO trial) and secondly more transfusions are administered in patients receiving low-dose platelet transfusions.[37, 38] Although prophylactic platelet transfusions likely prevent severe haemorrhages, no definitive quantitative strategy with regard to threshold or doses can be made based on these trials.

In contrast to the straight forward search for an "optimal" platelet transfusion strategy, the approach to develop the "optimal" platelet product evolved more complicated by a diversity of ways to manufacture and store platelet products. Trials studying product modifications use an array of endpoints, including transfusion efficacy, haemostatic efficacy and the incidence of adverse transfusion reactions. Platelets can be prepared using aphaeresis (AP) or a whole-blood method (WBD), of which the platelet-rich plasma (PRP) method is mostly used in the United States, whereas the buffy-coat method (BC) is used in Europe. On top of these different collection and production methods, a large diversity in platelet products arises if we also include a variety of storage bag systems, methods for leukoreduction, gamma irradiation, variations in storage time and several additive solutions, which all potentially affect efficacy. A simple calculation results in over a thousand different currently clinically used platelet products. This diversity resulted in the need for defining which requirements should ideally be met by new platelet products as stated in a draft guidance of the FDA in 1999 (table 1).[39]

Table 1: *FDA Guidance for industry.*

	Test	Subcategory	Type of tests
A	**Paired in vitro studies**	Morphology	Different levels of resolution, including EM
		Biochemical	ATP, glucose, lactate, pH and LDH
		PLT activation	P-selectin (CD62), CD63, fibrinogen binding (PA-Cl), B-thromboglobulin, Plateletfactor 3 and 4
		Physiological respons	HSR, aggregation and serotonin release
		Microparticles	
B	**Platelet survival**	Healthy volunteers	Radiolabeling of autologous platelets with ^{111}Indium and/ or ^{51}Chromium
C	**Clinical haemostatic efficacy**	Thrombocytopenic patients	Transfusion efficacy and haemostatic efficacy

PLT = Platelet; EM = Electron Microscopy; ATP = Adenosine Triphosphate; CD = Cluster of Differentiation;
HSR = Hypotonic Shock Response; FDA = Food and Dug Administration

This manuscript mainly deals with efficacy, although most new platelet products or changes in production are driven by safety and economics. An optimal platelet product should be easy to produce with constant quality parameters, should be stored for preferably longer period than the current 5 – 7 days maintaining this quality, clinically efficient in terms of bleeding prevention and without adverse reactions such as febrile and allergic transfusion reactions, transfusion related acute lung injury (TRALI), infectious

complications and alloimmunization resulting in refractoriness. Moreover, preferably we would have in vitro measures to predict clinical safety and efficacy. However, to date there are no in vitro tests predicting viability in terms of recovery and survival or haemostatic function after transfusion in patients.[40] The suggested performance of recovery / survival studies is hampered by the fact that the use of radiolabeling in volunteers is not operational in many countries, performed studies are small showing huge variation and more important the efficacy in healthy volunteers does not reflect the clinical outcome in thrombocytopenic patients. From 1970 to 2005 thirty-four RCTs have been performed testing one or more platelet product modifications (table 2).

Table 2: *RCTs with different products and/or product modifications up to 2005.* [43-55, 57-65, 67-81]

Type of modification	Endpoints	N trials
Production (PRP, BC, AP)	Alloimmunsation	6
	Adverse TRF reactions	
	Post transfusion recovery	
Leukodepletion I inactivation	Alloimmunisation	11
	Post transfusion recovery	
Plasmareduction	Adverse TRF reactions	6
	Post transfusion recovery	
HLA or ABO matching	Post transfusion recovery	4
	Bleeding	
Preincubation	Post transfusion recovery	2
Storage	Post transfusion recovery	2
Photochemical pathogen reduction	Post transfusion recovery	3
	Bleeding	
Total		34

TRF = Transfusion; Note: all the trials have been counted once, although some trials could have been included in more than one category.

Apart from studies investigating the clinical effect of storage time or production method (PRP, BC, AP), in historical order the following modifications have been subject of study: leukoreduction (centrifugation, filtration and UV irradiation), plasmareduction (concentration, additive solutions) and pathogen reduction (Amotosalen / UVA) (see also figure 1).

The incentives for leukoreduction, by far the most studied topic in platelet product RCTs, were mainly the prevention of alloimmunisation, platelet refractoriness and febrile non-haemolytic transfusion reactions (FNHTR) and CMV transmission. As the use of prophylactic platelet transfusions started to increase exponentially, the induction of HLA-antibodies with refractoriness as a consequence posed a major problem. Observations in mice by Claas et al supported previous clinical observations that the antibody response to platelets was enhanced by contaminating leukocytes acting as professional antigen presenting cells directly stimulating recipient T cells.[41,42] A first non randomised study subsequently showed a significant decrease in the incidence of alloimmunization, from 93% to 24%, in patients receiving platelet concentrates, depleted from leukocytes below 2×10^7 by centrifugation.[42] The first RCT using leukocyte-depleted platelet products was published in 1983. This study failed to show a reduction of alloimmunisation with leukocyte-depleted platelet products using a centrifugation technique, although a trend was noticed in

patients who where previously exposed to HLA antigens through pregnancy and or transfusions.[43] In contrast two other RCTs showed a significant reduction in the rate of HLA-alloimmunisation as well as platelet transfusion refractoriness. In these studies the platelet products were leukodepleted using a filtration method.[44, 45] In a study comparing centrifugation and filtration as methods for leukoreduction van Marwijk Kooy et al showed that filtration was more efficient in reducing HLA-immunization and refractoriness, probably due to a more consistent reduction in leukocytes.[46] Apart from the reduction of alloimmunization, leukoreduction by filtration was also shown to reduce the number of febrile non-haemolytic transfusion reactions (FNHTRs).[47] In the end of the eightees when sterile docking connecting two plastic tubings became possible, in Europe, the method of producing platelet concentrates changed from the PRP-method to the BC-method. After filtration BC derived PCs contain less leukocytes as compared to filtered PRP platelets and Oksanen et al showed that the use of filtered BC derived PCs (pre- as well as post storage alike) as compared to PRP platelets caused significantly fewer and milder adverse reactions.[48] The first study testing UVB irradiation for the leukocyte inactivation showed comparable efficacy in terms of corrected count increments (CCI) but did not show a significant reduction of alloimmunization.[49] The largest trial studying leukoreduction / inactivation is the TRAP trial. Essentially, this study in which 530 alloantibody negative patients with AML were randomised to receive standard non-leukodeleted pooled platelet concentrates, filtered pooled platelet concentrates, UVB treated pooled platelet concentrates or filtered platelets obtained by aphaeresis showed that, regardless the method used to reduce/inactivate leukocytes, leukoreduction resulted in a reduction of alloantibody-mediated refractoriness.[50] A meta-analysis of European studies and the TRAP study confirmed that leukoreduction reduced immunological refractoriness by almost 80%.[51]

In 1994 Heddle et al showed that the supernatant plasma component of stored leukocyte-containing PCs was more likely to cause severe reactions as compared to the platelet concentrates itself, and a strong correlation was observed between the reactions and the concentration of interleukin-1β an interleukin-6.[52] Chalandon et al showed that cytokines arise during storage of leukocyte-containing PCs, supporting that pre storage leukoreduction favours over post storage leukoreduction.[53] Another trial suggesting that plasma removal is more effective in preventing adverse transfusion reactions, despite cytokines accumulate during storage also in additive solutions, gave rise to the development of platelet additive solutions.[54] Up till 2005 only 1 RCT reported on the clinical efficacy of BCs stored in additive solution (AS). This study showed a significantly decreased transfusion efficacy of platelets stored in AS, however AS stored BCs significantly reduced the incidence of adverse transfusion reactions. No comments were made regarding the haemostatic efficacy.[55] Despite the reduced transfusion efficacy the results of this trial as well as similar results in a subanalysis of another trial led to the introduction of this platelet additive solution (PAS) in clinical practice in the Netherlands.[55, 56]

Platelets have to be stored in ambient temperature and small inocula of bacterial contaminants sometimes grow exponentially beyond 4 days. Moreover, emerging new and still occurring known transfusion transmittable infections have led to the development of pathogen reduction (PR) techniques using a photoactive substance in conjunction with UV irradiation. Up till the start of the trials reported in this thesis 3 RCTs investigated the clinical efficacy of platelets treated with amotosalen HCl (S-59) and ultraviolet A light (UVA), at that time point the only clinically available photochemical pathogen reduction technique.[57-59] These trials have shown a reduced transfusion efficacy of PR-treated platelets, but only one of these trials used haemostatic efficacy as a primary endpoint as

proposed by the FDA. Although this trial reported a high but similar proportion of patients with grade 2 or higher bleeding complications, patients supported with amotosalen-UVA treated platelets had a mean of 3.2 days with grade 2 or higher bleeding as opposed to 2.5 days in the control group (p = 0.02) and experienced more refractoriness not explained by lymphocytotoxic antibodies or significantly increased platelet consumption.[58]

Apart from leukoreduction / inactivation to prevent alloimmunization and plasmareduction to reduce adverse transfusion reactions, two items need to be discussed as they were cause for debate and trials the past two decennia: the effect of platelet storage and the clinical effects of the differently collected/produced platelets (Whole blood derived BC or PRP versus single donor AP). The incentive to use single donor aphaeresis platelets (AP) is to reduce the exposure to alloantigens and micro organisms. The first RCTs conducted before leukoreduction of PC, to study the effect on alloimmunisation comparing AP with whole blood derived, multi donor platelets were published in the early eighties. In contrast to a stable recovery of AP transfusions the recovery of multi donor platelet products declined each subsequent transfusion resulting in refractoriness after a mean of nine transfusions and it turned out that alloimmunisation was postponed, but not prevented.[60, 61] The first trial to compare the clinical effectiveness of the three available production techniques was published in 1995 by Bishop et al. The corrected count increment (CCI) after 24 hours was not different between the three products, although the 1-hour CCI was higher with single donor AP.[62] A similar trial published in 1996 did not find a significant difference in the 1- and 20 hour CCI of BC platelets as compared to AP, although the CCI of BC platelets declined with 30% during storage in contrast with AP, which did not show a significant reduction during storage.[63] Although the 1- and 20-hour CCIs of BCs, APs and PRPs did not differ significantly, transfusions with PRPs resulted in more FNHTRs.[64] A recent meta-analysis comparing the efficacy and safety of AP with WBD platelets concluded that AP platelets compared with PRP have better increments post transfusion and comparable increments as compared to BC. There was no difference in the occurrence of alloimmunization and refractoriness. Moreover provided the products were leukoreduced there were no differences in the occurrence of adverse transfusion reactions. More importantly there are no data comparing AP and WBD platelets with regard to haemostatic efficacy.[65] To date there are no RCTs comparing production techniques with regard to bacterial contamination, but in a recently published large non-randomised multicenter study bacterial contamination of pooled whole blood-derived platelets versus AP was studied. In this study the rate of confirmed-positive units was 0.06 and 0.09%, respectively p = n.s.).[66]

In these last three decades of technical advantages in the production of platelets has allowed for an increase in storage from 1 day in the late sixties to as long as 7 days nowadays provided that an adequate screening tool for bacterial contamination is instituted. Up till 2005 only three RCTs were published investigating the effect of storage time on clinical efficacy.[63, 67, 68] In the study of Shanwell et al 39 patients were randomised to receive either fresh single donor AP or APs stored for 2 – 5 days. No difference was shown.[67] In a study of 25 patients Rosenfeld et al studied platelet function as well as efficacy comparing 1-day stored APs with 4-day stored APs and showed an immediate increase in number and function of platelets post transfusion independent of storage time.[68] As compared to single donor APs, although keeping within an acceptable range, BCs showed a larger decrease in post transfusion recovery with increasing storage.[69]

During the design of our first RCT using corrected count increments as a primary endpoint, we used a non-inferiority model accepting a fairly large decrease in transfusion efficacy without really knowing what would be the biological or clinical relevance. The main concept was to convince haematologists in our regional academic centre to shift from plasma stored platelet products to platelets stored in additive solution as these had shown to reduce FNHTRs.[55] The trial used platelets produced with the BC method, were prestorage leukoreduced and patients were transfused at the conventional guidelines with regard to dose and threshold, in compliance with the evidence so far. The trial and its design do reflect the leading question today: should we keep improving safety at the expense of efficacy? Adverse transfusion reactions are rare and difficult to classify.[82] Alloimmunisation keeps occurring at a low level despite leukoreduction, moreover the far more frequent problem of non-immunological refractoriness (that may also be related to reduced product quality) seems to be an increasing issue in need for a solution. Last but not least the risk of transmittable infectious diseases is very low, making randomised studies testing the efficacy of preventive measures virtually impossible. With regard to efficacy as an endpoint for platelet transfusion recent years have shown increasing doubts and problems with the commonly used endpoints: (corrected) count increments and bleeding.

The (corrected) count increment is a ratio correcting for a measure of blood volume and the number of transfused platelets. This method has been challenged by several authors in the field with as main arguments a bias in favour of a preparation technique with fewer platelets combined with a not adequately estimated blood volume and doubts regarding the usefulness of the CCI as a surrogate outcome measure as it does not predict the clinical outcome bleeding.[83, 84] However the CCI is an easy quantitative and objective measure and far more standardized than the more obvious outcome measure bleeding. Apart from the lack of a validated tool for the scoring and grading of bleeding, measurement of bleeding is hampered by difficulties with regard to the methods, persons and timing of the bleeding observation in thrombocytopenic patients.[85, 86]

This thesis encompasses two RCTs encountering many challenges with regard to the studies endpoints but more importantly raises novel and old unsolved questions to be resolved in the near future.

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Chapter 2

In vitro comparison of platelet storage in plasma and in four platelet additive solutions, and the effect of pathogen reduction

Pieter F van der Meer[1], Jean-Louis Kerkhoffs[2], Joyce Curvers[3], John Scharenberg[2], Dirk de Korte[1], Anneke Brand[2], Janny de Wildt-Eggen[4]

[1]Sanquin Blood Bank North West Region, Amsterdam
[2]Sanquin Blood Bank South West Region, Rotterdam
[3]Sanquin Blood Bank South East Region, Nijmegen
[4]Sanquin Blood Bank North East Region, Groningen, The Netherlands

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ABSTRACT

Background

The introduction of platelet (PLT) additive solutions (PASs) and pathogen reduction (PR) technologies possibly allow extension of PLT shelf life. It was our aim to compare in vitro quality of white cell (WBC)-reduced PLT concentrates stored in various PASs with those in plasma during 8 days of storage. Also, the effect of PR was investigated. The study was performed in a nationwide, multicenter study design, where each center tested 4 of the 6 study conditions.

Study design and methods

In paired experiments (n= 12 per center), 20 AB0-identical buffy coats were pooled and divided into 4 products, to which various storage media were added. Plasma was used as reference in all 4 centers. Two centers used InterSol followed by PR (InterSol+PR) and InterSol without PR; other investigated PASs were T-sol, SSP+ and Composol. A rating system was used to judge PLT quality based on CD62P expression, annexin A5 binding and lactate production: a rating of 6 for good quality and 0 for poor quality.

Results

All PLT concentrates fulfilled release criteria (pH37°C>6.6; swirl present) until Day 8. Marked differences were seen for other in vitro parameters, including CD62P expression, which was 28±5; 31±7; and 39±9% for T-sol, Intersol+PR and without PR, respectively, which was significantly higher as the values found for Composol (12±3%), SSP+ (15±5%) and plasma (15±6%). Three in vitro parameters (CD62P, Annexin A5, and lactate concentration) were collapsed into one rating value; PLTs stored in plasma had a rating of 2.8±1.0, which was significantly higher as for PLTs in T-Sol (1.5±0.5), InterSol+PR (1.3±0.6) and without PR (1.7±0.5; all p<0.001 versus plasma). PLTs stored in potassium- and magnesium-containing PASs showed higher ratings as plasma, 4.3±0.5 for Composol and 3.8±0.8 for SSP+ (p<0.05).

Conclusion

PLT concentrates in plasma, SSP+ and Composol scored better using an arbitrary rating system as PLTs stored in T-Sol or InterSol; PR further impaired rating parameters. The applicability of these differences in rating for clinical effects needs a clinical study.

In vitro comparison of platelet storage in plasma and in four platelet additive solutions, and the effect of pathogen reduction

INTRODUCTION

Plasma is still the most widely-used medium for storage of platelet (PLT) concentrates. CPD plasma contains high levels of glucose as nutrient for the PLTs, and citrate to prevent clotting.[1] Other constituents are at near-physiological ranges, with the exception of a very low free calcium level, and a phosphate level approximately 3 times higher as in normal plasma.[2] Initially, a main motivation to investigate the use of PLT additive solutions (PASs) was an increased availability of plasma for fractionation of Factor VIII and other plasma-derived products. However, the use of PASs has more benefits. These include the fact that PASs can be manufactured sterile and pathogen-free, and have a standardized composition in contrast to donor-variations of plasma. Moreover, PLT concentrates in PAS contain less plasma proteins, reducing allergic reactions[3, 4], and have a lower titer of AB0 antibodies, thereby easier allowing AB0-mismatched PLT transfusions.[5] Theoretically, for the same reason of a 3- to 4-fold antibody dilution, PASs could decrease the risk of antibody mediated transfusion related acute lung injury (TRALI).[6, 7] Finally, PASs facilitate some pathogen reduction (PR) technologies that are inhibited by the presence of plasma proteins.[8] PASs can be introduced in currently used, plasma-based processing methods. PASs were initially developed in conjunction with the buffy coat method, where multiple (usually four to six) buffy coats are pooled, and instead of adding one unit of plasma, one bag with about 300 mL of PAS is added to this pool. After low speed centrifugation the PLT-rich supernatant is expressed to a PLT storage container. Due to the lower viscosity[9], PLT yield is generally a little less than when plasma is used. With apheresis, a highly concentrated PLT concentrate is collected which is diluted with PAS to produce a single-donor PLT concentrate that can be stored for 5 to 7 days.[10] With the PLT-rich plasma (PRP) method, the use of PASs is less easy to incorporate, although specific methods have been described.[11] All PASs require some residual plasma to maintain PLT quality and functionality.[12, 13]

Initial developments of PASs were done with PlasmaLyte-A, an infusion fluid licensed for use[14], and still under consideration for platelet storage.[15] One of the most widely used PAS in blood banks is T-sol, often referred to as PAS-II (Fenwal, Mont Saint Guibert, Belgium).[16] This solution is licensed for in vivo infusions in Europe, and it was developed for 5-day storage of PLT concentrates. With introduction of bacterial screening assays extension of the PLT storage time to seven days was allowed, but T-Sol could not always maintain the pH within acceptable limits.[17] Moreover, transfusions of PLTs stored in T-Sol resulted in lower increments compared to PLTs stored in plasma.[3, 4, 18] After T-Sol, a number of PASs have been developed that fulfilled in vitro quality requirements after 7-days PLT storage. Potassium and magnesium were added to some PASs to preserve PLT integrity throughout storage.[19] Also, specific PASs have been developed for PR technologies.[8] We compared a number of these newly marketed solutions, including: T-Sol, Composol-PS (Fresenius HemoCare, Emmer-Compascuum, The Netherlands), SSP+ (MacoPharma, Tourcoing, France) and InterSol (Cerus, Amersfoort, The Netherlands); plasma was used as reference. Because InterSol was specifically intended in combination with PR, we included PLT concentrates in InterSol after inactivation with amotosalen (Cerus, Concord, CA, USA). For this study, PLT concentrates derived from buffy coats were used. This in vitro study was conducted in conjunction with a phase III clinical trial in hemato-oncological patients, investigating the clinical effectiveness and safety of white cell (WBC)-reduced pooled random donor PLT concentrates, stored up to seven days in either PAS with and without PR, or in plasma.[20] For this preparative study many laboratory tests were applied to evaluate the quality of the new storage medium for PLTs in vitro, and run as a paired comparison with PLTs stored in approved containers.[21, 22] The benefits and pitfalls of such comparisons have been outlined before for recovery/survival studies[23]; in line with defining objective acceptance criteria for recovery and survival, we propose a rating system for in vitro PLT studies, which may allow a more objective interpretation of laboratory results.

MATERIALS AND METHODS

All four blood centers in the Netherlands participated in this study, designated center 1,
2, 3 and 4. Each center used their own materials and methods compliant with the Dutch
guidelines for preparation of platelet products, unless indicated otherwise.

Blood collection and processing

Blood was collected in quadruple bag bottom-and-top systems with an inline red cell
filter (from Fresenius HemoCare or from Baxter) on Day 0, and after rapid cooling to room
temperature stored overnight at this temperature. On Day 1, after hard spin centrifugation,
the units were separated into a unit of plasma, a buffy coat and a red cell concentrate using
an automated separation device (Compomat, Fresenius HemoCare, or Optipress II, Baxter).
Each center prepared 4 paired PLT concentrates by pooling 20 buffy coats in
a large container, mixed well, and split in equal parts over four buffy coat pooling sets
(from Terumo (Tokyo, Japan), Fresenius HemoCare, or from Baxter). All connections were
made with a sterile connection device (Terumo). Each center prepared one unit PLT
in plasma (derived from one of the whole blood units used for the buffy coat pool),
and 3 others by adding one container of PAS to the content of the pooling bag according
to the following scheme:

	Center 1	Center 2	Center 3	Center 4
A. plasma	●	●	●	●
B. T-Sol (300 mL)	●	-	-	●
C. Composol (300 mL)	●	●	●	-
D. SSP+ (300 mL)	●	●	-	-
E. InterSol (280 mL)	-	●	●	●
F. InterSol, followed by a PR step	-	-	●	●

The addition of the PASs resulted in a 35/65% ratio for plasma and PAS, respectively.
After soft spin centrifugation (adapted to the use of plasma or PAS) the PLT rich
supernatant was expressed through the WBC-reduction filter to the PLT storage container,
both part of the buffy coat pooling set. The units A through E were placed on a flat bed
shaker at room temperature in a climate-controlled cabinet at 60 strokes per minute.
Units F underwent PR with Amotosalen according to the manufacturer's instructions,
as described elsewhere.[24] These units were transferred to a storage container with a
Compound Adsorption Device (CAD) and placed on the flat bed shaker. On Day 2,
units F were transferred to the final storage containers present in the PR bag system.
At that time, all units A through F were weighed, and sampled for in vitro analysis.
Weighing and sampling was repeated on Day 6 and Day 8.

In vitro analysis

The volume of the units was calculated from the net weight and specific gravity of the
resuspension fluid (1.026 g/mL for plasma, 1.006 g/mL for PAS). The number of PLTs was
counted on a hematology analyzer; residual WBCs were counted by flow cytometry. pH
(measured and reported at 37°C), PO_2, and PCO_2 were measured with a blood gas analyzer,
glucose and lactate were measured on a blood gas analyzer or enzymatically. CD62P
expression[25] and annexin A5 binding[26] were determined with a flow cytometer. Swirl was
judged visually on a scale from 0 to 3. Regular surveys with identical specimens were

performed between the labs to estimate intra- and inter-laboratory agreement (PLTs, WBCs, pH, blood gases). Specific optimizations were done to reduce intra- and inter-laboratory variations for CD62P and annexin A5.[25]

Dutch product specifications required that the units had a volume between 150 and 400 mL, contained >250 x10^9 PLTs/unit in >95% of the units and <1 x10^6 WBCs/unit in >90% of the units. pH$_{37°C}$ should remain between 6.6 and 7.2 throughout storage.[27]

In vitro rating system

For the in vitro rating system we selected assays that could be performed shortly before transfusion of the PLTs, as endpoint measurement and to allow clinical evaluation of the rating system. We included three parameters in this rating system, each of which could play an independent role in predicting in vivo effectiveness of stored PLTs for reasons discussed below. First, we considered CD62P expression. This parameter of activation is, albeit contradicting, linked to PLT clearance[28-32] and there is evidence that a higher CD62P expression causes a faster PLT clearance. Impaired PLT survival in animals was found to be associated with the apoptosis marker annexin A5 binding[33,34] thus warranting inclusion of this parameter in our rating system. The third assay is the lactate concentration. The lactate production rate is considered as a good indicator of mitochondrial quality.[35] However, to calculate a lactate production rate over multiple days of storage, an additional baseline sample has to be taken immediately after production. In routine such sterile sampling before storage is not performed, making this (currently) unsuitable as endpoint measurement. The starting levels between units in plasma and PASs were slightly different (see Results), but the different production rates resulted in very different endpoint lactate concentrations, and so the lactate concentration prior to transfusion can be used as marker for lactate metabolism.

The in vitro outcomes of each of these three parameters were scored from 0 to 2, where 0 points would indicate a poor quality and 2 points a good quality. The combined rating then results in a value between 0 (poor quality) and 6 (excellent quality). For CD62P expression a value of 2 points was arbitrarily attributed to an expression <20%, 1 for 20-30% and 0 points for an expression >30%. For annexin A5, a value of 2 points for a binding <10%, 1 for 10-20% and 0 for all PLT concentrates with a binding >20%. Finally, lactate level >20 mM are known to indicate poor PLT quality [36], and we scored 2 points for a level <10 mM, 1 for concentrations between 10-20 mM and 0 points for a value >20 mM.

Statistical analysis

The results were analyzed with Instat (version 3.06, GraphPad software, San Diego, CA, USA). Results between groups were compared with a repeated measures analysis of variance (ANOVA) followed by a Tukey Kramer post test, or, if data were not normally distributed, with Dunn's post test. Differences between storage days were also compared with a repeated measures ANOVA, followed by Dunnett's test to compare with Day 2 values. A p value <0.05 was considered to indicate a statistically significant difference.

RESULTS

Composition of platelet concentrates

The composition of the PLT concentrates on Day 2 is summarized in Table 1. The volumes of
the PLT concentrates were significantly different amongst the groups, which was caused by
the different volumes of plasma (current routine, approx. 330 mL) or PAS (T-sol, Composol
and SSP+, 300 mL, InterSol, 280 mL) that had been added to the buffy coat pool. Additional
volume loss due to the PR procedure was observed. The number of PLTs per unit differed
significantly amongst the groups. The highest PLT counts were found in PLT concentrates
in plasma, while the lowest were found in PLT concentrates in pathogen reduced InterSol
concentrates. Though some units contained fewer than the required 250 x10^9 PLTs per unit
(see Table 1), still >95% conformed to this requirement; these units were not excluded from
evaluation.

None of the PLT concentrates in PAS contained >1 x10^6 WBCs/unit. On average, more
residual WBCs were seen in the units in plasma. This was caused by one center that
detected more WBCs in the units in plasma, but not in the units in PAS. Overall, 3/47 (6.4%)
of the units in plasma contained >1 x10^6 WBCs per unit, and therefore standard product
requirements were met.

Table 1: *Composition (on Day 2) and storage parameters of PLT concentrates in plasma and
in four different additive solutions (one with additional pathogen reduction), used to compare
storage characteristics (expressed as mean ± SD).*

	Plasma	T-Sol	Composol	SSP+	InterSol	InterSoi+ PR
	A	B	C	D	E	F
n	47	23	23	35	36	24
Volume, mL*	380±30	354±23	367±16	352±16	303±17	275±11
PL Ts, x10^9 †	380±64	373±28	344±52	330±41	319±45	300±47
PL T concentration, x10^9/ml ‡	1.03±0.16	1.06±0.12	0.94±0.11	0.94±0.10	1.06±0.17	1.09±0.16
PL Ts <250x10^9/U	0 (0%)	0 (0%)	0 (0%)	1 (3%)	1 (3%)	3 (13%)
WBCs, 10^6 |	0.23±0.43	0.03±0.06	0.04±0.05	0.06±0.06	0.05±0.05	0.08±0.06
Storage data						
Glucose consumption, mmol/10^{11} PLT/d ||	0.08±0.05	0.08±0.03	0.05±0.01	0.06±0.02	0.09±0.02	0.10±0.02
Lactate production, mmol/10^{11} PLT/d**	0.13±0.04	0.14±0.02	0.10±0.02	0.11±0.03	0.17±0.03	0.18±0.04

* all differences p<0.001 except A vs. CD, C vs. BD: n.s.

† B vs. D, C vs. F: p<0.05; A vs. C, B vs. E: p<0.01; A vs. DEF, B vs. F: p<0.001; all other differences n.s.

‡ B vs. CD, C vs. E: p<0.05; C vs. F, D vs. E: p<0.01; D vs. F: p<0.001; all other differences n.s

| A vs. BD: p<0.05; A vs. E: p<0.01; all other differences n.s.

|| A vs. CD: p<0.05; C vs. EF, D vs. EF: p<0.001; all other differences n.s.

** A vs. C: p<0.05; B vs. D: p<0.01; A vs. EF, C vs. BEF, D vs. EF: p<0.001; all other differences n.s.

Figure 1: *Figure 1 shows the various in vitro parameters of PLT concentrates in plasma and in 4 different additive solutions (one with additional pathogen reduction), stored for up to 8 days (shown as mean±SD; n: see Materials and Methods). Plasma ●, T-sol ●, Composol ●, SSP+ ●, Intersol ●, InterSol with pathogen reduction ●.*
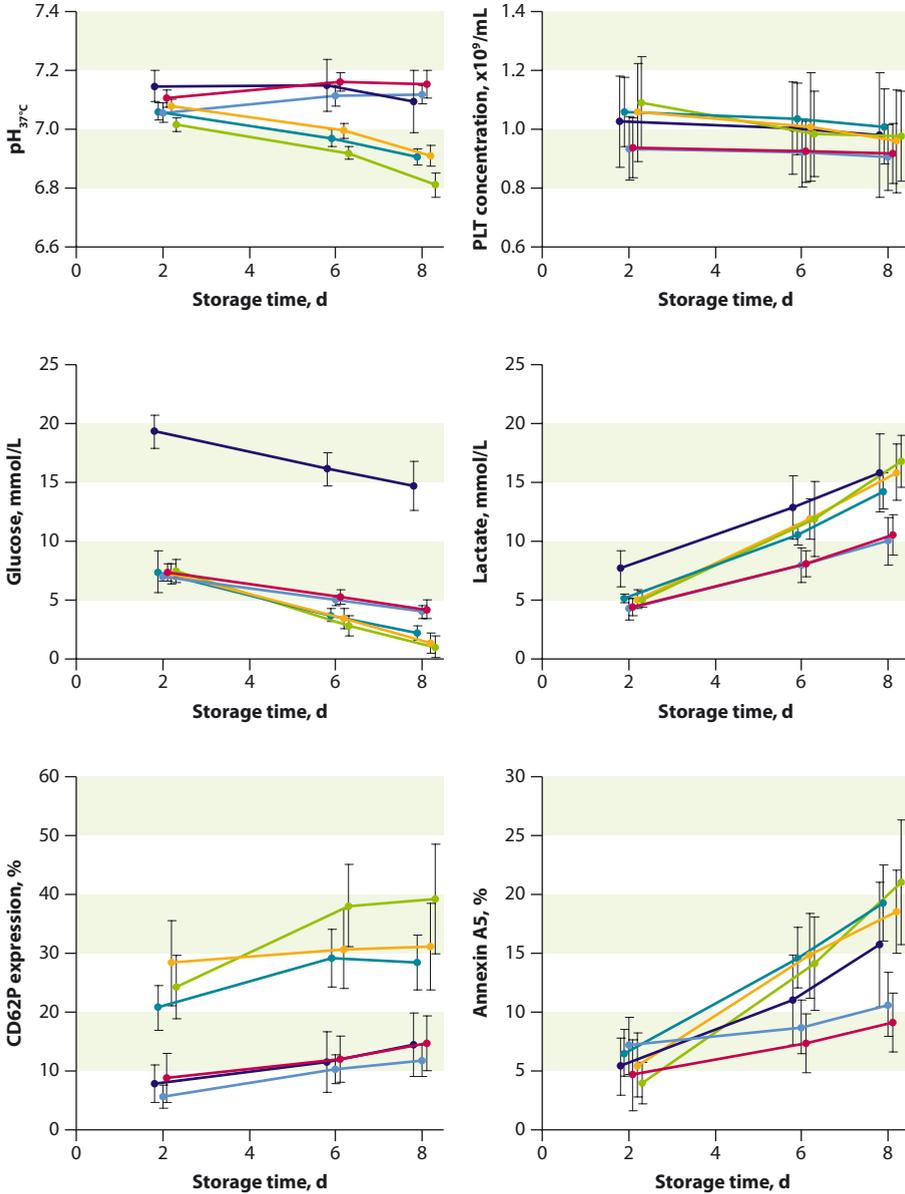
In vitro quality during 8-day storage

The results of various parameters during 8 days of storage are shown in Figure 1. All PLT concentrates were well able to maintain pH37°C >6.6. Plasma and the potassium- and magnesium-containing PAS (Composol and SSP+) showed constant pH throughout storage, while the others showed a decline over time. The PO2 increased during storage under all conditions, plasma having the lowest and InterSol+PR having the highest absolute levels (not shown). PCO2 decreased over time; throughout storage the PCO2 was highest for the units in plasma, while units in InterSol after PR had the lowest levels (not shown). Glucose levels in plasma were considerably higher in the PLT concentrates prepared with CPD-plasma as those made with PAS; all PLT concentrates showed a steady decline of glucose over the storage time.

The PASs with potassium and magnesium, Composol and SSP+, showed the lowest glucose consumption rates and lactate production rates, while InterSol (without and with PR) showed the highest. With respect to PLT activation, shown as CD62P expression, plasma, Composol and SSP+ had much lower expression rates throughout the storage period as the other three PASs tested. The initial PS expression (measured as annexin A5 binding) was similar for all tested conditions, but showed a steady incline for InterSol (without and with PR), T-Sol and for plasma; while PS exposure hardly changed for Composol and SSP+ over the whole storage time. Swirl remained present in all tested units irrespective of the storage solution used.

Rating results

The rating results for the PLT concentrates stored in various solutions are given in Table 2. After overnight storage following PLT processing, on Day 2 the PLT concentrates in plasma, Composol and SSP+ had a similar rating, while PLT stored in T-Sol, InterSol without or with PR had already significantly lower scores. These differences were mainly due to higher CD62P expression in the latter groups. From Day 6 onwards these differences between products became more pronounced, as the lactate concentrations increased more in the T-Sol and InterSol groups. By Day 6, Composol and SSP+ ended with higher scores over plasma, reaching statistical significance by Day 8, mainly as a result from to lactate production and higher annexin A5 expression for PLTs stored in plasma (compare with Figure 1).

Table 2: *Results of a rating system, based on CD62P expression, annexin A5 binding and lactate concentrations during storage. Results are shown as mean±SD.*

	n	Day 2	Day6	Day 8
Plasma	37	5.6± 0.6	3.7±1.0	2.8±1.0
T-Sol	17	4.2±0.6*	2.2±0.4†	1.5±0.5†
Composol	12	5.7±0.5	5.1 ±0.7	4.3±0.5*
SSP+	25	5.4±0.6	5.0± 1.0*	3.8±0.8*
InterSol	31	3.9± 0.6†	2.0± 0.5†	1.7±0.5†
InterSol+PR	24	4.0±0.8†	2.1 ±0.5†	1.3± 0.6†

* p<0.05 as compared with plasma

† p<0.001 as compared with plasma

DISCUSSION

This nationwide, multicenter comparison of PLT storage solutions revealed that all tested solutions maintained in-vitro PLT quality, allowing release to hospitals, for at least 8 days after blood collection: $pH_{37°C}$ was maintained above 6.6, and the swirling phenomenon continued to be visible. PLT concentrates in InterSol, and those that had undergone additional PR also fulfilled these criteria at day 8 with this limited set of requirements, but occasionally had low glucose levels. Though most preparation conformed to Council of Europe guidelines[37], additional assays displayed probably relevant differences in in vitro parameters between the storage solutions, underscoring the FDA instructions[21] for additional assays to be performed when evaluating new processing or storage methods. Unfortunately, the lack of correlation between a particular test result with clinical efficacy remains a significant limitation of most in vitro parameters. In the current study, PLT yield amongst the products differed; units in plasma had higher PLT numbers per unit than those in PASs. We explain this by the lower viscosity of the PASs which hampers to find good centrifugation conditions.[9] PR did not cause a decrease in the number of PLTs (since lower numbers would suggest PLT lysis), the lower PLT numbers in the final product were caused by volume loss alone.

When comparing between the PASs, a clear difference emerged between presence and absence of potassium and magnesium on the in vitro parameters. PLTs stored in PASs without potassium and magnesium (T-sol and InterSol) and had a lower pH, showed higher glucose and lactate metabolism, had a higher CD62P expression and higher annexin A5 binding as compared to solutes containing potassium and magnesium (Composol and SSP+). These results are consistent with other publications.[19, 38]

Glucose consumption and lactate production are stimulated by the presence of phosphate in the storage medium.[36] Consequently, plasma, T-Sol and InterSol showed significantly higher conversion rates as compared with Composol or SSP+. Because of the lower starting value for glucose in InterSol-units, this resulted in depletion of all glucose in part of the units on day 8. Composol contains no phosphate and showed lower conversion rates. Despite the presence of phosphate in SSP+, low conversion rates were seen similarly to those with Composol, indicating that presence of potassium and magnesium in this solution can counteract the effects of phosphate.[39] In T-Sol and in InterSol the higher production rate of lactate resulted in lower pH values during storage.

The units that underwent a PR procedure had lower pH, higher lactate levels, and higher CD62P and annexin A5 binding as control units that had not undergone this procedure suggesting additional PLT activation.

In general, all PLT preparations conformed to the requirements for release, despite detectable differences among other in vitro parameters. Usually, requirements are applied as pass/fail criterion, such as PLT number per pool above or below 250 x10^9 PLTs, pH at the end of storage below 6.6.[27] There is clinical support that some parameters are indeed dichotomous, for example a $pH_{22°C}$ value below 6.2 will result in poor recovery and survival of PLTs[40, 41], while any pH value above that level does not. However, other in vitro measures could have a more gradual effect. We therefore propose a rating system. This rating system is based on three parameters, reflecting different aspects of PLT storage, i.e. activation, apoptosis/cell death, and metabolism: CD62P expression, annexin A5 binding and lactate production. We considered all in vitro measures for inclusion into the rating model, but most were rejected. Blood gases were not included, because the levels are dependent both on PLT quality and on gas permeability of the storage container. For example, low CO_2 levels can indicate poor PLT quality, where little or no CO_2 is produced, but can also indicate that the gas permeability of the container is very high. The same applies for oxygen levels. As indicated earlier, pH is a dichotomous parameter, and thus not suitable for our rating. Furthermore, with current-generation PLT storage containers,

pH values <6.2 are rarely seen. As bicarbonate levels in blood are directly related to pH and CO_2, this parameter was also not included. For PLT storage, it is important that glucose becomes not depleted, and thus we considered glucose informative for inclusion in the rating. However, only levels <1 mM would indicate poor PLT quality[39], while any level above that is not indicative as quality marker. Moreover, as glucose is normally converted into lactate in a 1:2 ratio (as was the case in the current study), and lactate was already included, we decided not to include glucose. Swirl is believed to be a good predictor of PLT quality[32], and therefore in Dutch guidelines all PLT concentrates are checked for presence of swirl at the time of issue. We would therefore never find a PLT concentrate without swirl being transfused and thus it is not a useful marker in the PLT rating. Finally, hypotonic shock response shows good correlation with in vivo recovery and survival.[2] In the current study, two centers included HSR but we found large differences in absolute values, and therefore, before being included in our proposed rating system, further standardization of the test is necessary.

In our rating system all study groups were compared with PLT stored in plasma. As proposed by AuBuchon et al.[23], this gold standard was used to circumvent a "downward creep" when methods were compared amongst each other for recovery/survival studies. Validation of our proposed rating system against clinical outcomes is necessary, and this validation should indicate whether a trichotomous distribution is a good indicator of clinical efficacy, or that a continuous distribution is feasible. At this time, our rating system is based on conjecture and assumptions, and includes only a limited number of in vitro tests. It is intended as a starting point for discussing a combination of parameters, that are collapsed into one composite outcome. So far, there is no "ultimate in vitro test" for predicting in vivo recovery, survival and functionality, but possibly a combination of in vitro tests, as summarized in our rating system, may provide such a helpful tool.

In summary, this study shows that PLTs in plasma or in 4 different PASs, of which one included a PR step, all conformed to release requirements after 7 days of storage; additional biochemical and functional measurements do demonstrate differences amongst PLT preparations. The results were reproducible and comparable amongst 4 different blood centers. A rating system is proposed to incorporate additional in vitro measures to judge PLT quality with the aim to predict in vitro quality. This rating system summarizes multiple (activation, apoptosis/cell death, and metabolism) PLT storage characteristics into a single score, and facilitated interpretation of an otherwise complex study. For example, the outcome of the rating clearly demonstrated the benefit potassium and magnesium in the PAS. On the other hand, a number of questions remain: there is some evidence that the chosen parameters for the rating are related to platelet survival, recovery and functionality, but no formal prospective evaluation has taken place so far. Also, based on that evidence we postulate that there is a relation between the rating score and PLT recovery, survival and effectiveness, but we have to provide data to support this hypothesis. Therefore, validation studies for this rating system will be initiated; until that time, the rating system should be considered as a proposal that needs further support from clinical studies.

ACKNOWLEDGEMENTS

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Chapter 3

A Multicenter Randomized Study of the Efficacy of Transfusions with Platelets stored in Platelet Additive Solution II versus Plasma

Jean-Louis H Kerkhoffs[1,4], Jeroen C Eikenboom[2],
Martin S Schipperus[4], Rinie J van Wordragen-Vlaswinkel[1],
Ronald Brand[3], Mark S Harvey[5], Rene R.P. de Vries[5],
Renée Barge[2], Dick J van Rhenen[1], Anneke Brand[1,5]

[1]Sanquin Blood Bank South West Region, Rotterdam
[2]Department of Hematology, Leiden University Medical Centre
[3]Department of Medical Statistics, Leiden University Medical Centre
[4]Department of Hematology, HagaZiekenhuis, The Hague
[5]Department of Immunohematology and Blood Transfusion, Leiden University Medical Centre
The Netherlands

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ABSTRACT

Randomized studies testing the clinical efficacy of platelet additive solutions (PASs) for storage of platelets are scarce and often biased by patient selection. We conducted a multicenter, randomized study to investigate clinical efficacy of platelets stored in PAS II versus plasma, also including patients with clinical complications associated with increased platelet consumption. A total number of 168 evaluable patients received pooled buffy coat derived platelet concentrates (PC) suspended in either plasma (n = 354) or PAS II (n = 411), stored up to 5 days. Both univariate as well as multivariate analysis showed a significant effect of used storage medium in regard to 1- and 24 hour count increments and corrected count increments, in favour of plasma PCs. However, there were no significant differences between the groups regarding bleeding complications and transfusion interval. Adverse transfusion reactions occurred significantly less after transfusions with PAS II PCs (p = 0.04). Multivariate analysis showed no significant effect of the used storage medium on the incidence of 1- and 24-hour transfusion failure. We showed safety and efficacy of PAS II PCs in intensively treated patients, however plasma PCs show superior increments.

INTRODUCTION

The use of platelet concentrates (PC) for the prevention and treatment of bleeding complications in patients with thrombocytopenia, due to cytotoxic therapy or malignancies of the bone marrow, is generally accepted. Despite the use of prophylactic platelet transfusions, bleeding is a frequent complication and recommendations regarding the preferred transfusion regimen, the quantity and quality of transfused platelets and strategies to monitor efficacy differ and only a minority is evidence based.[1, 2]
In recent decades storage of platelets suspended in non-plasma media (additive solutions) evolved as a growing field of interest. Possible advantages of using additive solutions instead of plasma are an increase of plasma available for plasma products, a reduction of plasma related adverse reactions, improvement of storage conditions in order to increase the shelf-life of PCs and allowing photochemical pathogen reduction techniques.
In the Netherlands the National Blood Supply aims for harmonization of blood products used throughout the country. Currently, except when selected donors are required, all platelet products are prepared using the buffy coat (BC) method. On historical grounds two platelet products are used: Plasma stored platelet concentrates (Plasma PC) and platelet concentrates stored in Platelet Additive Solution II (PAS II PC, Trombosol, Baxter). However, there are no informative studies for a strong selection for one of these products. Although in vitro studies showed significant differences suggesting inferior quality in metabolic, functional and flowcytometric parameters in platelets stored in PAS-II as compared to plasma, platelets stored up to five days in PAS II stay within the range of minimal quality requirements.[3-6] The correlation of these in vitro parameters with clinical efficacy is inconsistent.[7-9] One paired radiolabelled platelet survival study showed a significant decrease in both recovery as well as survival of PAS II PCs compared to plasma PCs and PCs stored in PlasmaLyte A.[10] Data regarding the clinical transfusion response of platelets stored in PAS II are also limited. A small, non-randomized clinical trial did not show a significant difference between PAS II PCs and plasma PCs, and one small prospective, randomized study reported that corrected count increments (CCIs) after transfusion with PAS II PCs were significantly lower.[11, 12] Despite lower CCIs bleeding complications did not differ and the latter study reported a significant reduction in transfusion reactions.[11] Observational analysis of the transfusion response of PAS II PCs and plasma PCs, used in the control arm of a randomized trial evaluating pathogen inactivated platelets (EuroSPRITE), did not show significant differences.[13, 14] Major drawback of these studies was the exclusion of patients with clinical factors known to increase platelet consumption.[12-14] Because several studies show the importance of patient related factors in relation to platelet transfusion response we performed a randomized, controlled, double-blinded study to evaluate the therapeutic efficacy and safety of PAS II PCs in a non-selected patient population.[15-19]

PATIENTS AND METHODS

Patients and study design

The study protocol was approved by the hospital ethics committees and conducted according to the Guidelines of Good Clinical Practice. All patients > 18 year, who needed or were expected to need more than 2 platelet transfusions, were eligible. After informed consent patients were randomized to receive PAS II PCs or plasma PCs. Patients with HLA- and/or HPA-alloantibodies, active immune thrombocytopenia or an indication for CMV-negative blood products (CMV negative patients receiving stem cells of CMV negative, unrelated or HLA-mismatched donors) were excluded. Patients were enrolled at the Hematology departments of two hospitals. Inclusion was restricted to a period of maximal 30 days after the first PC transfusion or a maximum of 8 PC transfusions, whichever occurred first. The inclusion period ended in case of informed consent withdrawal, the occurrence of immunological refractoriness, after request of the patient or the treating physician, or in case of reaching 30 days after the first PC or 8 PC transfusions. In case of the latter two a second randomization was allowed. After randomization age, gender, length, weight, diagnosis, intended treatment, existence of an enlarged spleen (by physical exam and/or imaging techniques), medical history, transfusion history and medication were recorded. Blood samples were tested for ABO-RhD blood group, irregular red blood cell antibodies, hemoglobin, hematocrite, white blood count, platelets, HLA- and HPA-alloantibodies and anti-platelet autoantibodies. During the inclusion period platelet and red cell transfusions, transfusion-related adverse reactions (skin reactions, fever > 2˚C, dyspnoea, hypotension), bleeding complications, mucosal damage, fever, infections, and used medication were recorded. Bleeding complications were graded according to the World Health Organization criteria and mucosal damage was graded according to the Common Toxicity Criteria (version 2.0).[20, 21] Both parameters were reviewed on a daily basis. Infections were scored positive in case of positive cultures or if a focus was likely as shown by radiologic examination.

Platelet concentrates (PCs)

PCs were prepared from five-pooled whole blood BCs with the same ABO blood group.[22, 23] After collection of a unit of whole blood, BCs were prepared through high-speed centrifugation. Five BCs together with one unit of PAS II or one unit of plasma from one of the BC donors were coupled to a BC pool set (containing a transfer bag, leukocyte filter and a PL-2410 storage container, Baxter) through a sterile connection device. After connecting, the five BCs were pooled in the transfer bag together with the unit of PAS II or plasma. A low speed differential centrifugation was used to separate the platelet rich supernatant from erythrocytes and leucocytes, which subsequently was pressed through the leukocyte-filter into the storage container. A sample was obtained prior to storage to measure platelet content, pH and bacterial culture. The platelet content was measured using a Beckman Coulter Act-10 (Coulter Corp., Miami Florida, USA). The PCs were stored at 20-24˚C on a flatbed shaker up to 5 days. The PCs were γ irradiated with 25 Gy at time of issue in case of specific patient requirements for the prevention of transfusion-associated graft-versus-host disease.

Platelet transfusions and monitoring

The treating physician ordered platelet transfusions according to local hospital guidelines. In general, indications were divided in prophylactic trigger-based transfusions, prophylaxis prior to an intervention or treatment of bleeding complications. The transfusion trigger for uncomplicated prophylaxis was $\leq 10 \times 10^9$/l. In case of serious infections, anti-coagulant medication or administration of anti-thymocyte globulin (ATG) a trigger of $\leq 30 \times 10^9$/l was used. In case of surgical interventions or bleeding complications a platelet trigger of $\geq 50 \times 10^9$/l was used. Pre transfusion platelet count was measured one hour prior to transfusion. Platelet counts were measured from 10 minutes to 2 hours after transfusion and from 16 to 24 hours after transfusion to determine the 1- and 24-hour increment, respectively. Platelet counts in the participating hospitals were measured using a Sysmex XE-2100 (Sysmex Corp., Kobe, Japan). In case of a second PC transfusion within 4 hours, both transfusions were considered to be part of one transfusion. If the 24-hour increment exceeded the 1-hour increment, combined with other signs of haematopoietic recovery, then the 24-hour increment value was excluded from analysis. PC transfusion failure was defined as a 1-hour CCI < 7.5 and/or a 24-hour CCI < 4.5. Patients experiencing repeated episodes (≥ 2 subsequent PC transfusions) of PC transfusion failure, without an apparent non-immunological cause were tested for the existence of HLA- and/or HPA-antibodies. If available, PCs of ABO-identical donors were used, although both minor ABO incompatible (i.e. potential donor anti-A and/or B antibodies directed to the platelets/red cells of the patient) and major ABO incompatible PCs (i.e. potential patient anti-A and/or B antibodies directed to the donor platelets) were not excluded.

Study endpoints

The primary endpoints of the study protocol were the 1- and 24-hour CCI, calculated as follows: $CCI_{1/24h} = [($post transfusion count $(x\ 10^9$/l$)_{1/24h}$ – pre transfusion count $(x\ 10^9$/l$))$ x Body surface area (m^2)]/ Platelet dose (x 10^{11}). Secondary endpoints were transfusion interval, transfusion-related adverse reactions and bleeding complications.
The transfusion interval was defined as the calculated administrative time of two consecutive PC transfusions.

Statistical methods

The study was designed as a two-armed non-inferiority study. The sample size calculation was based on data of patients enrolled in the two randomized trials concerning non-plasma storage media.[12, 13] The standard deviation of the mean 1- and 24-hour CCI was estimated as 6.0. To detect a difference of 30% between the 1- and 24-hour CCI of PAS II PCs and plasma PCs using a 0.05 level 2-sided test, a sample size of 360 transfusions in each study arm provided a power of 90%. The statistical comparison of the CCIs of the two products was performed both as independent transfusion events as well as in a mixed linear model, assuming biological interdependence of consecutive PC transfusions in a patient (SPSS/PC+, Chicago, IL). Fisher-exact tests were used to compare patient characteristics. A multivariate analysis testing the effects on both count increments and CCIs as well as the occurrence of transfusion failure was performed including storage time, storage medium, gender, age, bodyweight, body surface area, diagnosis, therapy, history of prior platelet transfusions, fever (body temperature > 38˚C) at the time of transfusion, infection, splenomegaly and ATG, using a random effects logistic regression model (EGRET).

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RESULTS

Patient population

Between October 2003 and April 2005, 195 patients were randomized (plasma PC n = 95; PAS II PC n = 100). A total of 11 patients were excluded (plasma PC n = 7; PAS II PC n = 4) of which 6 patients had HLA-alloantibodies and 1 patient had HPA-alloantibodies prior to the first transfusion, 1 patient developed refractoriness with proven HLA-alloantibodies after the second transfusion, 2 patients acquired an indication for CMV-negative blood products and 1 patient was transferred to another department. Although patients were randomized based on expected platelet transfusions, 16 patients did not receive any platelet transfusion during the inclusion period (plasma PC n = 4, PAS II PC n = 12), resulting in 168 patients, in which platelet transfusions could be evaluated (plasma PC n = 84; PAS II PC n = 84). There were no significant differences between the two study arms (i.e. patients with evaluable transfusions) regarding demographic characteristics, diagnosis, treatment and transfusion history (table 1). The same applied for the excluded, non-transfused patients in both groups. Splenomegaly was present in 17 patients (10.1%). The mean time on study for patients receiving PAS II PCs and plasma PCs was 20.7 +/- 7.1 and 21.5 +/- 8.6 days (p = 0.54), respectively. Twenty-one patients were randomized more than once (plasma PC n = 11; PAS II PC n = 10). There were no significant differences in the occurrence of febrile episodes, proven infections and mucosal damage, most often localised to the digestive system (painful oral lesions and diarrhoea).

Table 1: *Patient characteristics.*

		Plasma PC	PAS II PC	p-value
		(n = 84)	(n = 84)	
	Male / female	53/31	56/28	0.85
	Age (Years ± sd)	51.4 ± 13.1	50.1 ± 14.6	0.54
	Body surface area (m² ± sd)	1.94 ± 0.22	1.92 ± 0.24	0.57
	Enlarged spleen	6 (7.1)[1]	11(13.1)	0.31
Diagnosis	AML / MDS	43(51)	44(52)	1.00
	ALL	7(8.3)	5(5.9)	0.77
	CML	5(5.9)	3(3.6)	0.72
	CLL	1(1.2)	3(3.6)	0.62
	Myeloma	14(17)	6(7.1)	0.09
	NHL	13(15)	21(25)	0.18
	Other	1(1.2)	2(2.4)	1.00
Therapy	Remission Induction	31(37)	31(37)	1.13
	Consolidation	9(11)	12(14)	0.64
	Allogenic transplant	18(21)	20(24)	0.58
	Autologous transplant	23(27)	19(23)	0.59
	TBI	17(20)	14(17)	0.69
	ATG	5(5.9)	7(8.3)	0.77
	Other	3(3.6)	2(2.4)	1.00
Transfusion history	RBCs[2]	66(79)	62(74)	0.59
	PCs	58(69)	52(62)	0.42
	Transplants	10(12)	5(5.9)	0.28

[1]*Number of patients (percentage of patients in study arm.* [2]*RBCs = red blood cell concentrates.*

Platelet transfusions: product parameters and increments

A total number of 765 PCs were transfused (plasma PC n = 354; PAS II PC n = 411). A total of 684 PC transfusions could be evaluated (89%; Plasma PC n = 311, PAS II PC n = 373). In the PAS II PC group the 1-hour and 24-hour CCI could be calculated in 337 (90%) and 334 (90%) transfusions, respectively. In the plasma PC group this was the case in 274 (88%) and 282 (91%) transfusions. The missing CCIs were a result of missing data regarding pre count, 1- and/or 24-hour post count.
In table 2 product parameters, dosage, count increments and CCIs are shown.
Although the mean platelet content of PAS II PCs was significantly lower than of plasma PCs, there was no significant difference in the mean dose per kilogram body weight per transfusion between the two groups. There was a significant difference regarding the pH. However, all products had a pH well above 6.8. Univariate analysis, assuming each platelet transfusion as independent event, showed a mean difference in 1-hour and 24-hour CCI between plasma PCs and PAS II PCs off 19.7% (95% CI 11.7 – 27.2%, p < 0.0001) and 17.8% (95% CI 5.9 – 31%, p = 0.004), respectively. We also analysed the CCIs in a mixed linear model for biological interdependence of consecutive PC transfusions (data shown in table 2). This analysis resulted in the same mean difference in 1-hour and 24-hour CCI between plasma PCs and PAS II PCs, but confidence intervals and p-values differed. In respect to the difference in 1-hour CCI the analysis showed a 95% CI between 6.5 and 32.9% (p = 0.004), and the difference in 24-hour CCI resulted in a 95% CI between –2.4 and 38.1% (p = 0.09). A multivariate analysis as described in the methods section showed an independent effect of the used storage medium with regard to both count increments and CCIs. Plasma PCs and PAS II PCs resulted in a sufficient 1-hour CCI in 81.3% and 69.1% respectively (p < 0.0001). The 24-hour CCI was sufficient in 70.7% and 65.7% (p = 0.16). Considering the difference in platelet content of plasma PCs and PAS II PCs, we also performed a linear regression analysis of count increments and platelet dose confirming significant lower 1- and 24-hour count increments after transfusion of PAS II PCs (data not shown). Gamma irradiation had no significant effect on the transfusion responses of both PCs.

Table 2: *Platelet product parameters, dosage and transfusion response.*

		Plasma PC	**PAS II PC**	**p-value**
		(n = 311)	(n = 373)	
Numbers of platelets/product	10^9 ± sd	412 ± 93	391 ± 119	p = 0.01
Storage time	days ± sd	3.5 ± 1.3	3.5 ± 1.1	n.s.
pH	± sd	7.12 ± 0.04	7.08 ± 0.04	p < 0.0001
Product volume	ml ± sd	356 ± 19	316 ± 11	p < 0.0001
Precount	10^9/l ± sd	13.3 ± 8.7	13.7 ± 10.5	n.s.
Platelet dose/kg body weight[1]	10^9/l ± sd	5.5 ± 1.7	5.3 ± 2.0	n.s.
Transfusion response[2]				
1-hour		n = 274	n = 337	
CI		32.2 ± 17.1	24.6 ± 14.8	p = 0.001
CCI		13.9 ± 7.0	11.2 ± 6.4	p = 0.004
24-hour		n = 282	n = 334	
CI		20.6 ± 16.0	16.3 ± 14.1	p = 0.028
CCI		8.4 ± 6.9	6.8 ± 6.4	p = 0.09

n = number of transfusions. [1]Per transfusion. [2]General linear mixed model acounting for within-patient-correlation of observations (repeated measurements).

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Bleeding complications, transfusion reactions and transfusion interval

The overall incidence of bleeding complications was 32.1%, consisting of 16.1% grade I, 14.3% grade II and 1.8% grade III. Grade IV bleeding was not observed. There were no differences between the two study groups. As a surrogate marker for bleeding we also calculated the mean transfused red cell concentrates per patient, no difference was observed.

A total number of 26 mild transfusion reactions were observed in 21 patients. Of these, 17 (5.5%) transfusion reactions were related to plasma PCs and 9 (2.4%) related to PAS II PCs (p = 0.04). Eight patients receiving PAS II PCs experienced transfusion reactions versus thirteen receiving plasma PCs (p = 0.35). One patient, receiving plasma PCs, complicated with repeated dyspnoea and wheezing, decided to end the study protocol and was further treated with plasma reduced hyper concentrated platelet products.

Table 3 shows an overview of platelet and red cell transfusions and the calculated transfusion interval. There were no significant differences with regard to transfused PCs, interval and required red cell transfusions per patient. However, the platelet transfusion interval is substantially determined by timing of blood sampling and varying (logistic) delays in PC administration after reaching a transfusion trigger.

Table 3: *Platelet transfusions, red cell transfusions and transfusion interval.*

	Plasma PC	**PAS II PC**	**p-value**
	(n = 84)	(n = 84)	
Number of RBC transfusions	452	475	
Mean RBC /patient (± sd)	4.8 ± 4.1	5.1 ± 3.8	0.62
Number of PC transfusions	354	411	
PC transfusion interval (days ± sd)	2.0 ± 1.0	2.1 ± 1.0	0.52
Mean PC/patient (± sd)	4.2 ± 2.7	4.9 ± 2.8	0.10
Cumulative platelet dose/kg (x 10^{11}/kg ± sd)	0.22 ± 0.15	0.23 ± 0.16	0.68

Effects of storage

Storage time had a significant effect on the transfusion response of both PCs (figure 1). Stored PAS II PCs as well as stored plasma PCs showed a decrease in 1-hour CCI compared to fresh PCs. For both PCs this difference became significant after two days of storage. Stored plasma PCs show a gradual decrease in 24-hour CCI, however no significance is reached compared to fresh PCs. The same is true for PAS II PCs, although after 4 days of storage the deterioration was significant (p = 0.02), showing that the effects of storage time were more pronounced in PAS II PCs.

Figure 1: *Figure 1 A and B shows the comparison of 1- and 24-hour CCI,respectively, related to storage time of plasma PCs and PAS II PCs. Both PCs show a significant decrease in 1- and 24-hour CCI during storage. Figure 2 A showes a significant difference between plasma PCs and PAS II PCs after 3 days of storage. Figure 2 B showes a significant difference beween the two products after 5 days of storage. SEM = Standard Error of the Mean; n = number of transfusions.*

A	day 1/2	day 3	day 4	day 5
● Plasma (mean)	15,6	13,2	14,4	13,2
● PAS II (mean)	13,8	11,3	10,0	9,8
p-value	0,11	0,07	0,0001	0,0001
● Plasma (n)	71	66	55	79
● PAS II (n)	83	76	84	87

storage time (days)

B	day 1/2	day 3	day 4	day 5
● Plasma (mean)	9,9	7,8	7,9	8,2
● PAS II (mean)	8,3	7,8	5,9	6,4
p-value	0,14	0,96	0,055	0,030
● Plasma (n)	73	66	53	87
● PAS II (n)	82	73	81	87

storage time (days)
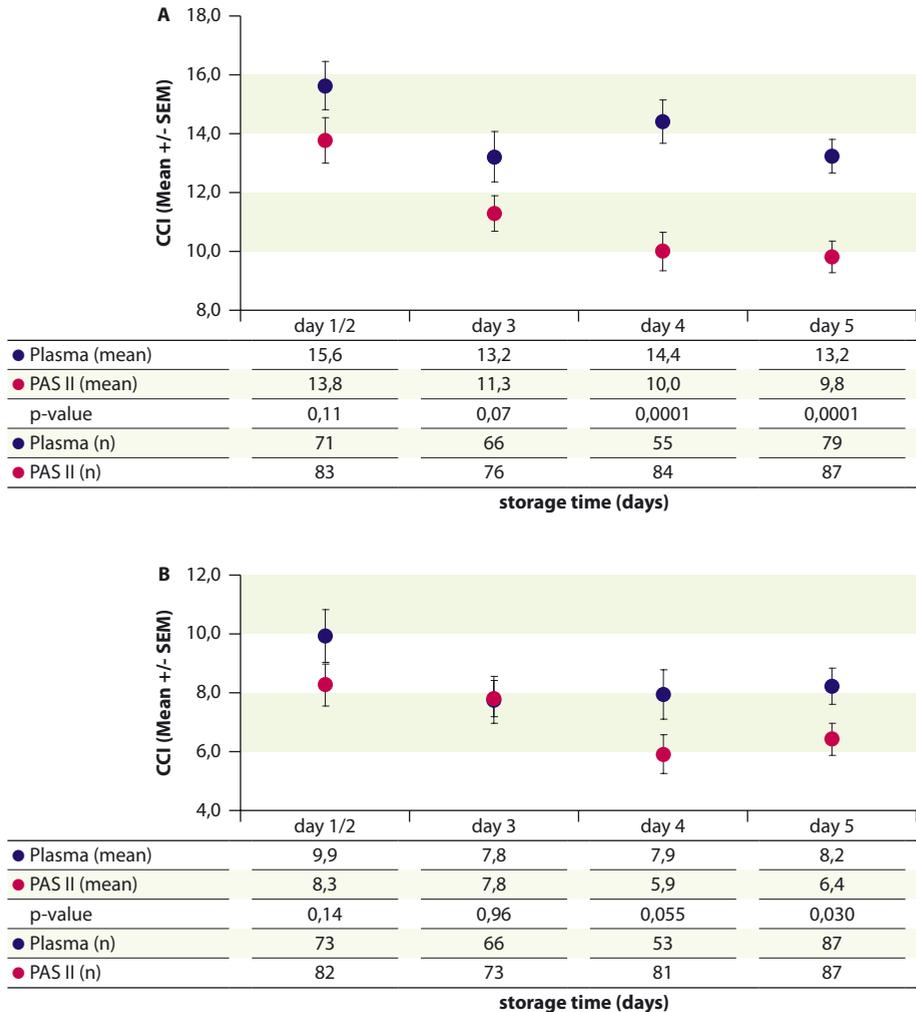
Effect factors of increased platelet consumption

Refractoriness, both immunological as well as non-immunological, remains an important clinical problem. In our study 34.5% of patients experienced one or more transfusions with a 1-hour CCI < 7.5, while 25% of all transfusions resulted in a 1-hour CCI < 7.5%. Of all transfusions 25 were major ABO incompatible (plasma PC n = 12, PAS II PCs n = 13), mostly patients with blood group O receiving a blood group A product. A 1-hour transfusion failure after two or more subsequent transfusions occurred in 34 patients (plasma PC n = 14, PAS II PC n = 20), in 7 patients (plasma PC n = 4, PAS II PC n = 3) not explained by obvious non-immunological factors. Testing these patients revealed only one patient with HLA-antibodies and none with HPA-antibodies. Two patients, without detectable HLA-antibodies, received a HLA-matched test transfusion, both without success.

A number of non-immunological factors have been associated with an increase in platelet consumption. Most patients with haematological malignancies experience complex clinical conditions and in our study only 25% of the transfusions were administered in the absence of factors known to increase platelet consumption. A multivariate analysis to evaluate transfusion efficacy in terms of 1- and 24-hour transfusion failure is shown in table 4. Factors independently influencing 1-hour transfusion failure were splenomegaly, ATG, fever and infection. Storage time showed a trend towards an effect, but the used storage medium did not significantly influence the occurrence of 1-hour transfusion failure. The 24-hour transfusion failure was determined by splenomegaly, ATG, fever and the age of the patient significantly contributed to the occurrence of 24-hour transfusion failure, whereas both storage time and used medium dissappeared as independent factors.

Table 4: *Multivariate analysis[1] of 1- and 24-hour transfusion failure.*

	Odds ratio 1-hour CCI < 7.5 (95% CI)	p	Odds ratio 24-hour CCI < 4.5 (95% CI)	p
Storage time	1.93 (0.95 – 3.93)	0.069	1.51 (0.82 – 2.79)	0.18
Storage medium	0.60 (0.25 – 1.42)	0.25	0.72 (0.30 – 1.69	0.46
Fever	1.41 (0.97 – 2.04)	0.071	1.88(1.33 – 2.66)	< 0.001
Infection	0.38 (0.17 – 0.84)	0.02	1.08 (0.57 – 2.05)	1.08
Enlarged spleen	26.7 (8.13 – 87.7)	< 0.001	7.55(2.35 – 24.2)	< 0.001
ATG	39.6 (7.81 – 201)	< 0.001	4.83 (1.14 – 20.5)	0.03
Age	*1.01 (0.98 – 1.04)*	0.47	1.04 (1.01 – 1.07)	0.023
Gender	0.59 (0.18 – 1.93)	0.39	0.71 (0.21 – 2.42)	0.58
Diagnosis	0.71 (0.71 – 1.31)	0.96	1.09 (0.82 – 1.43)	0.56
Therapy	1.16 (0.85 – 1.58)	0.36	1.06 (0.79 – 1.44)	0.68
Transfusion history	1.10 (0.41 – 2.90)	0.85	0.68 (0.46 – 3.35)	0.68
Bodyweight	0.97 (0.86 – 1.11)	0.69	0.90 (0.79 – 1.04)	1.04

[1]*Random effects binary logistic model for distinguisable data (odds ratios and p-values are corrected for within-patient-correlation of observations).*

DISCUSSION

With the intention to harmonise platelet products in the Netherlands and in anticipation to future product changes, we performed a randomised controlled trial comparing plasma PCs and PAS II PCs. With the exception of immunological refractoriness due to HLA- and HPA-antibodies no exclusion criteria regarding factors of increased platelet consumption were used. There is general agreement that changes in platelet products should be validated for their clinical quality. Because major bleeding complications are rare, platelet count increments and CCIs have been accepted as surrogate endpoints.[24] A draft guidance for testing and evaluating platelet components advises an array of in vitro tests, the use of in-vivo autologous radio labelled platelet survival studies and clinical trials, including haemostatic efficacy.[25] Currently, in Europe the requirements defined for quality control of platelet transfusion are minimal. In our study swirl, pH and platelet content were determined as in vitro parameters. Swirl was present in all transfused products. The platelet content of the products was measured directly after production as a previous study has shown a limited decline in platelet number during 5 days of storage.[4, 23] We found significant differences with regard to pH and platelet content of the two PCs. The lower pH of PAS II PCs is due to a lower intrinsic pH of PAS II, lower buffering capacity and higher lactate production.[23, 26] The lower platelet content of PAS II PCs can be explained by a viscosity-related difference in the platelet distribution during centrifugation, resulting in a less efficient separation.[23]

We showed that the 1- and 24-hour CCIs of PAS II PCs were lower as compared to plasma PCs, with a mean difference of 19.7% and 17.8%, respectively. This effect remained after correcting for possible confounders in a multivariate analysis. Although the platelet content in PAS II PCs was significantly (approximately 5%) lower as compared to plasma PCs, this small difference is not clinically relevant and the transfused dose per kilogram (or per square meter) in both groups was similar. Univariate analysis of the effect of storage time showed a significant decrease in 1-hour and 24-hour CCI in both products, more pronounced in stored PAS II PCs, in contrast to the results of the study of de Wildt-Eggen.[12] The mechanism of this storage effect is unknown. Increased P-selectin expression and structural changes have been suggested as possible mechanisms.[5, 6] Whether such in vitro changes explain for the inferior increments of PAS II PCs remains unclear.[8, 9]

To investigate the clinical relevance of the inferior CCI of PAS II PCs we compared the incidence of bleeding, transfusion interval, red cell concentrate usage and the occurrence of transfusion failure, the latter also in relation to patient factors. We did not observe significant differences with regard to bleeding complications or the consumption of PCs and red cell concentrates. Univariate analysis of transfusion failures showed a significant effect of PAS II PCs on the occurrence of 1-hour transfusion failure, but not on the 24-hour transfusion failure. A multivariate analysis showed that patient related factors overruled product defined factors as determinants of transfusion failure at 1- and 24-hour, with the exception of storage time, which showed a trend towards 1-hour transfusion failure. The only other randomised study conducted by de Wildt-Eggen[12] used a different transfusion threshold ($> 20 \times 10^9/l$) and excluded sick patients. It is likely that the differences in CCIs and transfusion failure between the two studies are caused by factors of increased platelet consumption in our study population, as several studies demonstrated the impact of patient factors on the occurrence of transfusion failure.[15-19] In our study over 75% of all PCs were transfused during episodes with clinical complications associated with increased platelet consumption and multivariate analysis showed that patient related factors annihilated the effects of the used storage medium in relation to transfusion failure.

Compared to other studies we found a relatively low percentage of transfusion reactions, although significantly less after transfusions with PAS II PCs (p = 0.04), confirming the results of de Wildt-Eggen.[12] Probably this percentage underestimates the real frequency due to the fact that most reactions are mild, whereas fever and chills are common symptoms in this category of patients.

In conclusion we showed that transfusion responses with PAS II PCs are inferior as compared to plasma PCs. The biological significance of this observation is not significantly exceeding a 30% deterioration. A multivariate analysis showed that patient related factors annihilated the observed differences and there were no significant differences with regard to bleeding complications or PC consumption. Transfusion reactions were mild, infrequent and significantly lower with PAS II PCs. As most patients in need of supportive care temporarily experience factors leading to increased platelet consumption, we propose that future clinical trials studying experimental platelet products should include these patients. We showed safety and efficacy of PAS II PCs in intensively treated patients, however plasma PCs show superior increments. To prevent a downward creep in future developed platelet products, we advise storage of platelets in plasma should be included as a reference in future trials.

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Chapter 4

Clinical effectiveness of leukoreduced, pooled donor platelet concentrates, stored in plasma or additive solution with and without pathogen reduction

J.L.H. Kerkhoffs[1, 5], W.L.J. van Putten[2], V.M.J. Novotny[3], P.A.W. Te Boekhorst[4], M. Schipperus[5], J.J. Zwaginga[6], E.C.M. van Pampus[3], G.E. de Greef[4], M. Luten[2], P.C. Huijgens[7], A. Brand[1, 6], D.J. van Rhenen[1] on behalf of the Dutch – Belgian HOVON cooperative group.

[1]Sanquin Blood Bank, Southwest Region, Rotterdam;
[2]Erasmus University Medical Centre, HOVON Data Centre, Rotterdam;
[3]UMC St Radboud, Nijmegen;
[4]Erasmus University Medical Centre ,Rotterdam;
[5]Haga Teaching Hospital, The Hague;
[6]Leiden University Medical Centre, Leiden;
[7]VU Medical Centre, Amsterdam, The Netherlands.

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4 Clinical effectiveness of leukoreduced, pooled donor platelet concentrates, stored in plasma or additive solution with and without pathogen reduction

SUMMARY

Pathogen reduction (PR) of platelet products increases costs and available clinical studies are equivocal with respect to clinical and haemostatic effectiveness. We conducted a multicentre, open-label, randomised, non-inferiority trial comparing clinical effectiveness of buffy-coat derived leukoreduced platelet concentrates (PC) stored up to seven days in plasma with platelets stored in platelet additive solution III (PASIII) without and with treatment with amotosalen-HCl/UVA photochemical pathogen reduction (PR-PASIII). Primary endpoint of the study was 1-hour corrected count increment (CCI). Secondary endpoints were 24-hour CCI, bleeding, transfusion requirement of red cells and PC, platelet transfusion interval and adverse transfusion reactions. Compared to plasma-PC, in the intention to treat analysis of 278 evaluable patients the mean difference for the 1-hour CCI of PR-PASIII-PC and PASIII-PC was -31% (p < 0.0001) and -9% (p = n.s.), respectively. Twenty-seven patients (32%) had bleeding events in the PR-PASIII arm, as compared to 19 (19%) in the plasma arm and 14 (15%) in the PASIII arm (p = 0.034). Despite the potential advantages of pathogen (and leukocyte) inactivation of amotosalen-HCl/UVA-treated platelet products, their clinical efficacy is inferior to platelets stored in plasma, warranting a critical reappraisal of employing this technique for clinical use.

Keywords:

Platelet, Buffy-coat, Amotosalen/UVA Pathogen Reduction, Efficacy.

For the generally accepted indications for treatment and prevention of bleeding, millions of platelet products are transfused yearly, warranting vigilance towards emerging logistical problems and safety issues (Slichter, 2007; Stroncek & Rebulla, 2007). Donor counselling and screening, including molecular techniques, have reduced the risk of transmission of hepatitis B, hepatitis C, HIV, HTLV-I and –II. However, despite the bacterial culture of platelet products, a risk of 1 in 25,000 platelet transfusions for transfusion-related sepsis still remains (Goodnough et al, 1999; Kuehnert *et al*, Dodd *et al*, 2002; Blajchman et al, 2005; Schrezenmeier *et al*, 2007). Availability of platelets and reduction of costs due to reduced outdating would benefit from extending the storage time of platelet products, which is hampered mainly by the risk of bacterial growth beyond 5 days of storage (Lee *et al*, 2003). Pathogen reduction (PR) has been shown to be very effective for the inactivation of several viruses and bacteria (Lin *et al*, 2004; Lin *et al*, 2005). Moreover, PR might also comprise a solution for emerging pathogens, CMV and an alternative for γ-irradiation for the prevention of graft-versus-host-disease (Grass *et al*, 1999; Lin *et al*, 2001). Several countries consider implementing PR as a standard for all platelet products, but concerns still exist with regard to clinical efficacy, potential long-term toxicity as well as uncertainty whether PR- platelet products can be stored longer than 5 days (Council of Europe, 2001; Simonsen *et al*, 2006). Although PR platelet products using amotosalen-HCl and UVA fulfil standard release criteria up to 7 days of storage, this treatment results in considerable metabolic deterioration, increased platelet activation during storage and inconsistent findings by in-vitro haemostatic assessment (van Rhenen *et al*, 2000; Picker *et al*, 2004; Janetzko *et al*, 2004; Jansen *et al*, 2004; Lozano *et al*, 2007; Apelseth *et al*, 2007; van der Meer *et al*, 2009). Nevertheless, transfusion in thrombocytopenic patients corrected prolonged bleeding times (Slichter *et al*, 2006). Radiolabeled, autologous 5 days stored amotosalen-HCI/UVA-treated platelets showed a significant lower recovery and reduction in survival time as compared to platelets stored in PASIII additive solution (Snyder *et al*, 2004). Three randomised controlled trials have been performed using amotosalen-HCI/UVA-treated platelet concentrates (PC) (van Rhenen *et al*, 2003; McCullough *et al*, 2004; Janetzko *et al*, 2005). In the SPRINT trial (645 patients), using aphaeresis PC stored in plasma as control, significantly lower post transfusion platelet increments were found, combined with a reduced transfusion interval and an increased rate of transfusion failure (McCullough *et al*, 2004). The EuroSPRITE trial (103 patients) reported no significant differences with regard to transfusion efficacy, however the control arm of this study used buffy-coat derived platelets stored in plasma as well as in additive solution (PASII) for approximately half of the transfusions (van Rhenen *et al*, 2003). In a previous RCT we have shown that PASII PC have a 20% lower corrected count increment as compared to plasma PC, which might have masked a relevant difference (Kerkhoffs *et al*, 2006). A third small trial with 43 patients showed a borderline significant reduction in transfusion efficacy (Janetzko *et al*, 2005). None of these trials reported inferior haemostatic efficacy. Before the implementation of pathogen reduced platelet products, extending storage time to 7 days while maintaining clinical efficacy is an important aspect to compensate for the additional costs of the procedure. We performed a multicentre open-label, randomised clinical trial to study the clinical efficacy in terms of transfusion response of pooled, random donor PC stored up to seven days in platelet additive solution (Intersol, Fenwal, Inc., Lake Zurich, IL, USA) without additional PR (PASIII) and with amotosalen-HCl/UVA photochemical PR (PR-PASIII, Intercept Blood System, Cerus Corporation, Concord, CA, USA), compared to platelets stored in plasma.

Clinical effectiveness of leukoreduced, pooled donor platelet
concentrates, stored in plasma or additive solution with
and without pathogen reduction

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METHODS

Study design

The study was designed as a prospective, randomised open-label non-inferiority
trial in haemato-oncological patients with thrombocytopenia or expected to be
thrombocytopenic caused by myelosuppression. Patients were included at the
haematology wards of eight Dutch hospitals. The study protocol and consent forms were
approved both by a central ethics committee as well as local institutional review boards.
The study was conducted according to the ICH-GCP guidelines and the declaration of
Helsinki. During the study all centres were audited and trial conduct was monitored by an
independent organisation. All patients older than 18 years, having a haemato-oncological
disease, were eligible for inclusion if they were expected to receive 2 or more platelet
transfusions. Exclusion criteria were immunological refractoriness to random platelet
transfusions due to HLA- and/or HPA-antibodies or clinical relevant auto-antibodies,
pregnancy (or lactating) and previous inclusion in this study. After informed consent
eligible patients were registered and randomised, stratified by centre, before start of
platelet transfusions in a 1:1:1 ratio to receive per protocol up to a maximum of 5 platelet
transfusions with Plasma-PC, PASIII-PC or PR-PASIII-PC in a period of maximal 42 days.
Off protocol platelet transfusions were allowed during the study period in case of non-
availability of the correct component. Apart from normal completion, reasons to go off
study were refusal to continue by the patient or treating physician, intercurrent death
and immunological refractoriness.

Platelet products, transfusions and monitoring

All products were produced by the Sanquin Blood Bank. PCs were prepared from 5 pooled
whole-blood buffy-coats (BC) with the same ABO-blood group using standard procedures
and with regard to pathogen reduction using manufacturer's instructions (van Rhenen
et al, 2003; Kerkhoffs *et al*, 2006). Samples were obtained prior to storage to measure
platelet content. Samples of all products were cultured for 7 days using the BacT/Alert
culturing system (BioMerieux, Boxtel, the Netherlands). All products were stored with
gentle agitation at 20–24˚C up to seven days. The PCs were γ-irradiated if requested by
the hospital.
Indications for platelet transfusions were divided into platelet count-based prophylaxis,
intervention related prophylaxis and treatment of bleeding. Generally accepted guidelines
were used as guidance for the indication of platelet transfusions. If or when a transfusion
was ordered was determined by the treating physician. In summary, in stable, non-
bleeding patients a platelet transfusion was advised to maintain the platelet count
$\geq 10 \times 10^9/l$ and $\geq 40 \times 10^9/l$ when these patients receive anti-coagulant therapy
or treatment with anti-thymocyte globulin. A transfusion trigger of $40 \times 10^9/l$ was
recommended in endoscopic evaluation of the gastrointestinal or respiratory tract,
when no biopsies are performed, diagnostic pleural or peritoneal puncture with a thin
needle, lumbar puncture, extraction of a central venous catheter and minor surgical
interventions. A trigger of $60 \times 10^9/l$ was recommended in case of bleeding, endoscopic
evaluation with biopsies, dental extractions, placement of a central venous catheter and
major surgical interventions, with the exception of neurosurgery and cardiac surgery.
In case of cerebral bleeding, diffuse alveolar haemorrhage, neurosurgery and cardiac
surgery a trigger of $100 \times 10^9/l$ was recommended. A pretransfusion platelet count
was preferably measured just before transfusion up till a maximum of 6 hours before

transfusion. A 1-hour posttransfusion platelet count was measured between 10 and 120 minutes after transfusion and a 24-hour post transfusion platelet count was measured between 16 and 28 hours after transfusion. The CCI was calculated as follows: $CCI_{1/24\,h}$ = [(post transfusion platelet count$_{1/24\,h}$ – pre transfusion platelet count (x 10^9/L)) x body surface area (m²)] / platelet dose x 10^{11}. Transfusions given shortly after one another without platelet counts between the transfusions are referred to as multi-dose transfusions and analysed as a single transfusion. If available, ABO-identical PC were used, although minor- and major incompatible PC were not excluded. Platelet transfusion failure was defined as a 1-hour corrected count increment (CCI) below 7.5 and/or and 24-hour CCI below 4.5 (Kerkhoffs *et al*, 2006). Immunological refractoriness was defined as the occurrence of transfusion failure of two consecutive ABO-matched random platelet transfusions combined with existence of HLA- and/or HPA-alloantibodies.

Study endpoints

The primary endpoint was the 1-hour CCI. Secondary endpoints were 24-hour CCI, bleeding, the transfusion requirement of red cells and PCs, platelet transfusion interval and adverse transfusion reactions. The following characteristics were recorded at entry: gender, age, blood group, haematological disease and treatment phase, WHO performance status, existence of enlarged spleen, transfusion history, treatment with anti-coagulation, medical history, medication, bleeding and presence of active infection. The following characteristics were recorded at each transfusion: the reason of the transfusion (trigger, bleeding or intervention), the blood group of the PC, presence of fever, presence of infection (graded according to the CTCAE), presence of mucosal damage, and use of acetaminophen, steroids or antihistamines. Patients were evaluated daily by trained personnel to observe, describe and grade bleeding complications at 8 defined sites according to the CTCAE under supervision of the local investigator (http://ctep.info.nih.gov/reporting/ctc.html). In short grade 1 or minor bleeding comprises petechiae, minimal or microscopic bleeding not requiring interventions. Grade 2 bleeding is defined as gross, symptomatic bleeding for which minimal intervention (i.e. aspiration, cauterisation, irrigation of the urinary tract) is indicated. Grade 3 is severe bleeding requiring red cell transfusions and/or major interventions. Generalized petechiae/purpura as well as retinal bleeding with visual impairment also is classified as grade 3. Catastrophic bleeding defines grade 4, as does CNS bleeding causing neurologic deficit or disability. Lethal bleeding is classified as grade 5. All major bleeding complications were reviewed centrally. Infections were scored in case of positive cultures or if a focus was likely as shown by clinical or radiological examination. Apart from haematological parameters, PT, aPTT and fibrinogen, were measured regularly. Some centres performed routine periodic serological testing of HLA- and/or HPA-alloantibodies, whereas other centres performed these tests only on indication.

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Reporting of serious adverse events and Data Safety Monitoring Board

Serious adverse events (SAE) for the purpose of this study were defined as any untoward medical occurrence that resulted in death, a life-threatening event or any other medical condition which might jeopardize the patient or required intervention to prevent more serious sequelae. SAE reporting was mandatory within 24-hours of the initial observation. An independent Data Safety Monitoring Board (DSMB) was installed before the start of the study. An interim analysis was planned after 300 transfusions. All serious adverse events (SAEs) were reviewed by the DSMB. Two criteria for early stopping of an experimental arm were defined: 1. A negative 24-hour CCI (decrement) not caused by immunological factors in more than 20% of the transfusions; 2. Statistically significant more bleeding complications (CTCAE ≥ 2) compared to the Plasma arm.

Power calculation and statistical analysis

The study was designed as a one-sided, non-inferiority study comparing the 1-hour CCI of the transfusions in the PR-PASIII arm and in the PASIII arm with the Plasma arm. Inferiority of an experimental arm was defined as a 20% lower mean 1-hour CCI compared to the Plasma arm. A mean 1-hour CCI of 15.6 and a standard deviation of 6.0 were used based on a previous study (Kerkhoffs *et al*, 2006). For a power of 90% and an alpha of 0.025 (multiple testing) 100 patients per arm were required. In case of multi-dose transfusions, the sum of the platelet content of the PC was used. If one of the PC products differed from the allocated arm, the multi-dose transfusion was considered as not according to protocol. The mean of the storage times of the PC in a multi-dose transfusion was used as the storage time. The 1 and 24 hour counts after the infusion of the last PC of a multi-dose transfusion were used for analysis. To account for the hierarchical structure of the data with a variable number of transfusions per patient, the data were analysed using mixed regression models with random effects for patient and transfusion number. Besides the CCIs, 1 and 24 hour posttransfusion counts were used as endpoints in regression models with as additional covariates besides arm, platelet dose, pretransfusion counts and body surface area of the patient (Davis *et al*, 1999). The data were analysed by intention to treat (ITT) as well as per protocol (PP). To assess safety, the incidence of bleeding complications and adverse reactions were analysed through tabulation. Pearson's chi-square test was used to compare categorical patient characteristics by arm and the Kruskal-Wallis test to compare ordinal or continuous characteristics by arm. A relation between storage time and the post transfusion counts and CCIs was assessed by adding this factor as covariate to the regression models. The association between the patient and transfusion characteristics mentioned above was assessed by adding each of these variables separately as covariate to the regression models. All statistical analyses were performed using Stata.

RESULTS

Patients and platelet transfusions

Inclusion of patients started March 2007. The inclusion of patients in the PR-PASIII group was halted after 92 patients in January 2009 on advice of the DSMB because of lower CCI's (p<0.0001) and more bleedings (p=0.045) compared to the plasma group. Inclusion of patients in the plasma and PASIII group ended May 2009 and overall 295 patients were randomised. There were 17 non-evaluable patients, resulting in a total of 278 evaluable patients and 1129 transfusion events (fig 1). There were no significant differences in the patient characteristics of the study groups (table 1). 302 transfusion events (27%) were not according to the allocated study arm, more frequent in both study arms. 85% of the off protocol PC were platelets stored in PASII, 15% were platelets stored in plasma. The study products had a lower platelet content, with a mean difference of 6% and 11% for PASIII-PC and PR-PASIII-PC as compared to plasma PC, respectively (table 1, p < 0.001).

Figure 1: *Figure 1 shows the randomisation scheme together with evaluable patients, transfusions and endpoints. n = number of patients, n PC = number of single platelet concentrates, n TRF = number of PC transfusion events (includes pooled transfusions). Of the 17 non-evaluable patients 4 patients were non-eligible due to anti-HLA antibodies and 13 patients did not receive any platelet transfusions, without differences between study groups.*
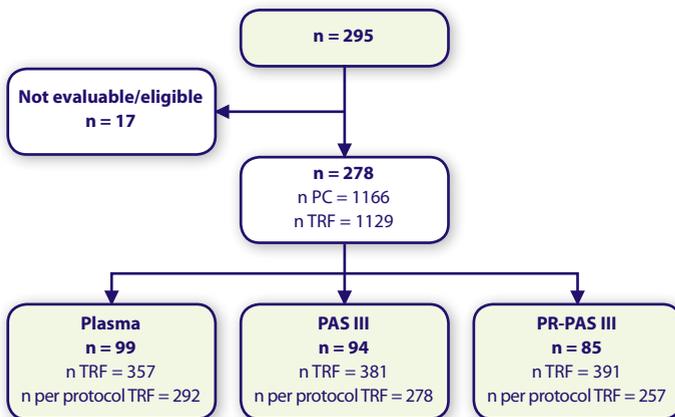
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Table 1: *Platelet transfusions, red cell transfusions and transfusion interval.*

	Plasma	PAS III	PR-PAS III
No. of patients	99	94	85
Male / Female	52 / 47	53 / 41	47 / 38
Age, years ± SD	54 ± 12	55 ± 12	53 ± 12
Body surface area, m² ± SD	1.93 ± 0.22	1.94 ± 0.19	1.96 ± 0.25
Enlarged spleen N (%)[1]	10 (10)	5 (5)	6 (7)
Diagnosis N (%)			
AML / MDS	42 (42)	52 (55)	44 (52)
ALL	9 (9)	4 (4)	3 (4)
Lymphoma	22 (22)	14 (15)	18 (21)
Multiple myeloma	22 (22)	21 (22)	17 (20)
Other	4 (4)	3 (3)	3 (4)
Therapy N (%)			
Remission induction	47 (47)	46 (49)	39 (46)
Consolidation	5 (5)	6 (6)	3 (4)
Autologous transplantation	32 (32)	31 (33)	33 (39)
Allogeneic transplantation	12 (12)	5 (5)	6 (7)
Other	3 (3)	6 (6)	4 (5)
Transfusion history N (%)			
RBC concentrates	55 (56)	59 (63)	43 (51)
PCs	48 (48)	61 (65)	41 (48)
No. of PC transfusion events	**357**	**381**	**391**
Product type according to protocol (%)	292 (82)	278 (73)[2]	257 (66)[2]
Multi-dose transfusion (%)	14 (4)	12 (3)	11 (3)
PC transfusion indication N (%)			
Prophylactic, trigger based	304 (85)	334 (88)	327 (84)
Intervention	38 (11)	25 (7)	44 (11)
Treatment of bleeding complication	11 (3)	19 (5)	16 (4)
Unknown	4 (1)	3 (1)	4 (1)
Platelet product content, mean x 10^{11} ± SD	3.9 ± 1.0	3.6 ± 0.8[2]	3.4 ± 0.8[2]
Storage time, mean days ± SD	4.0 ± 1.8	3.8 ± 1.8	4.0 ± 1.6
Pre transfusion PLT count x 10^9/L ± SD	18 ± 13	17 ± 13	16 ± 11[3]

[1]*Number (%) of evaluable patients and transfusions;* [2]*p < 0.001 as compared to plasma;* [3]*p = 0.04 as compared to plasma;*
AML = Acute myeloid leukaemia; MDS = Myelodysplastic syndrome; ALL = Acute lymphoblastic leukaemia; RBC = Red
blood cell; PC = Platelet concentrate. Major ABO-incompatibility occurred in only 6 PC transfusions.

Platelet transfusion efficacy

All efficacy analyses were done ITT as well as PP. The 1-hour CCI and 24-hour CCI were evaluable in 1004 (88.9%) and 1013 (89.7%) of the transfusion events, respectively. The single reason for a non-evaluable CCI-1/24 was failure to perform a platelet count after transfusion and with respect to these missing evaluations there were no significant differences between the study groups or between the per- and off-protocol transfusion events. All transfusion efficacy parameters show inferiority of transfusions with PR-PASIII-PC. There were no significant differences in transfusion responses between PASIII-PC and Plasma-PC (table 2). The proportion of 6 and 7 days stored PC was equally distributed across the arms, being 24%, 21% and 26% of transfused PC in the plasma arm, the PASIII arm and the PRPASIII arm, respectively.

Table 2: *Transfusion response parameters: ITT and according to protocol (PP).*

	Plasma	PAS III	PR-PAS III
No. of patients	99	94	85
ITT analysis			
CCI-1 hour, mean ± SD	17.1 ± 7.3	15.3 ± 6.5	11.4 ± 5.3[5]
Mean diff (97.5% CI)[1]		-9% (-22%; 4%)	-31% (-43%; -18%)
CCI-24 hour, mean ± SD	12.8 ± 7.8	11.6 ± 7.6	7.9 ± 5.3[5]
Mean diff (97.5% CI)[1]		-7% (-26%; 12%)	-34% (-52%; -17%)
PP analysis			
CCI-1 hour, mean ± SD	17.1 ± 7.3	15.3 ± 6.7	10.6 ± 5.0[5]
Mean diff (97.5% CI)[1]		-10% (-23%; 4%)	-36% (-49%; -24%)
CCI-24 hour, mean ± SD	12.5 ± 7.7	11.7 ± 7.6	6.8 ± 5.95
Mean diff (97.5%CI)[1]		-4% (-24%; 16%)	-42% (-61%; -23%)
Other response parameters (ITT)			
CI-1 hour, mean x 10^9/L ± SD	34 ± 15	29 ± 13	20 ± 10[4]
CI-24 hour, mean x 10^9/L ± SD	25 ± 15	21 ± 13	14 ± 10[3]
PC transfusions / patient, mean ± SD	4 ± 2	4 ± 3	5 ± 3[2]
TRF interval (hours), mean ± SD	81 ± 47	77 ± 44	61 ± 47[3]
Transfusion failure (ITT)			
N of Evaluable CCI-1	**314**	**340**	**350**
CCI-1 hour < 7.5 (%)[6]	48 (15)	66 (19)	97 (28)[5]
N of Evaluable CCI-24	**319**	**343**	**351**
CCI-24 hour < 4.5 (%)[6]	72 (23)	94 (27)	125 (36)[3]

The mean CCI and CI values were calculated as the mean of the average CCI/CI of all transfusions per patient.

[1]*Mean difference with 97.5% confidence interval of PAS III and PR-PAS III compared to Plasma derived from mixed model regression analyses.* [2]$p < 0.05$, [3]$p < 0.01$, [4]$p < 0.001$, [5]$p < 0.0001$ *as compared to plasma;* [6]*percentage of evaluable CCIs.*

Table 3: *Linear regression analysis 1- and 24-hour PLT counts.*

	1-hour PLT count		24-hour PLT count	
	Beta[1]	p-value	Beta	p-value
PASIII	-2.29	0.377	1.79	0.507
PR-PASIII	-9.63	0.001	-8.95	0.003
Storage time (days)	-1.55	<0.001	-1.24	<0.001
Body surface area (m²)	-15.4	<0.001	-10.1	0.002
Transfusion sequence number	-0.38	0.047	-0.08	0.686
Platelet product content (x 10^9)	0.09	<0.001	0.06	<0.001
Precount (x 10^9/l)	0.96	<0.001	0.96	<0.001

[1]*Random effects binary logistic model for distinguisable data (odds ratios and p-values are corrected for within-patient-correlation of 1Beta: regression coefficient. Multivariate linear regression analyses with patient as random factor and as dependent variables the 1-hour platelet (columns 2 and 3) and the 24-hour platelet count (columns 4 and 5). The factors included in the models are shown in the first column. The estimated regression coefficients are shown in the Beta -columns. The regression coefficients measure the strength of the effect per unit change of the corresponding factor; e.g the 1-hour platelet count decreases on average with 1.55 x 10^9/l with each additional day of storage, while an increase of the content of the platelet product with 1 x 10^9 results on average in an increase of 0.09 x 10^9/l of the 1-hour platelet count. The regression coefficients for PAS III and PR-PAS III indicate the average difference in the post transfusion counts as compared to Plasma.).*

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Both the 1-hour CCI as well as the 24-hour CCI decreased with longer storage time in all study groups. However both CCIs were significantly less in PR-PASIII-PC at each day of storage as compared to plasma PC (figs 2A&B). The 1- and 24-hour CCIs of PASIII-PC did not differ significantly to plasma PC up to 7 days of storage. Linear regression analysis of 1- and 24-hour platelet count showed a platelet dose independent effect of pathogen reduction (figs 2C&D, table 3). A number of product and patient related covariates were tested for an association with CCIs adjusted for arm (table 4). Storage time, enlarged spleen and fever were highly significantly associated with lower CCIs, while the use of steroids as premedication was associated with a higher 1-hour CCI and transfusion for a bleeding indication was associated with a lower 24-hour CCI.

Figure 2: *Figure 2 shows the fitted lines from linear regression analyses restricted to per protocol transfusions. Black, blue and red, respectively represent Plasma, PAS III and PR-PAS III. (2A/B) The 1-hr and 24-hr CCI as function of storage time for the three treatment groups. Point estimates with 95% confidence intervals and number of transfusions are indicated. The lines are the fitted lines assuming a linear relation between CCI and storage time for each group. (2C/D) Fitted 1-hr and 24-hr increments as linear functions of storage time for the three treatment groups for a patient with surface area 1.93, precount 12 and storage time of 4 days. Standard error bars are indicated. (See also supplementary table 1).*
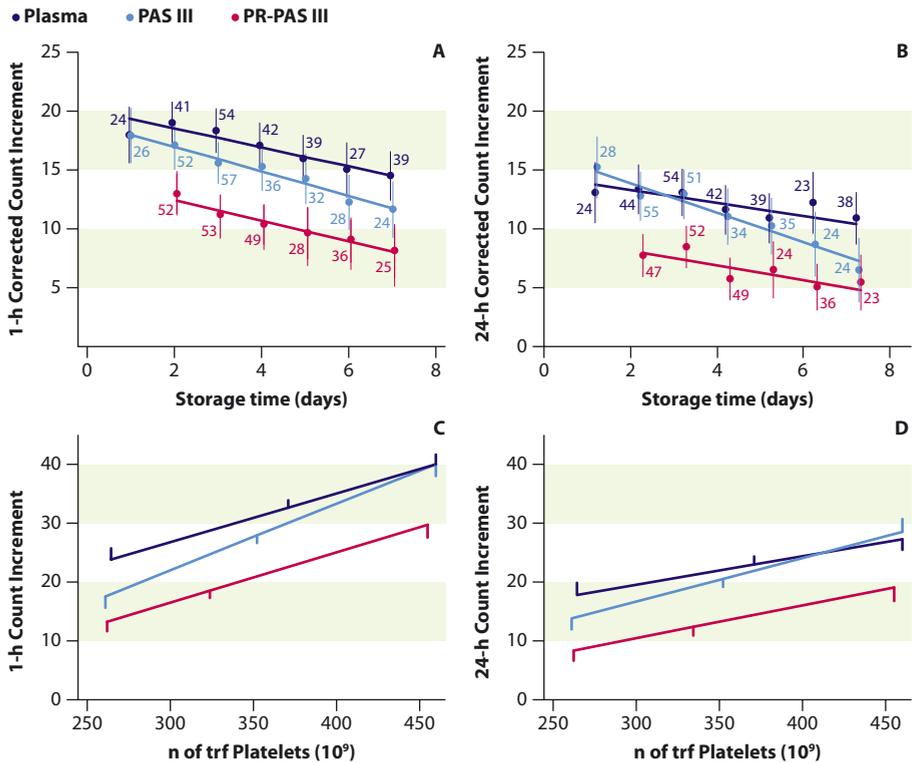
Table 4: *Relation between covariates and the CCI-1 and CCI-24 adjusted for arm.*

	1-hour CCI		24-hour CCI	
	Beta[1] (SE)	p-value	Beta (SE)	p-value
Storage time (days)	-0.9 (0.1)	<0.00001	-0.9 (0.1)	<0.00001
Spleen enlargement	-5.7 (1.4)	<0.00001	-6.5 (1.5)	<0.00001
Fever	-1.7 (0.4)	<0.00001	-1.5 (0.4)	0.0003
Steroids	2.6 (1.1)	0.02	1.0 (1.3)	0.43
Indication bleeding	1.1 (1.0)	0.29	-2.5 (1.1)	0.02
Indication Intervention	-0.6 (0.8)	0.39	-0.4 (0.8)	0.64
Age (years)	0.2 (0.3)	0.49	0.0 (0.3)	0.97
Sex	1.1 (0.8)	0.17	0.3 (0.8)	0.76
Prior PLT TRF	-1.0 (0.8)	0.22	-1.0 (0.8)	0.24
Prior RBC TRF	-0.9 (0.8)	0.25	-0.7 (0.8)	0.42
Prior TRF reactions	-2.4 (1.5)	0.12	-0.3 (1.7)	0.84
Infection	-0.5 (0.5)	0.33	-0.5 (0.5)	0.27
Mucosal damage	-0.1 (0.5)	0.82	0.1 (0.5)	0.82
ABO mismatch	0.2 (0.4)	0.68	0.4 (0.4)	0.33
Anti-histamines	-1.6 (1.3)	0.21	-1.8 (1.3)	0.16
Anti-coagulation	-1.3 (1.3)	0.31	-2.1 (1.4)	0.14
Acetaminophen	1.1 (1.3)	0.39	-1.3 (1.3)	0.31

Univariate random effects regression analysis adjusted for arm. [1]*Beta: regression coefficient; SE = Standard error;*

TRF = Platelet transfusion; RBC = Red blood cell concentrate. All covariates, with the exception of storage time

and patient age, are no/yes covariates.

Bleeding and other clinical complications

Sixty-seven new bleeding episodes (CTCAE grade 1-3) were observed in 60 patients during the on study period from the start of the first transfusion with significantly more (p=0.034) and higher grade (p=0.044) bleeding in the PR-PASIII group (table 5).

Distribution of bleeding sites was not different between the study groups. 14 of the bleeding patients were on anticoagulant therapy at the time of bleeding, without differences between the groups. We did not observe lethal bleeding complications in the on protocol period; however, one patient in the PR-PASIII arm deceased due to intracranial bleeding after going off protocol. We did not find an association between platelet dose, storage time or γ-irradiation and the occurrence of bleeding (all grades). There were no differences between the groups with regard to number of RBC transfusions received. The mean number of RBCs in the plasma group was 4±3 as compared to 5±3 and 4±3 in the PASIII and PR-PASIII group, respectively. Twenty-eight mostly mild transfusion reactions occurred in 25 patients, without significant differences between groups (table 5). Incidences of infections and SAE's were equally distributed among the groups. Three SAE's were possibly related to PC transfusion, one in each group. In the plasma group a patient developed a severe, generalized skin reaction, in the PASIII arm a possible TRALI was reported and in the PR-PASIII arm a patient developed acute glottis oedema treated successfully with antihistamines and steroids.

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Table 5: *Bleeding, transfusion reactions, infections and SAE's*

	Plasma	PAS III	PR-PAS III
No. of patients	99	94	85
Bleeding after first PC transfusion			
No of patients (%)	19 (19)	14 (15)	27 (32)[1]
No of episodes	19	16	32
Maximum grade (%)			
Grade 1	12 (12)	10 (11)	16 (19)
Grade 2	6 (6)	4 (4)	6 (7)
Grade 3	1 (1)	-	5 (6)
Patients with transfusion reactions, N (%)	11 (11)	8 (9)	6 (7)
No. of transfusion reactions	13	8	7
Severity of events			
No or minor morbidity	11	7	6
Moderate morbidity	1	-	1
Serious morbidity	1	1	-
Patients with infectious complications, N (%)	40 (40)	39 (41)	42 (49)
Maximum grade (%)			
Grade 1 (%)	1	-	-
Grade 2 (%)	3	5	6
Grade 3 (%)	30	29	28
Grade 4 (%)	6	4	8
Grade 5 (%)	-	1	-
Immunological Refractoriness, N (%)	2 (2)	-	2 (2)
Serious adverse events, N	7	3	5
SAE related to PC transfusion	1	1	1
Death, N	3[2]	1	3

Except for the number of bleeding episodes, the numbers in the table reflect numbers (percentage) of patients.

For the grades of bleeding and infections the maximum grade is used in case of more than one bleeding episode or more

than one infection. [1] p = 0.034 as compared to plasma; [2] 1 patient died in the plasma arm 24 days after the last transfusion

(the fifth) without SAE report. The cause of death was reported on the off study form as related to the treatment of

the underlying disease, with fever presumably due to sepsis.

DISCUSSION

In a non-selected population of thrombocytopenic haematology patients we studied the transfusion efficacy of PR-PASIII-PCs and PASIII-PCs in terms of increments, transfusion failures, PC consumption and transfusion interval as well as bleeding occurrence and adverse transfusion reactions, compared to plasma-PC. In accordance with the SPRINT trial but in contrast to the EuroSPRITE trial, we observed inferiority of transfusions with PR-PASIII-PC with regard to all transfusion efficacy-related endpoints (van Rhenen *et al*, 2003; McCullough *et al*, 2004). Moreover more patients in the PR-PASIII-PC arm experienced bleeding complications. As reported previously, both study products contained less platelets due to loss of platelets during the production process (McCullough *et al*, 2004; Kerkhoffs *et al*, 2006; Murphy *et al*, 2006; Pineda *et al*, 2006). As CCI might not adequately correct for dose differences between arms, we performed linear regression analysis of the posttransfusion platelet counts with covariates treatment arm, platelet content and storage time, also showing an independent effect of PR-PASIII PC (Davis *et al*, 1999). Using the linear regression analysis we estimate that a PR-PASIII-PC should on average contain 200×10^9 platelets extra (i.e. approximately 3 BCs) to achieve a comparable count increment. The relationship between storage time with both CCIs showed a constant difference at each incremental day of storage, suggesting a decreased viability of a fixed number of platelets and a normal disappearance of surviving platelets after treatment with this PR technique. To the same extend as plasma PC, PASIII PC show a decrease in transfusion efficacy up to seven days of storage and no difference in bleeding complications. Our results with regard to lower increments are in agreement with the SPRINT study. The discordance with the EuroSPRITE as well as with a large phase IV trial may be due to the usage of PC stored in PASII in approximately half of the reference group attenuating the results of the reference groups in these other studies (van Rhenen *et al*, 2003; Osselaer *et al*, 2009).

Patients in the PR-PASIII group experienced more and more grade ≥ 2 bleeding compared with both the other arms. The EuroSPRITE and the other smaller European RCT reported no differences between the study arms with regard to bleeding complications (van Rhenen *et al*, Janetzko *et al*, 2005). However in the extended safety report of the SPRINT trial the frequency of grade 2-4 bleeding appeared significantly higher in the PR-arm, 43% as compared to 34% in the control arm (p = 0.02) (Snyder *et al*, 2005). It is unlikely that the difference in bleeding complications is solely explained by a lower platelet dose resulting in lower post transfusion platelet peak levels. Estimating approximately one-third non-viable platelets in PR-PC, the platelet dose is still comparable with the low to medium dose applied in a recently presented platelet dose trial showing that bleeding complications did not differ between low, medium or high dose levels of platelets transfused (Slichter *et al*, 2010). Possibly, damage of platelet mitochondrial nucleic acids by PR may not only result in loss of viability of a proportion of platelets, but may impair haemostatic capacity as well (Keuren *et al*, 2006; Apelseth *et al*, 2007). We did not find significant differences in transfusion reactions as observed in larger trials using PR-PASIII PC (Osselaer *et al*, 2008a; Osselaer *et al*, 2008b).

This study has some shortcomings. The number of off-protocol transfusions in the PR-PASIII arm can be regarded as an important limitation of our study. However, performances of both an ITT as well as a PP analysis lead to similar conclusions.

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The open label aspect of our study is not expected to influence platelet counts, the primary endpoint of our study, although we cannot completely exclude bias with regard to evaluation of bleeding.

In conclusion, although there are clear advantages and arguments in favour of pathogen reduction techniques to increase transfusion safety, our results warrant a reappraisal of pathogen reduction techniques prior to routine implementation. The process of PR using amotosalen-HCl/UVA likely leads to decreased platelet viability and perhaps compromises haemostatic function, the primary goal of platelet transfusions in high risk patients. A comprehensive survey on the nature and consequences of amotosalen-HCl/UVA-induced platelet damage is needed to understand how this damage can be compensated for in routine transfusion practise.

ACKNOWLEDGMENTS

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Chapter 5

Clinical efficacy in thrombocytopenic patients of platelets stored in additive solutions as compared to plasma

Jean-Louis H. Kerkhoffs[1, 2], Wim L.J. van Putten[3],
Leo M.G. van de Watering[1], Jeroen CJ Eikenboom[4],
Rinie J. van Wordragen-Vlaswinkel[4], Anneke Brand[1, 4]

[1]Sanquin Blood Bank, Southwest Region, Rotterdam
[2]Haga Teaching Hospital, The Hague
[3]HOVON Data Centre, Rotterdam
[4]Leiden University Medical Centre, Leiden
The Netherlands

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5 Clinical efficacy in thrombocytopenic patients of platelets stored in additive solutions as compared to plasma

Background

The development and introduction of additives for the storage of platelet concentrates (PC) is proceeding steadily. In the Netherlands platelets stored in PAS II (T-Sol) up to 5 days are allowed for transfusion in contrast to platelets stored in plasma, which are allowed to be stored up to 7 days. A recent study suggested an adequate transfusion efficacy with platelets stored in PAS III (Intersol) up to 7 days.

Method

We reanalysed the data of the two RCTs in which plasma PC had been used as control arm and either PAS III PC or PAS II PC as study arms, respectively in order to compare the clinical efficacy of both additive solutions in relation to storage. Moreover, we calculated a combined Odds Ratio for adverse transfusion reactions.

Results

The CCI-1 of PAS II (stored up to 5 days) was 23.6% (95%CI 10.6; 36.5) lower as compared to plasma, whereas PAS III (stored up to 7 days) showed a reduction of 10.9% (95%CI -1.3; 23.2). The same effect was observed with regard to the 24-hour CCIs. Adverse transfusion reactions occurred less frequent after transfusion with platelets stored in an additive solution resulting in a risk reduction of 50% as compared to plasma (95%CI 10 – 72%, p = 0.025).

Conclusion

The use of additive solutions reduce the incidence of mild adverse transfusion reactions, an important advantage for patients, and the use of PAS III PCs, stored up to 7 days, for routine transfusion practice is an alternative for PAS II PCs.

Keywords:

platelet concentrates additive solution, efficacy, and adverse reactions

Since the first publication by Rock et al, the development and clinical use of synthetic additive solutions for the storage of platelets gained interest in many countries with as main incentives the recovery of plasma for other purposes, reduction of adverse transfusion reactions and the improvement of storage conditions to increase the platelet shelf-life.[1, 2] In the Netherlands, the vast majority of platelet products are pooled buffy-coat derived prestorage leukoreduced platelet concentrates (PC), stored either in plasma (Plasma PC) or in PAS II (PAS II PC, Trombosol, Baxter, Lessines, Belgium). Based on a study showing adequate in vitro characteristics and clinical efficacy, storage of plasma PC is allowed up to seven days.[3] Storage of platelets in PAS II is limited to a maximum of five days.[6] In a randomised study, comparing 1 - 5 days versus 6 - 7 stored PAS II PC in transplant recipients a significant decrease in transfusion efficacy of 6 - 7 days stored platelets was shown, without differences in hemorrhagic complications.[9] Although in vitro studies show acceptable quality parameters for PAS II PC during storage up to seven days and the in vivo autologous recovery and survival of 7 days stored PAS II platelets has been reported to be in acceptable ranges this illustrates the limited information of pre-clinical studies.[4] However, it is virtually impossible to compare all different platelet additive solutions in clinical studies. To improve storage conditions other additive solutions have been developed, using differing concentrations of acetate and phosphate, with or without the addition of potassium and magnesium.[10, 11] One of these solutions, PAS III (Intersol, Fenwal Inc., Lake Zurich, Il, USA), differs from PAS II only in the addition of phosphate, which besides increasing buffering capacity, may be superior to PAS II by protection against low adenine nucleoside levels during storage.[12, 13] PAS III PCs as well as PAS II PCs both fulfilled the standard release criteria (pH, swirling) stored up 8 days.[6] We have previously performed two randomised controlled clinical studies, one comparing 1-5 days stored platelets in PAS II with plasma PC and showing an approximately 20% lower efficacy of PASII PC, without a difference in bleeding complications and halving of transfusion reactions. A second RCT, a three-arm study, included Plasma PCs and PAS III PCs both stored up to seven days as control arms.[14] In this study, PAS III PC showed a minor reduction in transfusion efficacy. Instead of conducting a clinical study comparing PAS II with PAS III stored PC for their storage capacity, we analysed the data of these two RCTs in which plasma PC had been used as control arm and either PAS III PC or PAS II PC as study arms.[8, 14]

MATERIALS AND METHODS

Study design

The study design of both trials was very similar with respect to included patients, platelet transfusion policy and study endpoints. The first trial (Trial 1), conducted between October 2003 and April 2005, studied the clinical efficacy of pooled blood, buffy-coat derived platelet products, comparing plasma PCs and PAS II PCs stored up to 5 days.[8] The second trial (Trial 2), conducted between March 2007 and May 2009 compared PAS III PCs treated with pathogen reduction with plasma PCs and PAS III PCs without pathogen reduction.[14] For a detailed description of both trials we refer to the original publications. For both trials all products were produced by the Sanquin Blood Bank, prepared from five pooled buffy-coats with the same ABO-group. Samples of all products were obtained prior to storage to measure platelet count and culture using the BacT/Alert culturing system (Biomerieux, Boxtel, the Netherlands). PCs were stored with gentle agitation at 20 – 24 °C and γ-irradiated at request. There were a number of relevant differences between both trials (table 1). Most importantly, in Trial 1 patients were allowed to be randomised more than once, also more study transfusions were allowed in this trial. The primary objective of this analysis is an indirect comparison of the transfusion efficacy of platelets stored in PAS II (PAS II PC, Trombosol, Baxter, Lessines, Belgium) and platelets stored in PAS III (Intersol, Fenwal Inc., Lake Zurich, Il, USA). For the purpose of this comparison we abstracted the main patient characteristics as well as product characteristics and transfusion efficacy parameters (count increment, corrected count increment) of the first 5 according to protocol transfused PCs from the databases of both studies. We only included the first inclusions in trial 1 (figure 1). Adverse reactions were voluntary reported and classified according to Dutch Hemovigilance guidelines.

Figure 1: *Figure 1 schematically shows the selection of patients and transfusions included for this analysis. PC = Platelet concentrate; TRF = Transfusion; n = number of patients or transfusions.*
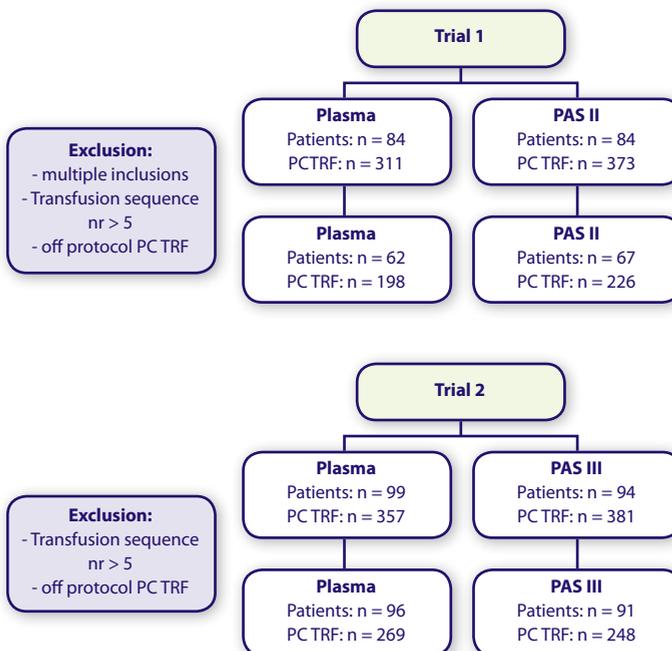
Table 1: *Trial and trial design overview.*

	Trial 1	**Trial 2**
Period	Oct 2003 – Apr 2005	Mar 2007 – May 2009
Type of trial	RCT, blinded	RCT, non-blinded
Stratification	Yes	Yes
N of study arms	2	3
N of participating centers	2	8
Primary endpoints	CCI-1 and 24-hour	CCI-1 hour
N of evaluable patients	168	278
Type of patients	Hemato-oncology	Hemato-oncology
Age	≥ 18	≥ 18
Exclusion criteria (main)	Auto- and/or alloimmunisation	Auto- and/or alloimmunisation
Multiple inclusions	Yes	No
N of PC transfusions	765	1129
Type of platelet products	BC	BC
Reference product	Plasma	Plasma
Study product	PAS II	PAS III +/- PR
Storage	1 – 5 days	1 – 7 days
N of study transfusions/patient	Maximal 8	Maximal 5

N = number; RCT = Randomised Controlled Trial; PC = Platelet concentrate; BC = Buffy coat; PR = Pathogen Reduction

Statistical analysis

Chi-square tests were used to compare categorical patient characteristics by arm, ordinal and continuous patient were compared using ANOVA. For the statistical comparison of pre- and post transfusion platelet count, count increments (CI) and corrected count increments (CCI) we used an averaged mean per patient to correct for interdependence of consecutive platelet transfusions within a patient. For each of both trials, we performed a multivariate analysis for the effect of several patient variables (sex, age, body surface area, enlarged spleen, pre transfusion platelet count and therapy) and product factors (storage medium, product platelet content and storage time) on post transfusion platelet count. Adverse transfusion reactions were analysed intention-to-treat both on patient as well on transfusion level using the full data set of both trials through tabulation and compared using a chi-square test. All statistical analyses were performed using SSPS (version 15.0 for Windows, Chicago, Il, USA). P-values < 0.05 were considered significant.

RESULTS

PC transfusion characteristics and transfusion efficacy

Randomisation in both RCTs led to well balanced patient characteristics in both studies
(table 2). The inclusion of only the PC transfusions of the first inclusion episode of a patient
in trial 1 and including only the first 5 on protocol PC transfusions in both trials resulted
in 424 PC transfusions to 129 patients in trial 1 and 517 to 187 patients in trial 2. By design
PCs in trial 1 had a mean storage time of 3.5 ± 1.2 days as opposed to 4.0 ± 1.9 in trial 2
($p<0.001$). Comparison of the transfusion efficacy of both plasma PC arms showed several
significant differences. As opposed to trial 1, PCs in trial 2 were transfused at a higher pre
transfusion platelet count (mean difference 4.2 (1.3 – 6.9)), plasma PCs contained less
platelets (mean difference 32 (14 – 51)) and resulted in a significantly higher 24-hour
post transfusion platelet count (mean difference 7.3 (2.5 – 12.1)) and 24-hour CCI (mean
difference 2.3 (0.3 – 4.4)). For this reason, we decided not to combine the plasma control
arms, but to compare both PASs to their respective controls (table 3, figure 2). Both the
1-hour and 24-hour CIs and CCIs of PAS II PCs were significantly lower as compared to
plasma. In contrast, only the 1-hour CI of PAS III was significantly lower than plasma.
Despite all PCs show a decreased efficacy with increasing storage time, in a multivariate
analysis storage interval was a non-significant futile factor in both trials. Only PAS II had
an independent negative effect on the 1-hour transfusion efficacy. As is shown in table 4,
pre transfusion platelet count and product platelet content are consistently associated
with higher post transfusion platelet increment. Body surface area and acute myeloid
leukaemia were associated with a decreased post transfusion platelet increment in both
trials, whereas an enlarged spleen remarkably only negatively affected the increments in
trial 1. Unfortunately we were not informed about the magnitude of the splenomegaly.

Table 2: *Patient and transfusion characteristics*

		Trial 1		Trial 2	
		Plasma	PAS II	Plasma	PAS III
n Patients		**62**	**67**	**96**	**91**
Sex	M/F	39 / 23	45 / 22	50 / 46	52 / 39
Age	Years ± SD	53 ± 14	49 ± 14	54 ± 13	54 ± 13
Body surface area	M² ± SD	1.9 ± 0.2	1.9 ± 0.2	1.9 ± 0.2	1.9 ± 0.2
Acute myeloid leukemia	N (%)	29 (47)	30 (45)	42 (44)	51 (56)
Remission induction Ctx	N (%)	28 (45)	28 (42)	46 (48)	44 (48)
Enlarged spleen	N (%)	5 (8)	8 (12)	10 (11)	5 (6)
n Platelet transfusions		**198**	**226**	**269**	**248**
Storage time	Days ± SD	3.5 ± 1.1	3.5 ± 0.8	4.0 ± 1.5	3.7 ± 1.4
Platelet content	10^9 ± SD	408 ± 62	390 ± 88	376 ± 50	355 ± 43[1]

Ctx = Chemotherapy; [1]p < 0.05 as compared to the respective plasma arm.

Table 3: *Transfusion efficacy.*

	Trial 1			24-hour CCI		
	Plasma	PAS II	p-value[1]	Plasma	PAS III	p-value[1]
Pre count	13 ± 7	14 ± 9	0.425	17 ± 11	15 ± 9	0.174
1-hour						
CI (10⁹/l)	33 ± 15	25 ± 12	0.001	34 ± 15	28 ± 13	0.012
CCI	15.7 ± 5.9	12.0 ± 5.5	<0.001	17.0 ± 7.4	15.2 ± 6.6	0.079
24-hour						
CI (10⁹/l)	21 ± 11	16 ± 11	0.017	24 ± 14	21 ± 13	0.189
CCI	10.0 ± 5.0	8.0 ± 5.0	0.026	12.3 ± 7.8	11.7 ± 7.7	0.605

[1]*Univariate p-value correcting for interdependence of consecutive PC transfusions using an Averaged mean per patient.*

Figure 2: *Figure 2 shows the estimated mean difference and 95% confidence interval for the 1- and 24-hour CCIs comparing both PASs to their own Plasma control. The dashed lines represent the non-inferiority margins as were used in both Trials.*
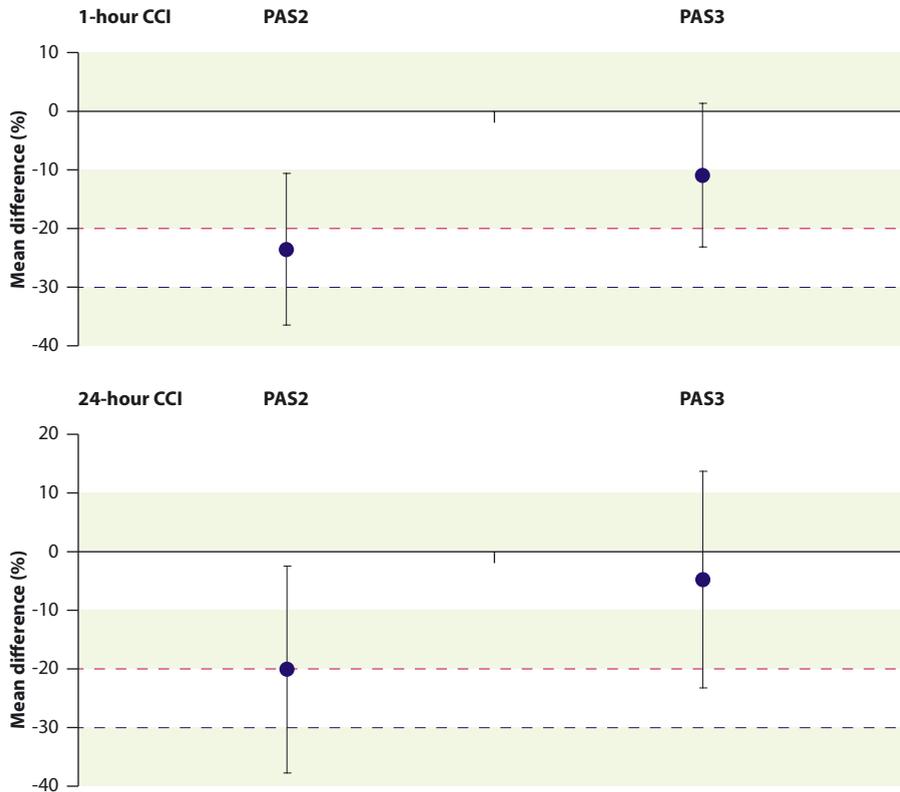
Table 4: *Multivariate analysis of post transfusion PLT count.*

Post count	Trial 1		Trial 2	
	1-hour	24-hour	1-hour	24-hour
Additive solution	-7.07 (-11.1; -3.00)	-3.02 (-6.72; 0.69)	-2.95 (-6.93; 1.02)	-2.31 (-6.36; 1.73)
Pre count	0.72 (0.46; 0.97)	0.90 (0.66; 1.13)	0.80 (0.59; 1.00)	0.90 (0.69; 1.11)
Storage time	-2.19 (-4.50; 0.11)	-0.609 (-2.69; 1.48)	-1.02 (-2.35; 0.31)	-0.49(-1.91; 0.93)
PLT content	0.05 (0.02; 0.08)	0.04 (0.02; 0.07)	0.08 (0.04; 0.12)	0.00 (-0.04; 0.05)
BSA	-20.2 (-28.9; -11.5)	-11.0 (-19.0; -3.06)	-24.2 (-34.1; -14.3)	-12.7 (-22.9; -2.58)
Female	-2.28 (-7.42; 2.87)	0.63 (-4.10; 5.35)	2.76 (-1.86; 7.38)	-0.90 (-5.82; 4.01)
Enlarged spleen	-11.4 (-18.2; -4.51)	-7.37 (-13.7; -1.09)	-1.22 (-3.26; 0.81)	-1.22 (-3.31; 0.87)
AML	-2.21 (-6.31; 1.90)	-4.16 (-7.87; -0.44)	-4.71 (-8.65; -0.77)	-4.37 (-8.47; -0.27)
RI Chemotherapy	3.04 (-2.04; 8.12)	1.65 (-3.00; 6.30)	1.87 (-2.65; 6.39)	-0.65 (-5.33; 4.03)

Beta: regression coefficient. Multivariate linear regression of the 1- and 24 post transfusion PLT count (averaged mean per patient in both trials). The estimated regression coefficient is shown in the columns, measuring the strength of the effect per unit of change of the corresponding factor. BSA = Body Surface Area; AML = Acute Myeloid Leukaemia; RI = Remission Induction.

Adverse transfusion reactions

Transfusion reactions were a secondary endpoint in both RCTs. In both trials the vast majority of adverse transfusion reactions were mild without significant morbidity. An intention-to-treat analysis, combining the results of both trials showed that 9.0% of the patients randomised to receive platelets stored in additive solution experience transfusion reactions, without differences between the type of PAS, as compared to 13.1% of patients randomised to receive plasma stored platelets (OR 0.7, 95%CI 0.3 – 1.3). In the combined additive arms 2.2% of the PC transfusions resulted in an adverse transfusion reaction as compared to 4.5% in the plasma arms (OR 0.5, 95%CI 0.3 – 0.9). Limiting this analysis to the selection of patients and transfusions evaluated in this study the OR for patients treated with additive stored platelets to experience a transfusion reaction is 0.4 (95%CI 0.2 – 1.0) and the OR for additive stored PCs to result in an adverse reaction 0.6 (95%CI 0.3 – 1.1).

DISCUSSION

For blood bank logistical and economical reasons, the use of an additive solution allowing for storage up to 7 days would be very attractive. By analysing the data of two trials, we have compared the transfusion efficacy of PAS II PCs and PAS III PCs relative to their own plasma PC controls. Because comparison of both control arms showed several significant differences we did not choose to pool the plasma controls, which would have enabled a direct comparison. The CCI-1 of PAS II (stored up to 5 days) was 23.6% (95%CI 10.6; 36.5) lower as compared to plasma, whereas PAS III (stored up to 7 days) showed a reduction of 10.9% (95%CI -1.3; 23.2). The same effect was observed with regard to the 24-hour CCIs. As was previously reported there were no haemostatic consequences of the observed decrease in transfusion efficacy, nor did the decreased efficacy lead to differences in transfusion interval or number of PC transfused.[8, 14] Mild adverse transfusion reactions occurred less frequent after transfusion with platelets stored in an additive solution in both trials and combining both trials results in an estimated risk reduction of 50% (95%CI 10 – 72%, p = 0.025).

A trial, comparing PAS II PCs stored 1-5 days with PAS II PCs stored 6-7 days in a paired fashion, showed that the mean 1- and 24-hour CCI of 6-7 day stored PAS II PCs was 7.4 ± 3.8 and 2.6 ± 2.6, respectively.[9] Both these mean CCI values could be conceived as transfusion failures. We did not study 6-7 days stored PAS II platelets, but we estimate by extrapolation of our data (estimated mean CCI-1 and CCI-24 for 6 – 7 day stored platelets in PAS II of 6.1 and 4.9 respectively), consistency with the data from Diedrich et al.[9] The results of our analysis strongly suggest that platelets stored in PAS III have superior clinical efficacy compared to PAS II stored PC and enable extension of storage time to 7 days without a clinically relevant decrease in transfusion efficacy.

The main limitation of this study is the indirect nature of the comparison; despite at first glance both trials appear very similar, there are a number of important differences potentially affecting efficacy such as pre-transfusion platelet count and platelet content of the product and these could not be corrected for by better matching and thus prohibited pooling of the plasma PC arms from the two RCTs.

Nevertheless the results support to replace PASII PC by PAS III PCs, stored up to 7 days, as an acceptable alternative for plasma PCs for routine transfusion practice. The development of additives with the addition of potassium and magnesium to PAS III are expected to further improve platelet storage conditions.[16] Lacking informative pre-clinical methods however, new platelet products need to be tested for their efficacy as well as haemostatic properties compared to plasma PCs, still gold standard, in clinical studies to avoid as formulated by Scott Murphy a downward creep.

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Chapter 6

The clinical impact of platelet refractoriness: Correlation with bleeding and survival

Jean-Louis H Kerkhoffs[1], Jeroen CJ Eikenboom[2],
Leo MG van de Watering[1], Rinie J van Wordragen-Vlaswinkel[3], Pierre W Wijermans[4], Anneke Brand[1,3]

[1]Sanquin Blood Bank, South West Region, Leiden;
[2]Dept of Hematology, Thrombosis and Hemostasis, Leiden University Medical Centre, Leiden;
[3]Dept of Immunohematology and Blood Transfusion, Leiden University Medical Centre, Leiden;
[4]Dept of Hematology, HagaZiekenhuis, The Hague, The Netherlands

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Background

Despite supportive care with platelet transfusions bleeding complications occur in a substantial number of patients with thrombocytopenia due to cytotoxic therapy. Moreover, refractoriness to platelet transfusions remains a frequently encountered problem. We investigated the clinical impact of platelet transfusion failure in 117 patients, part of a randomised platelet transfusion trial, which excluded patients with HLA- and/or HPA-alloantibodies.

Study design and methods

Between October 2003 and April 2005 a multicenter randomized controlled trial, testing the clinical efficacy of platelets stored in plasma as compared to PAS II, was performed. Using multiple regression analysis of observational data of patients randomised in one of the participating centres, the occurrence of platelet transfusion refractoriness was analysed for a relation with bleeding complications and patient survival.

Results

Platelet transfusion failure occurred at least once in 49.6% of the patients. Mild to moderate bleeding complications occurred in 19% of the patients. Platelet transfusion failure was, independently from thrombocytopenia, positively associated with bleeding complications (OR 3.4, 95%CI 1.1 - 11). Other independent risk factors were non-transplant related chemotherapy, severe mucosal damage and age. Moreover, patients experiencing one or more 24-hour platelet transfusion failures had, as compared to patients always showing a sufficient 24-hour increment, a significantly reduced median survival of 491 days (IQR 156-858) versus 825 days (IQR 355-996), respectively. In a Cox regression model the effect on survival was independent of therapy, diagnosis and age.

Conclusion

Our results suggest that platelet transfusion failure might be a sensitive clinical marker for the occurrence of bleeding and impaired patient survival. Platelet transfusion failure, bleeding complications and decreased survival could be manifestations of a more severe degree of endothelial damage. This poses a challenge to develop potential markers and improved treatment options in relation to the clinical efficacy of platelet transfusions.

Abbreviations

AML = Acute Myeloid Leukemia; ATG = Anti Thymocyte Globulin; CCI = Corrected Count Increment; CI = Confidence Interval; CTC = Common Toxicity Criteria; HLA = Human Leukocyte Antigen; HPA = Human Platelet Antigen; IQR = Inter-Quartile range; OR = Odds Ratio; NHL = Non Hodgkin Lymphoma; PAS = Platelet Additive Solution; PC = Platelet Concentrate; RBC = Red Blood cell Concentrate; RCT = Randomized Controlled Trial; TBI = Total Body Irradiation; TRAP = Trial to Reduce Alloimmunization to Platelets; WHO = World Health Organization.

Transfusion support with PCs is widely applied and accepted for the prevention and
treatment of bleeding in patients with thrombocytopenia, due to bone marrow diseases or
cytotoxic therapy, and thrombocytopathy.[1] Although not without limitations, the efficacy
of platelet transfusions usually is expressed as count increment, adjusted for platelet
dosage and patient size, resulting in recovery, survival or CCI.[2] Applying internationally
accepted, but arbitrary definitions, platelet transfusion failure occurs in 25 – 70% of
multiple transfused patients and up to 30 – 50% of platelet transfusions.[3-9] An analysis
of the outcome of 6379 transfusions in the TRAP study showed that prior pregnancies,
male gender, an enlarged spleen, bleeding, fever, infection, disseminated intravascular
coagulation, increasing height and weight, lymphocytotoxic antibody positivity, number of
platelet transfusions, heparin therapy or amphotericin treatment were related to decreased
posttransfusion platelet responses.[10, 11] The potential role of endothelial damage in relation
to increased platelet consumption in non-immunological platelet transfusion failure has
been suggested by several authors.[3, 8, 11] In a large study comprising 1402 patients after
bone marrow transplantation Nevo and colleagues found bleeding was significantly
associated with reduced survival, independent of type of transplantation, stem cell
source and diagnosis.[12] They conclude that the bleeding complications may be regarded
as a marker of multifactorial clinical deterioration. Interestingly, in a study evaluating
a restrictive platelet transfusion threshold, this group showed an association between
profound thrombocytopenia and reduced survival also in non-bleeding patients.
Exposure to profound thrombocytopenia was significantly increased when a restrictive
prophylactic threshold was used. A relation with increased platelet consumption due
to endothelial damage was argued.[13] These findings underline the need for the
identification of more specific risk factors for the occurrence of thrombocytopenia,
bleeding and survival, in order to improve supportive care strategies.[14, 15] We evaluated
whether platelet transfusion failure is associated with bleeding complications and
decreased survival and could serve as a signal for additional diagnostic tests and/or
adjustment of supportive care treatment .

MATERIALS AND METHODS

Between October 2003 and April 2005 a RCT, testing the clinical efficacy of platelets stored in plasma as compared to PAS II, was performed.[16] For the current analysis only patients randomised in the Leiden University Medical Centre were evaluated. All patients older than 18 years who needed or were expected to need more than two PC transfusions were eligible. Patients with HLA- and/or HPA- alloantibodies and active immune thrombocytopenia were excluded. An inclusion cycle was restricted to a maximum of 30 days and/or 8 platelet transfusions, whichever occurred first. In the RCT, patients were allowed to participate several cycles (i.e. during a remission-induction course, consolidation chemotherapy or stem cell transplantation). For the current analysis only the events during the first inclusion were used.

After randomisation, age, sex, height, weight, diagnosis, existence of an enlarged spleen, WHO performance status, medical history (including transfusion history), prior bleeding and medication (including treatment schedule) were recorded. During the inclusion periods platelet and red cell transfusions were monitored as well as transfusion related adverse reactions, mucosal damage (CTC, version 2.0, http://ctep.info.nih.gov/reporting/ctc.html), fever (defined as body temperature > 38°C), infections (defined as a positive culture and/or radiologic evidence for infection) and the occurrence of bleeding complications. Bleeding was graded according to the WHO criteria.[17] All parameters were reviewed on a daily basis by the attending physician and recorded on case report forms. Hospital records were used in regard to survival. The first day of the first inclusion cycle was considered day zero.

All PCs were prepared from 5 pooled whole-blood buffy coats, leukocyte depleted and subsequently stored up to 5 days in plasma or PASII. The treating physician ordered platelet transfusions according to hospital guidelines either for prophylactic or therapeutic indications, using a restrictive policy with a threshold of 10×10^9/l in non-bleeding, stable patients. The 1- and 24-hour CCIs were calculated according to the method described in our previous study.[16] Platelet transfusion refractoriness or failure was defined as a 1-hour CCI below 7.5 and/or a 24-hour CCI below 4.5. In patients experiencing repeated episodes of refractoriness without an apparent clinical cause, tests for the existence of HLA- and/or HPA- allo antibodies (PAK12, GTI Waukesha, USA) were performed.

Statistical methods

Fisher exact tests were used to compare the categorical variables. Continuous variables were tested using ANOVA or, when non-normally distributed, Mann-Whitney tests. Univariate analysis was performed to identify factors at randomisation and during the study cycle associated with the occurrence of bleeding complications. Factors with a p-value of ≤ 0.20 combined with known factors from the literature, e.g. prior bleeding, were included in a multivariate analysis.[11, 18] The data are presented as Odds ratios, with 95% confidence intervals. We performed a sequential analysis according to Wald and Barnard to investigate the temporal relationship of bleeding complications and platelet transfusion failure.[19]

To study the relation between platelet transfusion failure and survival, we performed a backward conditional Cox regression analysis initially correcting for patient age, diagnosis, and treatment schedule. All analyses were performed using SPSS/PC+ (version 14.0, Chicago, IL).

RESULTS

Patient characteristics

117 patients were randomized for a total of 151 inclusion cycles to receive either
plasma stored or PAS II stored PCs (n = 93 one cycle, n = 24 more than one cycle).
Table 1 summarizes the main characteristics, including events and complications during
the first inclusion cycle. The non-AML group consisted of patients with NHL (n = 26),
multiple myeloma (n = 14), acute lymphatic leukaemia (n = 10), chronic myeloid leukaemia
(n = 8), chronic lymphatic leukaemia (n = 3), aplastic anemia (n = 2) and one patient with
carcinoma of the testes. The vast majority of the remission-induction and consolidation
courses were anthracycline based, in patients with AML combined with cytarabine.
Most included patients with NHL were treated with busulfan, etoposide, cytarabine and
melphalan, followed by autologous stem cell transplantation. Patients with myeloma were
conditioned for autologous stem cell transplantation using high dose melphalan. A total of
31 allogeneic stem cell transplantations were performed, using both related and unrelated
stem cell donors, with a myeloablative scheme (cyclophosphamide / total body irradiation)
in 22 patients and 9 using a reduced intensity scheme based on ATG and fludarabine.
All allogeneic stem cells were in-vitro treated with alemtuzumab. Acute Graft versus
host disease was seen in 6% of the allogeneic transplantations. Most of the infectious
complications were bacterial (49%). 15% of the patients had a fungal infection of which
2 patients had pulmonary aspergillosis. Only one of these patients eventually received
amphotericine B. Combined infections were not infrequent. Severe mucosal damage was
encountered in 15% of the patients.

Red cell and platelet transfusions

During the first inclusion cycle the total number of transfused RBCs and PCs was 678
and 486, respectively. The mean consumption of RBCs and PCs per patient in the first
cycle is shown in Table 1. ABO major incompatibility was present in only 3.6% of the PC
transfusions. PC transfusion failures occurred at least once in 58 patients (49.6%). Of the
patients with PC transfusion failure, 31% experienced only one PC transfusion failure,
whereas in 69% failure occurred more then once. The mean use of both PCs as well as RBCs
was significantly higher in patients with PC transfusion failures, 6.4 ± 2.5 and 7.2 ± 3.6 as
compared to 2.9 ± 1.8 and 4.5 ± 3.7, respectively. Patients with prior bleeding, bleeding
complications, febrile and infectious complications were more likely to experience
PC transfusion failure. On the other hand, patients undergoing autologous stem cell
transplantation were less likely to experience PC transfusion failure (table 1, $p < 0.05$).

Table 1: *Patient characteristics, events and complications during first inclusion.*

	Patients	Without failure	With failure
	n = 117	n = 59	n = 58
At inclusion			
Male/female	78 / 39	38 / 21	40 / 18
Age (Years, mean ± sd)	48.9 ± 13.4	47.8 ± 14.1	49.9 ± 12.3
Age categories			
< 40 years	29	15	14
40 – 60 years	63	31	32
≥ 60 years	25	13	12
Body surface area (m^2, mean ± sd)	1.94 ± 0.23	1.94 ± 0.23	1.94 ± 0.23
Prior bleeding	17	4	13
Enlarged spleen	11	4	7
Number of inclusions			
1 inclusion	93	49	44
> 1 inclusion	24	10	14
Inclusion time (days, mean ± sd)	20.5 ± 7.6	20.4 ± 7.8	20.6 ± 8.0
Diagnosis			
AML	53	22	31
Non AML	64	37	27
Chemotherapy	60	24	36
Stem cell transplant	57	35	22
Allogeneic transplantation	31	17	14
Autologous transplantation	26	18	8
ATG	10	3	7
TBI	22	15	7
During follow up			
Complications			
Fever	71	28	43
Infections	68	27	41
Mucosal damage	88	43	45
Severe mucosal damage (grade III-IV)	18	5	13
Bleeding complications	22	5	17
n days with PLT counts ≤ 10 x 10^9/l			
0 days	35	16	19
1-3 days	58	39	19
≥ 4 days	24	4	20
Red cell and platelet transfusions (mean ± sd)			
Red cell transfusions	5.8 ± 3.9	4.5 ± 3.7	7.2 ± 3.6
Platelet transfusions	4.3 ± 2.4	2.9 ± 1.8	6.4 ± 2.5
Follow up and survival			
Follow up (months, mean ± sd)	20.9 ± 12.5	22.9 ± 11.8	19.0 ± 13.0
Lost to follow up	2	1	1
Survival	56	33	23

Numbers of patients, unless otherwise specified.

Bleeding complications

During the first inclusion cycle bleeding occurred in 22 patients (19%), mostly grades I and II. No lethal bleeding occurred during the first inclusion cycle, however during follow up 4 patients died with severe bleeding complications. Two patients died of an intracranial haemorrhage and two with recurrent AML and graft versus host disease, with concomitant severe bleeding. 45% of our study population consisted of patients with AML, mostly receiving remission-induction chemotherapy. In this category bleeding incidence was 39%. Although we could not find a direct correlation between platelet count and bleeding complications, patients with bleeding had more days of mean platelet counts below $30 \times 10^9/l$ (p < 0.01, figure 1). In the PC transfusion failure group there were significantly more patients with a platelet count of $\leq 10 \times 10^9/l$ for ≥ 4 days. Table 2 shows the risk for bleeding complications according to patient characteristics. In the multivariate analysis PC transfusion failure remained significantly associated with bleeding complications (OR 3.4, 95% CI 1.1 – 11), as were non-transplant related chemotherapy, severe mucosal damage and age. The level of thrombocytopenia was not significant in the multivariate analysis. Sequential analysis showed no temporal pattern between the occurrence of PC transfusion failure and bleeding complications (data not shown). The mean RBC consumption was 7.9 ± 4.0 in bleeding patients, versus 5.3 ± 3.7 in non bleeding patients (p < 0.01). The mean PC consumption was 6.0 ± 2.1 and 3.9 ± 2.3, respectively (p < 0.01).

Figure 1: *Figure 1 shows the distribution of daily mean platelet count expressed as a percentage of all daily mean platelet counts over different strata of platelet counts for bleeding (285 days in 22 patients) and non-bleeding patients (1202 days in 95 patients). The same distribution was seen in the daily minimal platelet counts. The median number of days with minimal platelet counts $\leq 10 \times 10^9/l$ was 1 day(0 – 8 days) in non-bleeding patients as compared to 3 days (0 – 10 days) in bleeding patients (p = 0.034). The median number of days with minimal platelet counts $\leq 30 \times 10^9/l$ was 4 days (0 – 18 days) and 7 days (0 – 17 days), respectively (p = 0.12).*
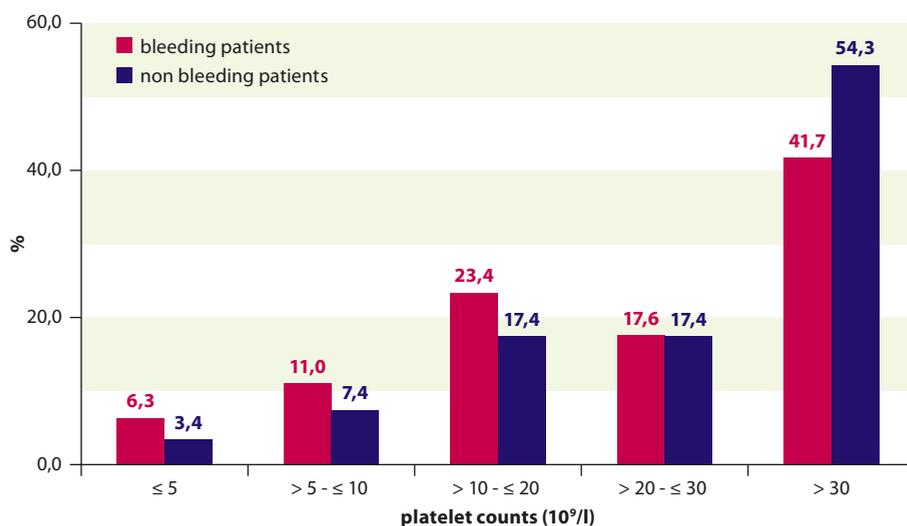
Table 2: *Relative risk for bleeding according to patient characteristics.*

|	Univariate analysis			Multivariate analysis		
	OR	95% CI	P	OR	95% CI	P
Chemotherapy[1]	5.7	(1.8-18)	0.003	12.5	(2.4-65)	0.003
PC transfusion failure	4.5	(1.5-13)	0.006	3.4	(1.1-11)	0.04
Severe mucosal damage	3.6	(1.2-11)	0.023	5.0	(1.3-19)	0.021
Age ≥ 60 years	1.6	(0.8-3.2)	0.194	2.7	(1.1-6.7)	0.038
Fever	3.6	(1.1-11.3)	0.031	2.2	(0.6-8.5)	0.243
Enlarged spleen	2.8	(0.7-11)	0.130	2.2	(0.39-12)	0.369
AML	2.0	(0.8-5.1)	0.154	0.3	(0.1-1.2)	0.091
Male sex	1.4	(0.5-4.0)	0.505	1.7	(0.5-5.9)	0.401
Prior bleeding	1.3	(0.4-4.4)	0.687	0.5	(0.1-2.3)	0.381
≥ 4 days PLT < 10 x 10^9/l	3.7	(1.3-10.2)	0.012	1.1	(0.3- 4.0)	0.867

[1]*Non-transplant related chemotherapy, i.e. remission induction and consolidation chemotherapy.*

PC transfusion failure and survival

For this analysis we only used the 24-hour CCIs. In 5 of the 117 patients there were insufficient PC transfusion data to calculate the percentage of 24-hour transfusion failure. Two other patients were lost to follow up. Of the remaining 110 patients 61 did not experience 24-hour transfusion failure versus 49 patients, experiencing one or more episodes of transfusion failure. The 100-day survival in both groups was 98% versus 83%, respectively (p < 0.01). The median survival in both groups was 825 (IQR 355 – 996) days and 491 (IQR 156 – 858) days, respectively (p = 0.032). Figure 2 shows the survival curves of patients with and without 24-hour transfusion failure. The difference between both groups remained significant, using backwards conditional Cox Regression analysis correcting for age, diagnosis, treatment and level of thrombocytopenia.
The effect was more pronounced in patients with acute myeloid leukemia. If we perform the analysis for the effect of 1-hour transfusion failure, there was only a less outspoken trend towards reduced survival (p = 0.11).

Figure 2: *Figure 2 shows the survival of patients with and without 24-hour platelet transfusion failure, during the first inclusion cycle. A Cox regression model was used to adjust for age, diagnosis, treatment and level of thrombocytopenia.*
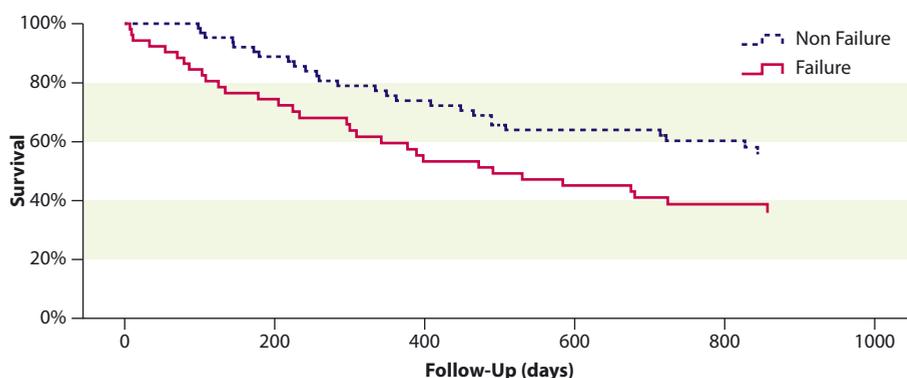
DISCUSSION

We analyzed the relationship between the occurrence of platelet transfusion failure(s), bleeding complications and patient survival. Our results confirm the frequent occurrence of non-immunological platelet refractoriness. In the first analysis of this study we already showed that patient-related factors were the main determinants of transfusion failure, independent of product factors such as storage medium and storage time.[16] This is in agreement with a recently published study showing that storage duration of both buffy-coat PCs as well as apheresis PCs only explained for less then 4% of the variation in CCI and transfusion interval.[20]

The relationship between platelet count and bleeding has been reported with conflicting results. A large study in 2,942 patients did not show a relationship with platelet counts and bleeding.[24] Until now, only one previous study reported an association between PC transfusion failures and bleeding complications. In that study bleeding complications were significantly associated with an increased risk of 1- and 24-hours PC transfusion failure (Hazard ratio 2.0).[11]

The overall bleeding incidence in our study is less than reported in the literature, presumably related to the relatively short observation period including only the first randomisation cycle.[12, 21-23] We could confirm an association between PC transfusion failures and bleeding complications. As shown in figure 1, bleeding patients had more pronounced thrombocytopenia as compared to non-bleeding patients, consistent with other studies, although this difference is relatively small.[22, 23] In the multivariate analysis the level of thrombocytopenia did not independently correlate with bleeding complications. Pathophysiological mechanisms as to how clinical factors contribute to decreasing transfusion efficacy and bleeding complications are not unravelled, but endothelial vascular damage caused by cytotoxic treatment or disease-related complications has been proposed as a potential mechanism.[8, 11, 24] This vascular damage could at least in part explain the not hitherto reported association of PC transfusion failure and patient survival. Impaired patient survival was previously observed in patients experiencing bleeding complications after stem cell transplantation and profound thrombocytopenia in non-bleeding patients.[12, 13]

In our study, we found no correlation between patient survival and bleeding complications or profound thrombocytopenia in non-bleeding patients. The most likely explanation for this discrepancy is the small number of patients and transfusion events in our study. Moreover our study not only included transplant procedures, but also remission-induction and consolidation chemotherapy. It might be that PC transfusion failure stronger predicts systemic platelet destruction caused by epithelial and endothelial cell damage, as compared to bleeding complications or the occurrence of profound thrombocytopenia.

Both in vitro, as well as in vivo evidence of endothelial cell damage and/or activation due to cytotoxic and irradiation therapy exists.[25-29] Clinically, endothelial cell damage can be the cause of bleeding complications, capillary leakage and proteinuria, as opposed to endothelial activation, which may be more related to thrombotic complications. All of these complications occur in patients treated with cytotoxic therapy. For instance, treatment with VEGF-inhibitors in oncology patients resulted in an increased incidence of both bleeding as well as thrombosis.[30, 31] Alas, all of these studies lack details on platelet transfusions and transfusion efficacy.

Recognizing the small sample size and retrospective nature of our study, we have shown an association between PC transfusion refractoriness and the occurrence of bleeding and a reduced survival. Obviously, neither PC transfusion failure nor bleeding is causally related to death. Rather the associations found could be related to the level of endothelial cell damage, which in turn contributes, by an unknown mechanism, to decreased survival. Incorporation of markers for endothelial damage or activation may be helpful to explain transfusion refractoriness and may ultimately lead to treatment options.

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Chapter 7

The observation of bleeding complications in hemato-oncological patients; results of a pilot study.

Paula F Ypma[1], Jean-Louis H Kerkhoffs[1, 2],
Joost A van Hilten[2], Rutger A Middelburg[3],
Miriam Coccoris[2], Okke Eissen[2], Rinie J van Wordragen-
Vlaswinkel[3], Jaap J Zwaginga[3], Erik M Beckers[4],
Rob Fijnheer[5], Pieter F van der Meer[2], Anneke Brand[2, 3]

[1]Dept of Hematology, HAGA teaching Hospital Den Haag;
[2]Sanquin Research, Dept of Transfusion Medicine, Leiden;
[3]Dept of ImmunoHematology and Blood transfusion service,
Leiden University Medical Centre, Leiden;
[4]Dept of Hematology, Maastricht University Medical Centre, Maastricht;
[5]Dept of Internal Medicine, Meander Medical Centre, Amersfoort;
The Netherlands

7 The observation of bleeding complications in hemato-oncological patients: results of a pilot study

Background

The reported percentage of patients experiencing bleeding complications is highly variable, ranging from 5 – 70%, posing a major problem for designing clinical platelet transfusion trials using bleeding complications as a primary endpoint. A pilot study was performed to access the percentage of patients with WHO grade 2 or higher bleeding in preparation of a large randomised controlled trial.

Study design and methods

We performed a prospective, observational study using a rigorous bleeding observation system. Endpoints of the study were the percentage of patients and days with bleeding WHO grade ≥ 2. The results were compared the previously reported HOVON study. Moreover, the impact of scoring successive days with stable skin bleeding was assessed.

Results

Bleeding grade ≥ 2 occurred in 37 patients (54%). The percentage of days with bleeding of grade > 2 was 18%. The administration of chemotherapy was the strongest predictor of grade > 2 bleeding. The vast majority of bleeding complications occurred mucocutaneously, largely explaining the difference with the HOVON trial. Censoring for stable skin bleeding had a profound effect on bleeding incidence per day.

Conclusion

The results of this study confirm the bleeding frequency reported in literature reporting on platelet transfusion studies provided a rigorous bleeding observation strategy is used. However, the clinical relevance of bleeding as an endpoint remains a matter of debate.

INTRODUCTION

Patients with hemato-oncological diseases receiving myelosuppressive chemotherapy or undergoing haematopoietic stem cell transplantation are supported with platelet transfusions to prevent or treat bleeding complications.[1] Despite several policy-driven trials the prophylactic platelet transfusion strategy has remained stable without major changes in the last four decades. In contrast, safety as well as economical concerns has led to several changes in the production of platelet products. Although several endpoints have been used as an endpoint for platelet transfusion trials, the Guidance for Industry For Platelet Testing and Evaluation of Platelet Substitute Products, published by the FDA in 1999 prescribes the recording of bleeding outcomes as a necessary activity.[2] However, as a consequence of the several different methods for the observation and grading of bleeding complications, passive versus active reporting, frequency of bleeding complications and differences in patient populations, the reported percentage of patients experiencing bleeding complications is highly variable.[3] A review of literature shows that the reported incidence of major bleeding (WHO grade ≥ 2) varies from 5 to 70% (figure 1).[4, 5, 6, 7, 9, 12-32]

Figure 1: *Figure 1 shows reported percentages of patients with grade 2 – 4 bleeding complication. The error bars reflect the 95% confidence interval. The filled markers represent randomised controlled trials, whereas the open markers represent observational studies. 1 = Blumberg et al[14]; 2 = Sensebe et al[15]; 3 = Kerkhoffs et al[7]; 4 = Oka et al[16]; 5 = Tinmouth et al[17]; 6 = Gil-Fernandez et al[18]; 7 = Zumberg et al[19]; 8 = Kerkhoffs et al[6]; 9 = Mirasol[20]; 10 = Diedrich et al[21]; 11 = Wandt et al[22]; 12 = Rebulla et al[12]; 13 = Gmur[23]; 14 = Nevo et al[9]; 15 = Wandt et al[24]; 16 = Sagmeister et al[25]; 17 = Murphy et al[26]; 18 Pihush et al[27]; 19 = Higby et al[28]; 20 = Navarro et al[29]; 21 = Heddle et al[5]; 22 = McCullough et al[13]; 23 = Lawrence et al[30]; 24 = Friedmann et al[31]; 25 = Slichter et al[4].*

Grade 2-4 bleeding in literature
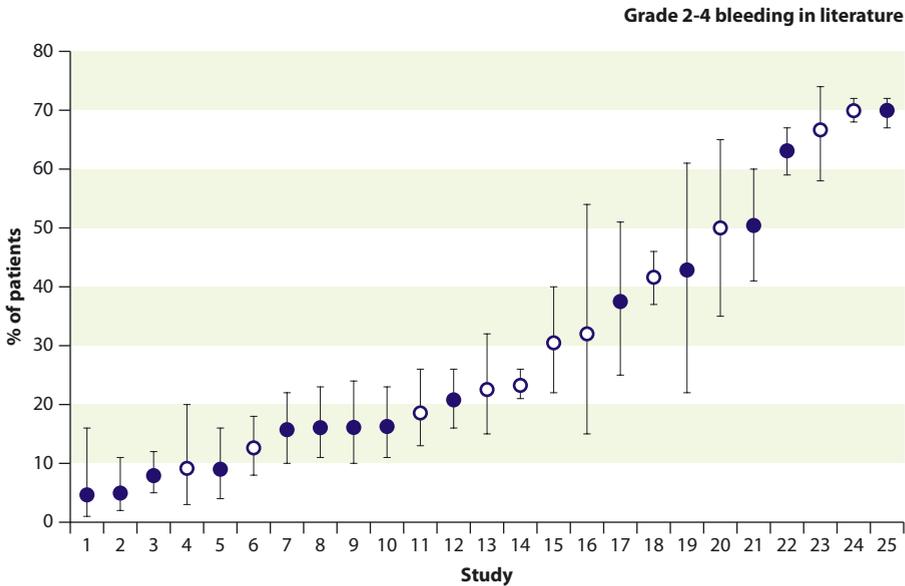
This variability poses a major problem for designing clinical platelet transfusion trials using bleeding complications as a primary endpoint. In studies using a rigorous bleeding observation and adjudication process to assess bleeding, at least one episode of major bleeding (WHO > grade 2) is reported in up to 70% of patients and on 16% of thrombocytopenic days.[4, 5] In contrast, studies that relied on physician's bedside assessment of bleeding complications reported much lower incidences of major bleeding.[6, 7] Recently, we initiated a platelet transfusion trial studying the haemostatic efficacy of transfused platelets treated with an alternative pathogen reduction technique (PREPAReS; Pathogen Reduction Evaluation & Predictive Analytical Rating Score). As bleeding complications are the intended primary outcome of this study, we performed a pilot study to investigate the percentage of patients with major bleeding complications in the Dutch situation using a rigorous bleeding observation system. Secondary endpoints were the percentage of patients experiencing any bleeding complication (WHO grade 1-4) and the percentage of days with WHO grade ≥ 2 bleeding complications. Moreover we have compared the data from this pilot study with the bleeding data from a previous study (HOVON) testing pathogen reduced platelet products.[7]

METHODS

We performed a prospective observational study, including patients ≥ 18 years, admitted to the hospital for receiving high dose chemotherapy or stem cell transplantation for hemato-oncological disease expected to need platelet transfusions during their admittance. Exclusion criteria were suspicion of microangiopathic thrombocytopenia, the use of anticoagulant drugs or active bleeding (grade > 2) at the time of inclusion. The study protocol and consent forms were approved both by a central ethics committee and local institutional review boards. In total, four haematological centres participated in the study, two academic medical centres and two large general hospitals (HIC, Haematological Intensive Care centres). Hospital discharge, death or patients' refusal to continue were reasons to go off protocol. Daily assessment of bleeding symptoms for 8 World Health Organisation (WHO) defined sites (oral, nasal; skin, soft tissue, musculoskeletal; gastrointestinal; genitourinary; pulmonary; body cavity; central nervous system; invasive sites) was performed by trained physicians, nurses or research staff members in each of the four hospitals including physical examination and a patient's interview. Adjudication of grades of bleeding according to the WHO criteria was performed by two clinicians, independently.[8] Platelet transfusions were administered in general if the morning platelet count was below the trigger (10×10^9/L) or at the treating physicians' initiative if the patient experienced bleeding or using a higher trigger in certain clinical circumstances. Platelet transfusions consisted of a mean of 350×10^9 buffy-coat derived platelets. Red cell transfusions were administered below an age-dependent trigger as described in national or local transfusion guidelines. Besides bleeding, each day a blood cell count and transfusion requirements were recorded. For the comparison with the previously published HOVON study, we extracted all bleeding data from the case report forms of that study.[7] Primary endpoint of the study was the percentage of patients experiencing bleeding WHO grade ≥ 2. Secondary outcome measures were the percentage of patients experiencing bleeding of any grade and the percentage of days with bleeding WHO grade ≥ 2.

Statistical analysis

Categorical patient characteristics and bleeding complications are reported as percentages. Continuous data are presented as the mean with standard deviation (STD) for normally distributed variables and the median with (inter)quartile range (IQR) for other continuous variables not normally distributed. A univariate comparison of patients with and without WHO grade ≥ 2 bleeding was performed. A multivariate logistic regression analysis was performed including all parameters associated with bleeding ($p<0.1$) in the univariate analysis. Variables considered as possible consequences of bleeding rather than causes (number of platelet and red cell transfusions), were not considered for multivariable analyses. All statistical analyses were performed using SSPS (version 15.0, Chicago, IL). P values < 0.05 were considered statistically significant.

RESULTS

Patients

A total of 68 patients were enrolled at four sites (centre A, B, C and D) with a median follow up period of 20 days. The patients were not equally divided: centre A enrolled 10 patients, centre B 13 patients, centre C 28 patients and centre D 17 patients. Patient characteristics are summarized in table 1. More men than women were included and almost half of the patients suffered from acute leukaemia. The composition of the included patients does not differ substantially from the patients included in the HOVON trial. A small number of patients treated with a reduced intensity conditioning regimen did not reach a level of thrombocytopenia indicating a platelet transfusion; they were however not excluded from this analysis.

Table 1: *Patient characteristics.*

		N=68
Male / female	n / n	46 / 22
Age	years, mean(SD)	55 (±13)
Diagnosis		
acute leukemia	n (%)	31 (45%)
myeloma	n (%)	14 (21%)
lymphoma	n (%)	12 (18%)
other	n (%)	11 (16%)
Therapy		
chemotherapy	n (%)	30 (44%)
stem cell transplantation	n (%)	35 (52%)
other	n (%)	3 (4%)
Follow-up	days, median (IQR)	19 (14 - 26)
Mean hemoglobin	mmol/l, median (IQR)	5.9 (5.7 - 6.3)
Mean platelet count	10^9/L, median (IQR)	46 (30 - 74)
Platelet nadir	10^9/L, median (IQR)	7 (5 - 10)
Red blood cell transfusion (units)	n, median (IQR)	3.5 (1.3 - 7.8)
Platelet transfusion*	n, median (IQR)	2.0 (1.0 - 5.8)

*prepared from 5 buffy coats, pooled and prestorage filtered. n = numbers of patients or units of RBC/PC.

Primary endpoint

One or more episodes of grade ≥ 2 bleeding were experienced by 37 patients (54%).
Five patients (7%) suffered from a grade 3 or 4 bleeding and 1 patient died from a bleeding
complication. Fifty-nine (87%) patients experienced one or more bleeding complications
of any grade. Table 2 summarizes the bleeding complications by different bleeding sites
as defined by the WHO criteria. The vast majority of bleeding complications occurred on
mucocutaneous sites as expected. Figure 2 shows the comparison for the several bleeding
sites as reported in the HOVON studies. The striking difference in grade ≥ 2 bleeding
complications is almost completely explained by the difference in mucocutaneous
bleeding complications.

Table 2: *Bleeding complications (maximal score) by site.*

Bleeding site	Grade 1 bleeding	Grade ≥ 2 bleeding
	n	n
Oral cavity and nose	41	2
Skin, soft tissue and musculoskeletal	25	20
Digestive tract	-	14
Urogenital tract	-	-
Respiratory tract	-	6
Body cavity	-	3
Central nervous system / retina	-	4
Invasive site	-	3

n = number of patients

Figure 2: *Figure 2 shows the comparison between the BOP trial with the HOVON trial of
patients with bleeding WHO grade ≥ 2 grouped by the anatomical sites. The bars reflect 95%
confidence intervals.*
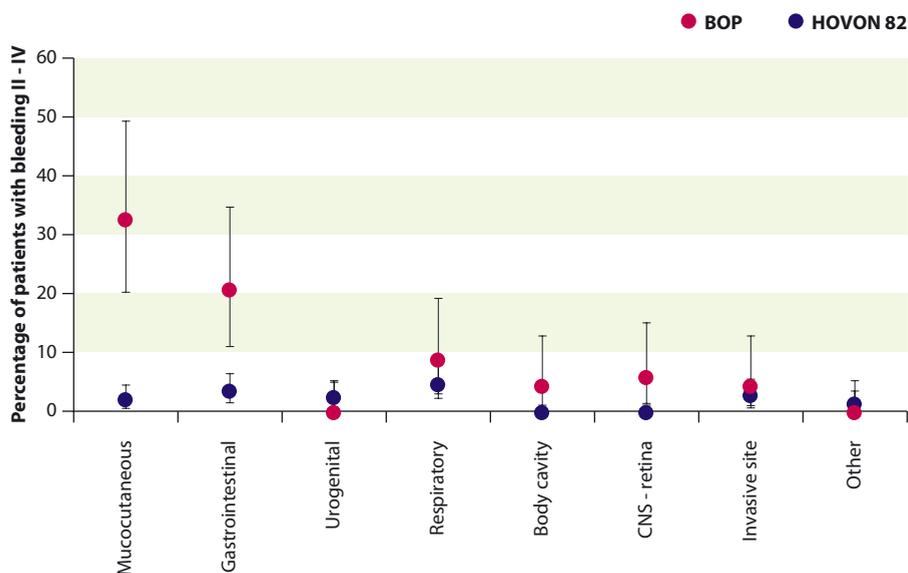
Secondary endpoints

The percentage of thrombocytopenic days with bleeding of any grade during the observation period was 42%. The percentage of days with bleeding grades> 2 for patients on study was 18%. Most of the grade > 2 bleedings occurred on the skin.
Including only newly perceived haematomas, the percentage of days with bleeding of grade > 2 was only 7.8%. Figure 3 shows the relation between platelet counts and percentage of days with grade II – IV bleeding with or without excluding persisting haematomas, again illustrating the effect of mucocutaneous bleeding on the total incidence of bleeding.

Figure 3: *Figure 3 shows the percentage of days with bleeding grade ≥ 2 with the 95% confidence interval according to the platelet count categories based on the days with both a platelet count measurement and information on bleeding (n = 901 days). The closed symbols represent the percentage of days with bleeding if all the events are included. The open symbols show the censored percentage after exclusion of successive days with stable skin bleeds.*
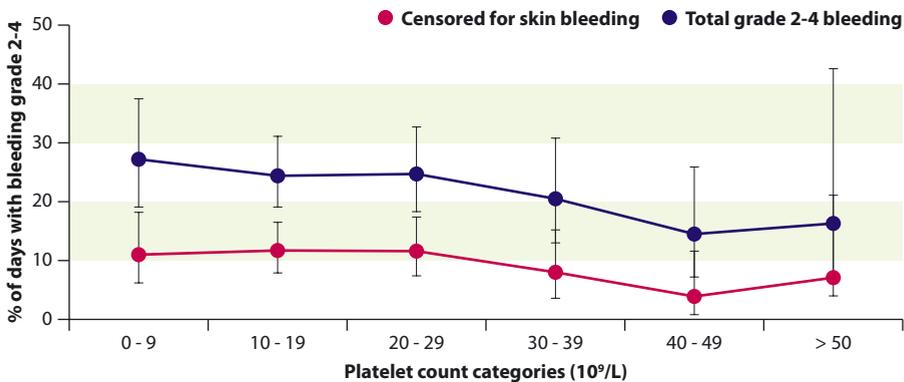
Univariate analysis indicated that patients in academic centers experienced less bleeding episodes as compared to patients in general hospitals (p = 0.05). Acute leukaemia as indication for treatment (compared to other haematological disorders) and the administration of chemotherapy (as opposed to transplantation therapy) resulted in significantly higher bleeding frequencies (table 3). A multivariate analysis showed that the administration of chemotherapy (remission–induction and consolidation chemotherapy) was the only independent predictor of grade > 2 bleeding in patients with hemato-oncological diseases receiving myelosuppressive therapy. Although maintained as a factor in the model of the backward multivariate analysis, a low mean platelet count only showed a trend of an association with bleeding (p = 0.08).

Table 3: *Statistical comparison of patients with bleeding grade ≥ 2 versus no or grade 1 bleeding.*

Bleeding site	No or grade 1 bleeding	Grade ≥ 2 bleeding
	n = 31	n = 37
Male / female; n / n	23 / 8	23 / 14
Age, years; median (range)	57 (19-70)	59 (23-77)
Acute Leukemia; n (%)	8 (26)	23 (74)[#]
Stem cell transplant; n (%)	25 (81)	13 (35)[#]
Academic center; n (%)	18 (58)	12 (32)
Mean Hb, mmol/L; median (range)	6.0 (5.2-7.5)	5.8 (5.2-8.4)
Mean PLT, 10^9/L; median (range)	67 (14-151)	39 (4-148)[#]
PLT Nadir, 10^9/L; median (range)	8 (2-134)	7 (1-22)
n of RBC; median (range)	2 (0-16)	4 (0-17)[#]
n of PC; median (range)	1 (0-16)	5 (0-18)[#]

*Hb = Hemoglobin; PL T = Platelets; RBC = Red blood cell concentrate; PC = Platelet concentrate; n = number of patients
or units; [#]p < 0.05 (univariate); in the multivariate analysis only stem cell transplantation was independently associated
with a decreased bleeding incidence (p < 0.01).*

DISCUSSION

This study was designed to explore a rigorous bleeding observation and adjudication
strategy in the setting of the preparation of a multicenter platelet transfusion trial.
We observed an incidence of WHO grade > 2 bleeding of 54% in our patient population
and in 18% of the days, which was in the range of other studies applying rigorous bleeding
assessment.[4, 5] The vast majority of the observed major bleeding complications consisted
of mucocutaneous bleeding. Moreover, we showed that a different way of assessment by
taking only newly appearing skin haemorrhages into account had a large impact on the
percentage of bleeding days. Comparison with the HOVON study, published in 2010, shows
that the main difference in bleeding score is explained by the difference in mucocutaneous
bleeding.[7] There was some concern that despite the defined rigorous bleeding observation
strategy variation between centers persisted, as revealed by univariate analyses, but this
factor disappeared in the multivariable analyses. Likely, different patient populations as
well as differences in treatment explain for the variation between academic and non-
academic centers. Indeed patients with a newly diagnosed acute myeloid leukemia often
receive their remission-induction and consolidation therapy in a non-academic center,
while post remission transplant procedures were only performed in the academic setting.
Acute leukaemia on treatment with chemotherapy was associated with the highest risk
of bleeding.
The relevance of skin bleeding in a daily observation and scoring system requires special
consideration. Although the presence of a grade > 2 bleeding of the skin might be relevant
in the above mentioned perspective, it seems less appropriate to include every day a skin
bleeding is visible as a "bleeding day" in the scoring system. Bruising of the skin takes
several days to weeks to disappear and counting all those days forms a distorted picture
of the bleeding tendency. As is shown in figure 3, excluding successive days with stable
haematomas affects only the percentage but does not affect the curve of the line. There is
no difference in the percentage of days with grade > 2 bleeding when platelet counts are
0-9 x 10^9/L or 10-19 x 10^9/L or 20-29 x 10^9/L. Also with higher platelet counts a considerable
percentage of days remain to be reported where patients suffer from grade > 2 bleedings
in agreement with the platelet dose trial reported by Slichter et al.[4]

Although bleeding complications were found to be associated with survival, the clinical relevance of reporting of grade 2 bleeding for platelet transfusion study purposes has been called into question.[9] Recently, in a commentary Heddle et al discuss the use of grade 2 bleedings in transfusion studies. This type of bleeding is likely to neither represent a valid surrogate for an effect of the intervention (bleeding occurs despite adequate prophylactic platelet transfusions), nor a valid composite outcome according to methodological criteria lacking reproducibility and accuracy.[10]

Our pilot study underscores that slight differences in assessment have a great impact on the estimation of bleeding. Although we completely agree on the need for better (surrogate) criteria to evaluate platelet transfusion therapy, the pilot study we performed had the purpose of investigating the soundness of scoring bleeding tendency in anticipation of a large multicenter randomised controlled trial. In this perspective the occurrence of an effect on any bleeding tendency, albeit not a surrogate for clinical outcome, might be informative. Indeed, there is evidence that certain modifications of the platelet product have an adverse effect on the haemostatic capacity.[11]

To summarize, we have found a comparable incidence of WHO grade ≥ 2 bleeding scores, supporting the power calculation of a recently initiated platelet transfusion trial. However there are still a number of unresolved issues. In this respect, the term "clinically relevant" gives rise to most of these issues. From a patient's perspective relevance is without doubt different, but more important is the discrepancy of the "relevance" concept between the clinicians taking care of the patients and blood bank scientists aiming to improve platelet products. Every clinician knows the difference between a "wet" patient and just an incidental haematoma albeit smaller or greater than 1 inch. Perhaps it is time to re-evaluate the current bleeding scales in relation to these different purposes.

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Chapter 8

Summary and Discussion

Summary and Discussion

THROMBOCYTOPENIA, BLEEDING AND SURVIVAL – THE END OR START OF A CHAPTER?

Introduction

The use of platelet concentrates (PC) is generally recommended for the prophylaxis and treatment of haemorrhagic complications in patients with thrombocytopenia due to myelosuppression.[1–4] Based on the observations of Gaydos et al as well as two post mortem studies, the concept of prophylactic platelet transfusions became standard practice since the late sixties and early seventies of the past century.[5–7] Indeed, after the introduction of platelet transfusions, the incidence of lethal haemorrhages steadily declined from more than 60% in the sixties to less than 5% in the last two decades.[6–8] Although it has to be noted that other supportive care measures as well as leukaemia treatment without doubt contributed major to this decline, a Cochrane analysis, which included these older studies, showed a small but significant effect of prophylactic platelet transfusions in the reduction of severe haemorrhages as compared to therapeutic transfusions.[9] Over the past three decades several endpoints have been engaged in successive trials starting with more patient centred endpoints such as bleeding prevention efficacy and the incidence of adverse transfusion reactions (e.g. alloimmunisation, febrile non-haemolytic transfusion reactions), as well as transfusion efficacy, a more transfusion product oriented endpoint. This thesis is based on the results of two product-based randomised trials, looking at transfusion efficacy in terms of increments, with adverse transfusion reactions and haemostatic efficacy as secondary outcome measures.[10, 11] This discussion reviews the most relevant outcomes of these trials and debate the clinical relevance of these (surrogate) endpoints, focussed on refractoriness and bleeding incidence.

The trials

The first randomised controlled trial (RCT) we performed aimed to investigate the clinical efficacy of a platelet additive solution as compared to conventionally plasma stored platelets in non-selected, thrombocytopenic patients in two large hospitals. Despite platelets stored in platelet additive solution (PAS2, Trombosol) had a significantly lower 1- and 24-hour corrected count increment, the wide chosen 30% margin of non-inferiority, as well as the fact that a comparable transfusion interval and no difference of hemorrhagic consequences were observed has not led to abandoning of platelets stored in PAS2 for up to five days in clinical practice (Chapter 3).[10] The transfusion efficacy results are in line with the findings of three other studies using this additive solution.[12-14] Adverse transfusion reactions occurred less frequently after transfusion with platelets in additive solution, consistent with the concept of plasma as important factor in FNHTRs. Platelet transfusion refractoriness defined as a 1-hour CCI < 7.5 and/or a 24-hour CCI < 4.5 occurred more frequent after transfusions with PAS2-PCs, however a multivariate analysis showed that patient factors like enlarged spleen, fever and infection and not product factors like storage time and storage solution determined the incidence of non-immunological refractoriness.[10]

As a consequence of the growing awareness of transfusion-transmitted infections along with a public debate, the second randomised trial investigated the transfusion efficacy of pathogen-reduced platelet products (PR-PAS3PC, Intercept) as compared to two non-pathogen-reduced platelet products (PAS3 PC, Intersol; plasma PC) in a comparable

non-selected hospitalised patient population as the first trial. With the chosen 20% margin of non-inferiority, inferiority was shown for pathogen-reduced platelets as compared to both control arms. Moreover, patients treated with pathogen-reduced platelets had significantly more bleeding complications and was reason for the Data Safety Monitoring Committee to stop the study arm PR-PAS3PC (Chapter 4).[11] Although the overall bleeding incidence, scored by clinicians in this trial, was considerably less as compared to other trials in which bleeding was scored according to rigorous prescriptive protocols; the reported outcome is consistent with these trials.[15-17] Whether the increase in haemorrhagic complications was due to a difference in platelet dose or impaired platelet function could no be determined. Platelet dose, which was significantly lower in the pathogen reduction arm, has been suggested as possible explanation for the increase in haemorrhagic complications.[18] A recently published large trial, however, testing 3 different platelet doses showed no difference with regard to the bleeding incidence.[19] In vitro studies reported that platelets treated with pathogen reduction, especially after prolonged storage, show reduced aggregation capacity, reduced glycoprotein expression, increased expression of annexin V as well the activation marker P-selectin, suggesting functional alterations that may play a role in an increase in bleeding complications.[20, 21] Apart from this unresolved issue, the outcome of this second trial has lead to both a debate with regard to bleeding as an endpoint as well as enormous (legal) consequences of reporting an adverse outcome and consequently stopping a study arm. The outcomes of the second trial as well as the intention to include bleeding as a primary outcome measure in current trials have led to a number of questions. To what extend is an increase in haemorrhagic complications acceptable using manipulated platelets to reduce the already very low incidence of transfusion-transmitted infections? And, if so what margin of non-inferiority is acceptable? What are the clinical consequences of bleeding complications?

THE ENDPOINTS: REFRACTORINESS AND BLEEDING

Refractoriness

Although still incompletely understood, the association of refractoriness with several clinical factors has been noted and investigated by several studies (table 1). Bishop et al showed that the recovery of platelets is affected by several clinical factors, including diffuse intravascular coagulation (DIC), administration of amphotericin B, splenomegaly and HLA antibodies. Antibiotic therapy, bleeding and temperature were less important factors.[22-24] Norol et al studied the impact of platelet storage time in the context of clinically "stable versus unstable" patients (i.e. patients with bacterial infection, GVHD, veno-occlusive disease as well as patients with splenomegaly) showing that only stored platelets performed worse as compared to fresh platelets in "unstable" patients.[25]
Bock et al demonstrated that fever as well as the use of certain antibiotics, either causal or as confounder (amphotericin B, ciprofloxacin, vancomycin), significantly influenced platelet increment.[26] The relation of fever with refractoriness has been explained by circulating cytokines, among others IL-1 and TNF, which induce activation of endothelial cells leading to the expression of adhesion molecules and induction of procoagulant activity.[27, 28]
In line with this,, the association between a higher dose of total body irradiation with refractoriness also suggests that endothelial damage could play an important causal role.[27] Heim et al showed in a large group of haemato-oncology patients that patient age, female sex and the administration of antithymocyte globulin (ATG) were associated with better post-transfusion platelet recovery, whereas increasing storage time, ABO-mismatched platelets, additive solution (T-Sol), allogeneic haematopoietic stem cell transplant (HSCT),

transfusion sequence > 40 and fever before transfusion had a significant adverse affect.[29] Similar results were found in an analysis using the data of the TRAP trial. In this analysis refractoriness was defined as 2 sequential 1-hour post transfusion increments (recovery) of less than 11×10^9/L (equalling an average CCI of 5.0). The strongest negative impact on 1- as well as 24-hour increment had male sex and females with previous pregnancies. Other negative factors were enlarged spleen, amphotericin B, bleeding, fever, infection, weight, height and transfusion sequence number. Platelet product factors negatively influencing 1- and 24-hour increments were ABO-incompatibility and storage time. By estimation, patient factors determined over 80% of the variation of both the 1- and 24-hour increment. The authors suggested that decreasing transfusion efficacy with increasing platelet transfusions might be related to progressive endothelial damage with increased platelet adherence and thus a more rapid loss from circulation.[30, 31] In our first RCT platelet transfusion failure occurred at least once in 49.6% of the patients. Platelet transfusion failure was, independent from thrombocytopenia, positively associated with bleeding complications, non-transplant related chemotherapy, severe mucosal damage and age.[10, 32] To summarize, non-immunological refractoriness, a frequent observed complication of platelet transfusions, is largely determined by several patient factors including bleeding complications, infectious complications and chemo- and radiotherapy induced tissue damage, whereas product factors such as storage time and storage solution play a minor role. In this sense platelet refractoriness may be a consequence as well as an indicator of increasing endothelial activation and damage.

Table 1: *Factors associated with refractoriness*[22, 24, 25, 26, 29, 31, 32]

	Bishop	Norol	Bock	Heim	Slichter	Kerkhoffs
Gender				●	●	●
Age						●
BSA					●	
Enlarged spleen	●	●			●	●
Bleeding	●				●	●
Infection		●			●	●
Fever	●		●	●		●
VOD		●				
GVHD		●				
DIC	●					
Mucosal damage						●
TBI		●				
ATG						●
Stem cell Tx				●		
Chemotherapy						●
Antibiotic therapy	●		●		●	
Transfusion sequence				●	●	
Storage time		●			●	
ABO mismatch				●	●	
Additive solution				●		

BSA =Body Surface Area; VOD = Veno Occlusive Disease; GVHD =Graft versus Host Disease; DIC = Diffuse intravascular Coagulation; TBI =Total Body Irradiation; ATG =Anti Thymocyte Globulin; Tx = Transplantation

Bleeding

The reported incidences of grade 2 – 4 bleeding varies from 5 – 70% (figure 1). Clinical platelet transfusion studies evaluating platelet products report major bleeding > grade 1 in 48% of the patients, whereas the incidence of major bleeding in AML RCTs evaluating patient outcome is only 7%, comparable with the percentage found in our second RCT. These figures reflect the subjectivity of the grading of bleeding. Active versus passive reporting of bleeding complications together with the frequency and timing of bleeding observation are the main factors explaining for the enormous variation in reported bleeding incidences in literature.[33] Apparently the difference between platelet product driven studies and clinical outcome studies in AML trials is explained by the perception of "clinically relevant", i.e. the perception of "what is meaningful".[34] Adapting the active and rigorous bleeding observation of the PLADO trial, which encompasses an active bleeding observation of 8 WHO defined sites by trained personnel and an independent adjudication, the Bleeding Observation Pilot (BOP) study showed bleeding grade 2 – 4 in 54% of the 68 participating patients on 18% of the observed days, which is in agreement with reports using similar bleeding score systems.[19, 35] Comparing the data of the BOP trial with our second RCT shows that the large difference in bleeding incidence is mainly due to the underreporting of skin- and mucosal bleeding events, underlining the impact of clinical perception (Chapter 6) .

Figure 1: *Figure 1 shows reported percentages of patients with grade 2 - 4 bleeding complication. The error bars reflect the 95% confidence interval. The filled markers represent randomised controlled trials, whereas the open markers represent observational studies. 1 = Blumberg et al[71]; 2 = Sensebe et al[72]; 3 = AML Trials 83 = Kerkhoffs et al[11]; 4 = Oka et al[16]; 5 = Tinmouth et al[17]; 6 = Gil-Fernandez et al[18]; 7 = Zumberg et al[19]; 8 = Kerkhoffs et al[10]; 9 = Mirasol[76]; 10 = Diedrich et al[77]; 11 = Wandt et al[78]; 12 = Rebulla et al[52]; 13 = Gmur[79]; 14 = Nevo et al[55]; 15 = Wandt et al[80]; 16 = Sagmeister et al[81]; 17 = Murphy et al[82]; 18 Pihush et al[53]; 19 = Higby et al[83]; 20 = Navarro et al[84]; 21 = Heddle et al[35]; 22 = McCullough et al[16]; 23 = Lawrence et al[85]; 24 = Friedmann et al[46]; 25 = Slichter et al[19].*

Grade 2-4 bleeding in literature
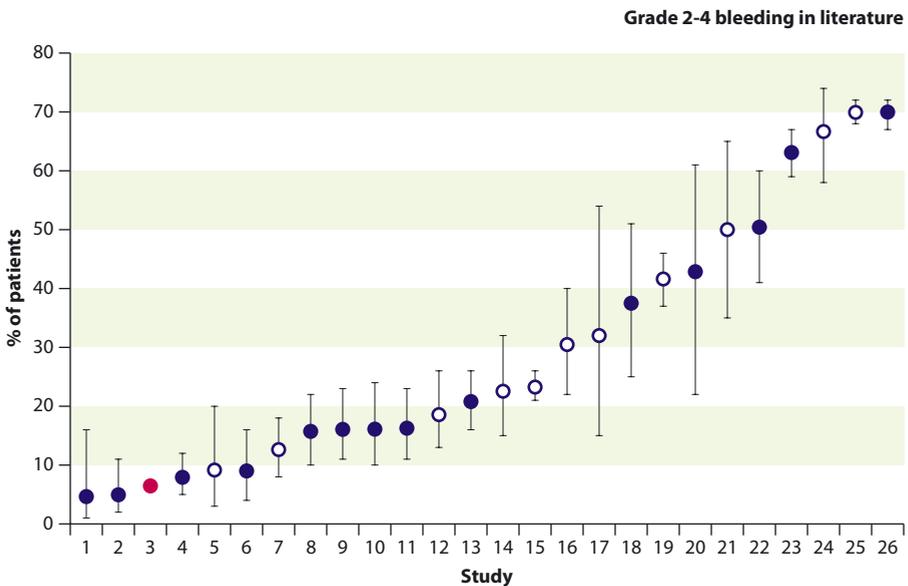
The use of bleeding as an endpoint assumes the level of thrombocytopenia to be an important etiological factor as well the potency of transfused platelets to correct this level to decrease bleeding complications. Despite a preventive platelet transfusion policy major bleeding is occurring in half of the patients. We still lack the tools in understanding which patients are going to have bleeding complications and to what extend platelet transfusions are aiding in their prevention or even may be harmful in certain clinical situations.[36, 37]

Experimental studies in thrombocytopenia

In rabbits with severe thrombocytopenia flattening and increased fenestration of the endothelium has been shown using electron microscopy.[38] Also an increased leaking of red cells associated with the level of thrombocytopenia was shown in thrombocytopenic rabbits.[38, 39] The relation between thrombocytopenia was explored using radio labelled red cells in stable thrombocytopenic patients. Substantial faecal loss of radio labelled red cells only occurred at platelet counts less than 5 x 10^9/L.[40] In a landmark study using radio labelled platelets an endothelium supportive role for platelets was suggested with an average consumption of 7 x 10^3/μL/day.[41] Although these studies show that platelets play an important role in the maintenance of endothelial integrity, it is likely that increased fenestration and flattening of the endothelium causes petechiae and mucosal bleeding, but it is unknown whether this explains for the major bleeding complications.
Using mice experiments, Ho-Tin-Noé et al showed mice deficient of β-integrines, which have decreased neutrophil infiltration capabilities, were protected from thrombocytopenia-induced tumor hemorrhage. The same group showed that platelet adhesion in it self is not required to maintain vascular integrity and that in the absence of platelets hemorrhage only occurred in an inflamed microcirculation.[42, 43]

Bleeding susceptibility

Gaydos et al first described the relationship between thrombocytopenia and haemorrhage, although no threshold could be recognized and most of the lethal cerebral haemorrhages described in this study occurred in patients with cerebral leukaemia involvement. Moreover aspirin was frequently used as an antipyretic agent.[5] Estey et al studied the causes and risk factors of remission induction failure in 378 previously untreated AML patients in the period 1973 – 1979. Only 22% of these patients were primary chemotherapy resistant, the majority of patients failed coming into remission due to infectious complications and in 33% of the patients failure occurred due to fatal haemorrhages despite prophylactic platelet transfusion support. The main risk factor for fatal hemorrhage were an initial white blood count of ≥ 25 x 10^9/L (OR 2.7; 95%CI 1.3 – 5.3) and the incidence of death from haemorrhage was highest during the initial 2 weeks of treatment. In the discussion an etiologic role for leukaemia infiltration of vessel walls is considered as also reported by Freireich.[44, 45] Friedmann et al studied clinical and laboratory features predicting for severe hemorrhage in 2942 patients. 368 patients (12.5%) suffered severe bleeding complications. Uraemia, hypoalbuminemia, recent BMT, platelet transfusion, the administration of aminocaproic acid and recent bleeding were associated with increased bleeding. Platelet count was not significantly associated with bleeding in untransfused patients. The main limitation of this retrospective study was the lack of information regarding the temporal relationship between platelet count, platelet transfusion and bleeding.[46] Studying the relationship of thrombocytopenia with bleeding post stem cell transplant, Nevo et al compared 321 bleeding BMT patients with 287 non-bleeding matched controls. There was a small but significant increased risk for bleeding in patients

with more days of platelet counts ≤ 10. However profound thrombocytopenia was present in only 8.6% of bleeding patients. Pulmonary hemorrhage was significantly associated with thrombocytopenia in contrast to bleeding from other sites.[47] This might be associated with GVHD and endothelial damage, as has been shown by a post-mortem study.[48]

In line with the report of Gil-Fernandez et al, who identified high platelet consumption factors (VOD, fever, treatment with amphotericine B and mucosal damage) in most cases of bleeding in two transfusion trigger groups (10 vs. 20 x 10^9/L), Nevo et al showed that profound thrombocytopenia was not the primary cause of bleeding in both groups.[49, 50]

In contrast, in the re-analysis of the Rebulla trigger trial, including 255 patients with acute myeloid leukaemia, six variables were multivariate associated with grade I-II bleeding: administration of antifungal medication, steroid administration, a higher platelet count and platelet transfusion decreased the risk, whereas the presence of infection and fever increased the risk. Grade II – IV bleeding was associated with fever as well as platelet count. Grade III – IV bleeding was associated with the administration of antifungal therapy (increased risk!). The presence of a grade I bleeding was associated with at 2.6 times increased risk of grade II – IV bleeding (95%CI 1.18 – 5.49) and grade I – II bleeding was associated with a 3.1 times higher risk of grade III – IV bleeding (95%CI 1.17 – 7.95).[51, 52] Pihusch et al studied hemorrhagic and thrombotic events in 447 transplant patients (autologous n = 83; allogeneic n = 364). Haemostatic (thrombotic as well as haemorrhagic) events occurred in 83.2% of the patients. Severe haemorrhage occurred in 41.5% of the patients and 3.6% suffered lethal bleeding. Intracranial haemorrhage was observed in 21 patients (4.7%) and associated in a majority of patients with infection. Allogeneic BMT patients had a higher bleeding incidence as compared to autologous BMT patients. Patients with GVHD > grade I had a significantly higher incidence of bleeding. A strong correlation was found between the duration of thrombocytopenia and bleeding events. GVHD and duration of thrombocytopenia were the only "predictors" for the occurrence of bleeding. Also a thrombotic event such as microangiopathic haemolytic anemia (MAHA) was more frequent in the allogeneic group. Interestingly in 23.5% of the MAHA cases a shortened aPTT was found indicating endothelial perturbation and activation. In the discussion authors suggest a role for TNF-α, essential in the pathogenesis of GVHD, known to modulate endothelial haemostatic function and enhance the production of Plasminogen activator inhibitor-1 and Tissue Factor as well as down regulating Tissue factor pathway inhibitor.[53, 54]

To summarize (see also table 2), bleeding is a frequent but variably reported complication in patients with thrombocytopenia due to myelosuppression. Apart from the level of thrombocytopenia, also inflammation and vascular damage are associated with an increased risk of bleeding. It could be postulated that both the dynamics of thrombocytopenia as well as hemorrhage are determined by endothelial activation and damage, associated with inflammation.

Table 2: *Factors associated with increased bleeding (severity)*[5, 44, 46, 47, 51, 53, 32]

	Gaydos	Estey	Friedman	Nevo	Gil	Rebulla	Pihush	Kerkhoffs
Age								
WBC	●	●						
Uraemia			●					
Hypalbuminemia			●					
Bleeding			●			●		
Fever					●	●		
Infection						●	●	
VOD					●			
GVHD							●	
Mucosal damage					●			●
Platelet count	●			●		●	●	
Stem cell Tx			●				●	
Chemotherapy								●
Antifungal Rx					●	●		

WBC = White Blood cell Count; VOD = Venoocclusive Disease; GVHD = Graft versus Host Disease; Tx = Transplantation; Rx = Therapy

Refractoriness and bleeding in relation to survival

In a study in 1,402 patients, the first 100 days post transplant, Nevo et al showed that bleeding was both for allogeneic as well as well autologous patients associated with reduced survival.[55] Bleeding severity correlated with GVHD severity in 463 allogeneic transplant patients studied by Nevo et al. A significant association was found with gastrointestinal bleeding, hemorrhagic cystitis and pulmonary bleeding. Acute GVHD occurred early in the course post transplant and in 88% of the patients with bleeding and GVHD, bleeding episodes started after GVHD initiation. Both bleeding as well as GVHD were significantly associated with a reduced survival. Also, survival in non-bleeding patients was significantly reduced in patients with more pronounced thrombocytopenia perhaps suggesting more extensively injured endothelium.[47, 56, 58] The association between hemorrhagic complications, reduced survival and GVHD was also shown in another study of 807 allogeneic HSCT patients.[57] In the study by Pihush et al, haemostatic events (both thrombotic as well as hemorrhagic) were associated with an increased mortality risk (RR 1.7; 96%CI 1.0 – 3.2).[53] Intrigued by these findings, we studied the association of platelet refractoriness with patient survival. Surprisingly, patients experiencing one or more 24-hour platelet transfusion failures had, as compared to patients always showing a sufficient 24-hour increment, a significantly reduced survival, independent of therapy, diagnosis and age (Chapter 6).[32] As it is unlikely, that bleeding and refractoriness are directly causally related to the reduced survival, it is hypothesised that these are both confounders for vascular damage and /or microthrombosis.

Damaged endothelium: a common pathway?

Haemorrhagic complications, platelet refractoriness and deep, prolonged thrombocytopenia are repeatedly observed to define a category of patients with reduced survival. Without a logical direct causality, this leads to the hypothesis that these patients have more pronounced damage of the vascular endothelium (figure 2). As has been mentioned above, although thrombocytopenia in itself leads to endothelial flattening and fenestration, in the absence of an inflammatory process thrombocytopenia does not lead to haemorrhage in animal models unless deep thrombocytopenia which is associated with leakage of erytrocytes. Endothelial damage is regarded as a pathologic hallmark of vascular complications after HSCT, such as veno-occlusive disease of the liver, thrombotic microangiopathy, and capillary leak syndrome. In GVHD, the vasculature is sequentially affected. Endothelial damage is caused by the conditioning regimen, followed by neovascularisation and recruitment of inflammatory cells with in the third phase alloreactive T-cells targeting the endothelium.[59] The intensity of the conditioning regimen positively correlates with endothelial damage as measured by plasma levels of vWF, sVCAM-1 and sTNF receptor I.[60] Cyclic GMP, also a marker for endothelial damage, was a negative predictive factor for survival after HSCT.[61] Pericapillary hemorrhage was shown in areas EC lesions in severe intestinal GVHD and associated with severe hemorrhagic enterocolitis.[62] Circulating endothelial cells (ECs) are increased with endothelial damage and in patients after myeloablative conditioning an increasing number of ECs was found.[63, 64] In patients with GVHD significantly more EC microparticles were found as compared to patients without GVDH as are vWF and thrombomodulin.[65, 66] Moreover, factors like interleukin-1 and TNF-α have been shown to induce ultra structural changes in the blood-retina barrier.[67] Apart from GVHD, which might be a model to study bleeding susceptibility also for other conditions, several cytotoxic agents have been shown to cause endothelial damage and interestingly treatment with vascular endothelial growth factor inhibitors in oncology patients resulted in an increased incidence of both thrombotic as well as haemorrhagic complications.[68-70]

In conclusion deep aplastic thrombocytopenia causes endothelial fenestration and capillary leakage of erythrocyts. Its association with skin and mucous membrane bleeding is obvious, but an association with major bleeding is not proven. In contrast high blast counts, chemotherapy, irradiation, infection and GVHD associated endothelial damage are recognized to be associated with bleeding at varying degrees, as well as with thrombocytopenia. Enhanced endothelial damage due to these causes leads to increased consumption of both autologous as well as transfused platelets, haemorrhagic complications as well as ultimately a decreased patient survival. It is unclear to what extend transfused platelets are preventing these complications, although it seems unlikely that we will be able to prevent these complications just by transfusing platelets. Future studies are needed to test novel grading systems for the bleeding complications, preferentially distinguishing between just lack of endothelial repair and endothelial damage associated with activation. Endothelial maintenance benefits from platelet substitution and a relatively low number of platelets is sufficient. Endothelial damage may need another approach and it is questionable whether increase of the transfusion threshold and increasing the transfusion dose is the answer. It is challenging to validate the corrected count increment as a surrogate outcome parameter for survival as well as trying to make an IPSS-like scoring system to predict hemorrhagic complications to improve the platelet transfusion strategy on a patient level.

Figure 2: *Figure 2 summarizes the hypothesis of endothelial damage in relation to increasing platelet (PLT) consumption, haemorrhagic complications and survival. The figure represents segments of vascular endothelium showing normal endothelium (segment A), flattened and fenestrated endothelium occurring in thrombocytopenia (segment B), activated endothelium due to disease, cytotoxic agents, irradiation, GVHD and/or infectious agents (segment C) and damaged, apoptotic endothelium (segment D).*
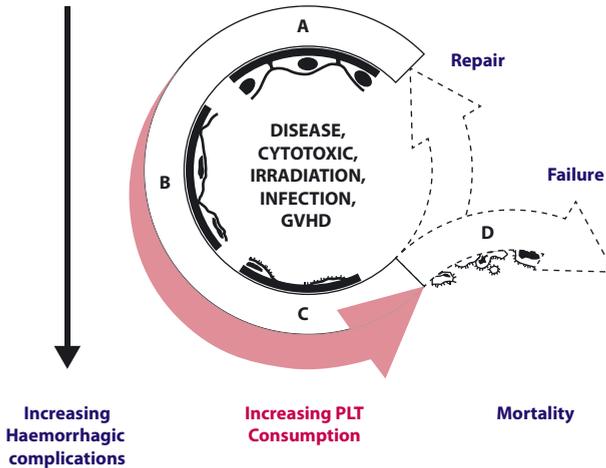
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Chapter 9

The continuing story:
The PREPAReS study

9 The continuing story: The PREPAReS study

Clinical effectiveness of standard versus pathogen-reduced buffy coat-derived platelet concentrates in plasma in hemato-oncological patients

The PREPAReS Study: Pathogen Reduction Evaluation & Predictive Analytical Rating Score

A phase III study PROTOCOL

R

Arm A

Plasma stored
platelet concentrates
(Plasma-PCs)

Arm B

Pathogen-reduced
plasma stored
platelet concentrates
(PR-plasma-PCs)
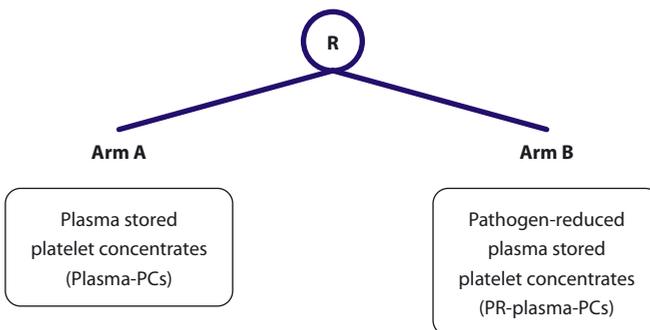
INTRODUCTION

Background

Platelet transfusions are extensively used for treatment and prophylaxis of bleeding in thrombocytopenic patients. Bleeding still is a frequent complication and recommendations differ regarding the preferred transfusion regimen, the quantity and quality of transfused platelets and strategies to monitor efficacy.[1-3] These recommendations regard the platelet transfusion triggers for prophylaxis, intervention and bleeding. However, except for the upper level of the trigger for prophylaxis, a minority of the recommendations is evidence-based.

Several platelet products are in use. Most variations concern the donor origin (random or matched), way of collection (whole blood or apheresis), production (platelet rich plasma-derived, pooled buffy coats, white cell-reduction methods), storage solution (plasma or platelet additive solution) and storage duration. For most of these variations, even major ones such as prolongation of the storage time and replacement of plasma by additive solutions, clinical comparative studies were not or scarcely performed.

The gap between platelet product developments and even platelet substitutes on one hand, and clinical evaluation on the other, was noticed by regulatory bodies, such as the FDA. Progress in this respect is however slow, due to a poor correlation between in vitro quality tests and clinical efficacy. The FDA has therefore suggested a number of biological guidelines. These include documenting the viability of manipulated (autologous) platelets in normal volunteers. Using radioisotope studies, recovery should be 67% of fresh platelets and survival 58% or more, as compared to a gold standard defined as a fresh (<1 day) plasma stored platelet product.[4] With these minimal requirement strategy, the goal is to protect against the risk of a "downward creep" in quality. There is now general agreement that substantial changes in platelet production should also be validated for their clinical quality, including assessment of bleeding. Recently it is possible to subject platelet products to a pathogen inactivation step. Apart from obvious bacteriological and logistic advantages as well as possible immunologic advantages of pathogen-reduced and extended stored products as eminent new developments, these new products clearly need clinical validation for their haemostatic effectivity. Policymakers, product providers, and investigators agree that clinical platelet transfusion studies are essential.

The Dutch situation

In line with international developments, Sanquin Blood Bank explores emerging issues as extending storage (storage up to 7 days) of platelet concentrates (PCs) in plasma and additive solutions, development and use of additive solutions and methods of pathogen inactivation. Recently extended storage for plasma-PCs to 7 days has been approved for clinical use requiring a post marketing surveillance phase. This approval is based on one trial investigating extended storage of plasma-PCs in a selected population of thrombopenic patients.[5] In the Netherlands all platelet products undergo aerobic and anaerobic culture and approximately 0.44% is found bacterially contaminated.[6]

Experimental platelet product to be investigated in the proposed study

Both in vitro and clinical studies have been done with the Mirasol system, using riboflavin. Riboflavin is a naturally-occurring vitamin (B2) and is postulated to interact with nucleic acids which undergo a chemical reaction when exposed to UV light.[7] Extensive toxicology, mutagenicity, carcinogenicity, photo toxicity, and pharmacological studies established an adequate safety for photo-chemically treated PCs.[8] The technical file has been evaluated by KEMA Notified Body, and they have accepted the file.[9] The Mirasol Pathogen Reduction System for Platelets has been classified as a Class IIb device and is CE marked. Riboflavin-based photo-chemical treatment has shown to be effective against selected pathogens, including HIV, WNV, gram positive and gram negative bacteria, obtaining a more than 4 log10 reduction, except for S. aureus and B. cereus[10] (see Table 1A, 1B and 1C). Spiking studies in apheresis platelet concentrates showed a high effectiveness of inactivating various bacteria, including complete inactivation of the spore-forming B. cereus, despite only 1.9 log reduction. A. baumannii showed only partial inactivation, despite being spiked at low bacterial dose.[11] Although in vitro studies of treated platelets show functional[13] and metabolic alterations[14-16] during storage up to five days, minimal requirements (pH, swirl) for issuing PCs are preserved.

Seven-day storage of platelet concentrates is an important issue in the Netherlands. There is limited clinical experience with 7 day-stored Mirasol-treated platelets; laboratory data of buffy coat-derived PCs stored for 6 days show comparable in vitro quality as apheresis platelets that have been stored for (the currently licensed) 5 days (see Table 2). There are differences among the treated versus untreated PCs, but the treated units nevertheless conform to current Dutch blood product specifications (which requires a pH37°C>6.3)[17], with pH22°C>6.8, [Canadian Blood Services, unpublished observations] and swirl present. These data show that buffy coat-derived platelets maintain better in vitro quality as apheresis platelets during storage and that shelf-life of buffy coat-derived platelets can likely be extended to 7 days. The experimental platelet product investigated in the current study has to comply with the CCMO (Central Committee on Research Involving Human Subjects) guidelines, and the WMO (Medical Research Involving Human Subjects Act) is applicable.

Prevention of allo-immunization and Graft versus Host disease

The Mirasol treatment is likely to be effective in reducing allo-antibody formation and prevention of transfusion-associated graft versus host disease (TA-GvHD) in transfusion recipients. Currently the standard guideline for prevention of TA-GvHD is gamma irradiation. As compared to gamma irradiation, Mirasol treatment is more effective abolishing the proliferation of lymphocytes as allogeneic responder cells in a mixed lymphocyte culture, whereas the reduction of lymphocyte proliferation is the surrogate assay for assessment of radiation dose to prevent GvHD.[18] The TRAP trial[19] indicated that UV-B irradiation (1480 mJ/cm^2) alone was able to reduce the incidence of HLA-antibody mediated refractoriness from 13% in patients receiving unfiltered PRP-derived PCs, to 5% in patients that received UV-B irradiated PRP-derived PCs. Leukoreduction by filtration of the PCs gave a similar rate of 3% refractoriness. In this study the formation of lymphocytotoxic antibodies, not leading to platelet transfusion failure, reduced from 45% to 20%, whereas in patients with prior pregnancies the antibodies fell from 65% in the control group to 33% in the group that had received UV-B treated platelets. This suggests that the primary immune response in naïve individuals and the booster stimulus in primed individuals are impaired. A recent study[18] suggests that the Mirasol treatment, that uses a UV-B dose of 530 mJ/m^2 in addition to the presence of riboflavin, induces loss of surface expression of HLA class II and co-stimulatory molecules in peripheral blood mononuclear cells, similarly as obtained with higher doses of UVB. Furthermore, Mirasol treated mononuclear cells had a significant reduction in surface expression levels of a number of adhesion molecules as compared to untreated cells and showed virtual absence of cell-cell conjugation in vitro. The observed loss of immunogenicity was nearly complete and UV irradiated antigen presenting cells (APCs) barely induced measurable IFN-γ production and no detectable STAT-3, STAT-5, or CD3-ε phosphorylation in allospecific primed T cells. These results suggest that defective cell-cell adhesion prevents UV irradiated cells from inducing T cell activation.

Clinical studies

Transfusion of up to 5 day-stored, riboflavin/UV-B-treated apheresis PCs to normal subjects revealed that recovery was 50±19%, which was significantly lower as that of control units at 67±13%. Also, survival time was shorter, 104±26 h for the treated group versus 142±26 h for the reference group.[15] Although recovery and survival of treated platelets in healthy volunteers is impaired, the PCs performance falls within the range as delineated by the FDA for new products. One randomized study is available, although not yet peer reviewed. In spite of the lower recovery and survival data in volunteers, the Miracle trial, evaluating corrected count increments of apheresis- or buffy coat-derived plasma-PCs, showed acceptable 1-hour CCI values for both methods of preparation: 15.7±1.0 for buffy coat and 13.0±0.6 (mean±SE, p=0.02 [unpublished results]). An unresolved issue was however a sudden drop in CCI with riboflavin-UV-B-treated buffy coat platelets halfway the study. Prior to the interim analysis, the 1-h CCI was 11.9±0.6 (versus 14.6±1.3 in the untreated group), and this value dropped to 7.3±0.5 after the interim analysis (versus 17.4±1.6 in the untreated group). This trial showed no difference in red cell or platelet usage, but there was a significant difference in the average number of days between the first 8 on-protocol transfusions in the Mirasol subjects and Reference subjects: 2.4±0.8 days and 3.3±1.5 days, respectively (p<0.001). For greater than 8 on-protocol transfusions, the average number of days between transfusions in the Mirasol group was 1.2±0.9 days, versus 2.2±0.9 days in the Reference group (p=0.1).

Rationale for this study

Currently some pathogen-reduced platelet products (PR-PCs) have passed phase III studies, are in progress or can be expected in the near future. At present some transfusion centers throughout Europe have implemented PR-PCs, but as yet PR-PCs are not formally accepted as a standard product that should be applied nation-wide. Because many uncertainties currently exist on the "optimal" platelet product, it is in the interest of patients, health care providers and the transfusion provider (Sanquin) to decide on evidence. With all the current safety measures remaining in place, pathogen reduction provides a safety benefit by reducing the number of transfusions of platelet concentrates contaminated with bacteria, but which were missed by the screening method. In the Dutch situation, morbidity is estimated to be 1:14,000 platelet concentrates.[20] In this publication, two cases of transmission of B cereus by a platelet transfusion are reported, where both patients experience a life-threatening sepsis, but recover eventually. Cases of bacterial transmission however often go unnoted, so a frequency as low as 1:130,000 has been reported.[21] The same is true for mortality; this value ranges from 1:50,000 [22] to 1:500,000.[23] A more precautionary benefit is protection against known and unknown pathogens. It is difficult to estimate the actual risk, and consequently to estimate the benefit for the patient. While in The Netherlands no epidemics have occurred against which no screening tests could be developed, including Q-fever,[24] there is a small but real risk that an epidemic can wipe out the blood supply in a country. This has happened in La Réunion, where an epidemic of chikungunya virus urged import of blood products from abroad, followed by rapid introduction of a pathogen reduction technology to ensure the blood supply.[25] An outbreak of this virus in Italy resulted in suspension of blood collections in an affected area, which led to a low blood inventory as well as a reduced delivery of plasma to fractionation institutes.[26]

As mentioned above, appreciating the difficulties of extrapolating in vitro tests towards in vivo efficacy, platelet products should be tested in clinical trials. Of note, radiolabeling techniques in volunteers as required by the FDA, are not used in the Netherlands. For major product variations in the Netherlands we depend on studies in patients. Extending storage for logistic purposes, combined with maintaining or even improving the safety of platelet products, and maintaining clinical efficacy are the main features in the development of new platelet products. In this study protocol we aim to investigate transfusion efficacy of two different platelet products: plasma-PCs, and pathogen-reduced (PR)-plasma-PCs, combining extended storage with or without treatment with a photochemical pathogen reduction technique. Prior to the start of the clinical study an in vitro study of the product has been performed, showing that the study product meets the current in vitro quality requirements for release for transfusion. However, on site implementation validation still has to take place.

Refractoriness to platelet transfusions and bleeding complications are the main clinical problems in intensively treated hemato-oncological patients and are essential endpoints for transfusion studies as well. In this trial bleeding will be scored according to the World Health Organization (WHO) scale as a primary endpoint. Refractoriness is defined as a 1-hour CCI <7.5 and/or a 24-hour CCI <4.5 after ABO compatible platelet transfusions on at least two successive occasions. Known causes of non-alloimmune refractoriness are included in this trial because for the purpose of generalization, relevant to develop a national product, testing transfusion efficacy of new platelet products should imply all patients in need of a preventive support with platelet transfusions. The 1- and 24-hour CCI are commonly used to evaluate platelet transfusions and, albeit not without discussion, currently the platelet count is the only parameter in trigger-based transfusion policy.

The ratio of both the 1-hour and 24-hour CCI mirrors both platelet recovery immediately after transfusion as the 1-hour CCI, and platelet survival one day after transfusion as the 24-hour CCI. Other secondary clinical endpoints of the trial will be transfusion requirement (red cells and platelets), transfusion interval to next transfusion and adverse reactions.

Relation between in vitro measures and clinical outcomes

Besides a low pH, resulting from an increased lactate production, there are no laboratory measures available that accurately predict platelet recovery, survival or hemostatic function.[12] In vitro data showed that, despite pH and swirl being unaffected, other in vitro measures of platelet activation, function and metabolism may show considerable differences between products. We hypothesize that a combination of metabolic, activation and functional parameters of PCs, combined into one 'rating' score, may predict either the 1-hour and/or the 24-hour CCI. For such an in vitro rating value we selected assays that can be performed shortly before transfusion of the PLTs, to be able to associate the laboratory values with clinical endpoints. We selected three parameters for this rating system, each of which could play an independent role in predicting in vivo effectiveness of stored PLTs for reasons discussed below. First, we considered CD62P expression on the platelet surface. This reflects activation of platelets and a higher CD62P expression has been associated with enhanced PLT clearance from the circulation.[27-31] Impaired PLT survival in animals was found to be associated with the apoptosis marker annexin A5 binding[32,33] thus warranting inclusion of this parameter in our rating system. The third assay is the lactate concentration (as surrogate for lactate production). A low lactate production rate is considered as a good indicator of mitochondrial function.[34,35] However, to calculate a lactate production rate over multiple days of storage, a baseline sample has to be taken immediately after production. In reality, this baseline sample shows only a small variation, and so the lactate concentration prior to transfusion can be used as being representative for lactate metabolism. The in vitro outcomes of each of these three parameters can be scored from 0 to 2, where 0 points would indicate a poor quality and 2 points a good quality, rated on an arbitrarily chosen linear basis. The combined rating then results in a value between 0 (poor quality) and 6 (excellent quality). For CD62P expression a value of 2 points can be attributed to an expression <20%, 1 for 20-30% and 0 points for an expression >30%. For annexin A5, a value of 2 points for a binding <10%, 1 for 10-20% and 0 for all PLT concentrates with a binding >20%. Finally, lactate levels >20 mM are known to indicate poor PLT quality,[35] and score of 2 points is proposed for a level <10 mM, 1 for concentrations between 10-20 mM and 0 points for a value >20 mM.

By sampling and analyzing the PCs prior to transfusion and relating the in vitro outcomes with CCI values, the relation between a combination of metabolic, activation and functional parameters of transfused PCs, and the usefulness of combining these into one 'rating' value, will be evaluated. Such a rating system may enhance flexibility to search for improvement of products by preclinical studies.

In this study, a PC sample will be taken prior to transfusion, and the above in vitro measures will be performed on platelet products that are issued from Monday to Friday, so that in vitro analysis can take place on week-days. These will be determined by investigators blinded for the product and clinical results and made available to the statistician for comparison with the 1-hour and 24-hour CCI and analyzed in a multivariate model. Likely, the dichotomous nature of the proposed rating score can be replaced by a more continuous scale, and both will be validated. This adapted rating model will be validated with the existing database.

Immunological effects

As mentioned, Mirasol treatment is expected to reduce primary and secondary HLA allo-antibody formation probably because of the lack of cell-cell interaction. It is as yet unknown whether such treated mononuclear cells, not recognized by recipient T cells, may show a prolonged survival of donor cells.[36] A prolonged survival may enhance indirect antigen presentation leading to more delayed alloimmunization, while also the establishment of regulatory T cells may be affected. To further investigate this hypothesis in the current study, samples will be obtained from patients who are negative for HLA-antibodies prior to transfusions, and analyzed according to primed and naive immune status, to investigate the formation of HLA-antibodies during a longer follow-up period. These samples will be collected weekly up till day 28, and then on day 56 and tested in the Luminex assay for presence of single antigen HLA-antibodies. Specifically, EDTA samples will be centrifuged and the plasma will be aliquoted in 0.5 mL samples and frozen. Part of these (blinded) samples will be shipped to the Blood Systems Research Institute (San Francisco, CA, USA) on dry ice for antibody detection and identification. In a selected cohort of primed and unprimed patients, following the first on-protocol transfusion, the 1-h samples will be analyzed for induction of HLA class II molecules on T cells of donor and recipient and at day 56 blood will be collected and processed for evaluation of persisting donor cells. The buffy coat fraction of the EDTA samples will be frozen to study immunological effects in the white cell fraction comparing patients who show increased antibody titers with controls.

STUDY OBJECTIVES

Primary objective:

To assess the non-inferiority of PR-plasma-PCs compared to plasma-PCs up to a storage interval of 5 days in terms of WHO bleeding complications ≥ grade 2. The first 8 transfusions with PCs that have been stored for 1-5 days will be used to assess the incidence of bleeding complications ≥ grade 2.

Secondary objectives:

1. To assess the transfusion failures defined as 1 hour CCI < 7.5 and 24 hour < 4.5, 1 and 24 hour CI and CCI of 1-7 days stored platelets of all platelet transfusions, and in relation to the transfusion number.
2. To assess the percentage of days that bleedings ≥ WHO grade 2 occur.
3. To evaluate whether clinical factors interact with the different study products leading to a difference in platelet refractoriness.
4. To assess the safety (adverse reactions).
5. To assess the transfusion requirement (red cells and platelets).
6. To assess the transfusion interval.
7. To assess the incidence of adverse reactions.
8. To assess the rate of HLA allo-immunization.
9. To evaluate whether in vitro measures relate to in vivo outcomes measures as the 1-hour and the 24-hour CCI.

STUDY DESIGN

The study is a prospective, randomized multicenter trial for the evaluation of platelet products in hemato-oncological patients with thrombocytopenia or expected to become platelet transfusion dependent due to myelosuppressive therapy or malignancy-related myelosuppression. In this trial patients will be randomized to receive one of two platelet products during a transfusion episode with a maximum of 6 weeks or a total of 8 platelet transfusions according to protocol, whichever comes first.

Because the Mirasol-treated platelet products show a color difference not allowing an appropriate placebo, the study will be single-blinded for investigators evaluating the CCI and bleeding score. Products will be stored up to 7 days. The primary endpoint is restricted to 5 days storage as this implies the most relevant information. Secondary endpoint evaluation requires that the patient continues treatment in the assigned study arm.

Arm A: Plasma stored platelet concentrates (Plasma-PCs)
Arm B: Pathogen reduced plasma-stored platelet concentrates (PR-plasma-PCs)

STUDY POPULATION

Inclusion criteria

- Age ≥ 18 years.
- Expected ≥ 2 platelet transfusion requirements.
- Signed informed consent.
- Having a hemato-oncological disease
- Exclusion criteria
- Micro-angiopathic thrombocytopenia (TTP, HUS) and ITP
- Bleeding > grade 2 at randomization (after treatment, the patient can be randomized in the study after 2 or more weeks after the last transfusion that was used to stop the bleeding)
- Known immunological refractoriness to platelet transfusions.
- HLA- and/or HPA-allo immunization and/or clinical relevant auto-antibodies.
- Indications to use hyper-concentrated (plasma-reduced) platelet concentrates, i.e. patients with known severe allergic reactions and documented transfusion-associated circulatory overload (TACO)
- Pregnancy (or lactating)
- Prior treatment with other pathogen-reduced blood products
- Known allergy to riboflavin or its photoactive products

Platelet transfusions

Indications for platelet transfusions are distinguished into platelet count-related prophylaxis (PP), intervention related prophylaxis (IP) and treatment of bleeding (TB). For each transfusion the indication shall be recorded. The CBO guidelines1 will be used as guidance for the indication of platelet transfusions, these imply: trigger for PP 10 x 10^9/L; for IP 50 x10^9/L; and for TB stopping of bleeding or at PLT counts >100 x10^9/L, although the treating physician determines if or when a transfusion is ordered.

All products are produced by Sanquin Blood Banks. Logistics will be organized to assure a seven day coverage of availability for all study products. All platelet products will fulfill standard quality requirements prior to release. PCs are prepared from pooled BCs.
The pooled PCs are leukoreduced by filtration. Platelets will then be resuspended in a unit of plasma from one of the buffy coat donors who has not been pregnant or has received prior transfusions. In case of photochemical pathogen reduction, 500 mM riboflavin is added to the leukoreduced plasma-PCs within 28 hours of platelet collection, mixed and exposed to UV-B light (wavelength 265-370 nm) during five to ten minutes (depending on the volume of the PC) with constant agitation at 120 rpm, giving a total dose of 6.2 J/mL. All platelet products will be stored with gentle agitation at 20-24°C up to 7 days. Products will be γ-irradiated if indicated by the requesting center.
Apart from routine testing (platelet count, swirl and BacT/Alert screening), PCs of each arm will be subjected to additional quality control (QC) tests immediately before transfusion with respect to platelet metabolism, activation markers and platelet function.
The four blood bank divisions have determined the inter-laboratory variation of these QC parameters by regular contingency exercises. A swirling effect is present and the bacterial screening "negative to date".

END OF PROTOCOL TREATMENT

Reasons for going off protocol treatment are:
• Transfusion independency > 7 days or hospital discharge whatever occurs first.
• No compliance of the patient (especially refusal to continue).
• Intercurrent death.
• Serious adverse transfusion reactions necessitating other products (see paragraph 13).
• Immunological refractoriness.

ENDPOINTS

Primary endpoint

• Incidence of WHO bleeding complications ≥ grade 2 after transfusion of PCs that are 1-5 days old.

Secondary endpoints

• 1-hour and 24-hour CCI, based on PCs that are 1-7 days old
• WHO bleeding grade, see appendix A
• Transfusion requirement, red cells and platelets
• Platelet transfusion interval
• Adverse events according to CTCAE version 3.0

Sample size and power considerations

This one-sided, non-inferiority study will compare the mean incidences of WHO bleeding complications ≥ grade 2 in patients receiving platelets stored in plasma with or without pathogen reduction. Results of a previous study37 show that on average 50% of the patients had bleeding complications ≥ grade 2. The margin of non-inferiority is set at an absolute difference of 12.5%: thus from 50% increasing to 62.5%. With alpha at 0.025, a power of 80% and tested one-sided, the required number of patients to demonstrate non-inferiority is at most 618 patients (309 patients per arm, which includes a 7% drop out rate). A flexible study design, based on a pre specified alpha and beta spending function is used, which allows early study termination at any of the 9 interim analyses currently scheduled using the above mentioned parameters. Interim analyses will be performed after inclusion of each one-tenth of the number of patients. However, due to the alpha and beta spending functions, additional interim analyses do not jeopardize the design, for example when requested by the DSMB. Both efficacy and safety will be tested.

At each interim analysis the hypothesis (H0) will be tested. If the difference in outcome between the groups favors the treatment group sufficiently (see upper boundary specification in the figure below) the study can be terminated early by showing efficacy (non-inferiority in this case). On the other hand, if the treatment group is sufficiently worse than the control group (the difference is crossing the boundary for futility) than the trial can be stopped because non-inferiority is very unlikely to be reached when continuing the trial. The table included provides estimates of the likelihood of an early stopping of this trial both when the null-hypothesis is true (inferiority) and the alternative is true (in the current study implying equality of the treatments). If neither boundary is crossed, the trial continues until the next interim analysis or the final one when the total maximum required number of 618 patients has been accrued.
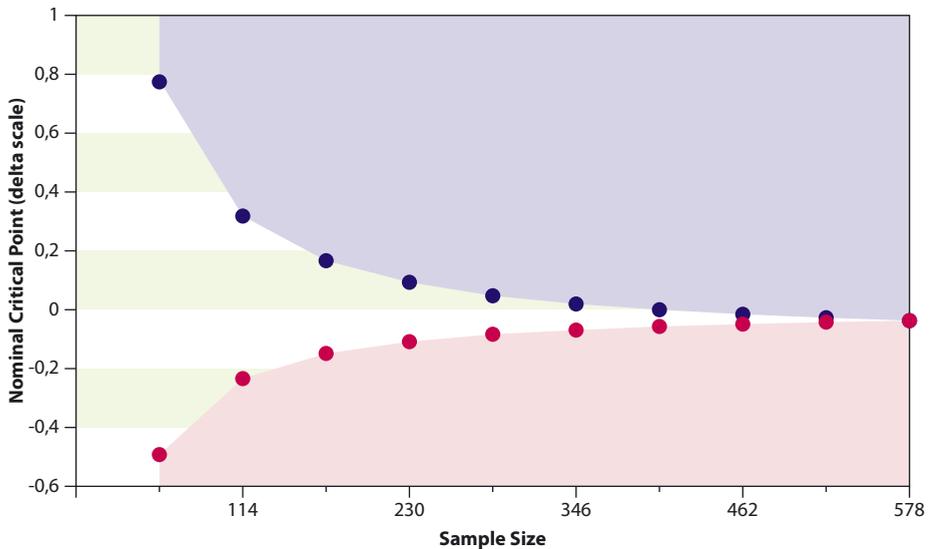
Decision rules, based on percentage difference in the bleeding score:
 The study will be terminated (see above):
1. When the effect at interim analysis meets the pink boundary area showing that the study has proven inferiority beyond the boundary of 12.5%.
2. When the interim analysis demonstrates an effect within the blue area (i.e. demonstrated non-inferiority).

The final analysis yields an effect estimate and its associated 95% confidence intervals. In the case of a confidence interval of which the left hand side not only exceeds the boundary of 12.5% inferiority but actually exceeds the 0% difference, we will claim superiority. Given an average of eight protocolled transfusions per patient and the continuation in the assigned arm for secondary endpoint analysis, the minimum number of platelet transfusions will be n=5,000.

Prior to the PREPAReS study, a pilot study will be conducted (the Bleeding Observation Pilot Study, BOPS) to accurately assess bleeding in patients that received Sanquin's current standard platelet products. The rationale is that in the hitherto published studies,[37,38] the percentage of ³ grade 2 bleeding in patients is considerably higher (49.2%[37] to 69%[38]) as in earlier studies conducted by Sanquin (16.1%[39]). The reason for this discrepancy is not entirely clear, but the thoroughness of assessing the bleeding sites is a likely explanation. In this pilot study, bleeding will be assessed actively by trained nurses. If this pilot study shows that the percentage of bleeding is more than 10% different from the 50% value obtained from the SToP trial, then a new power calculation will be conducted.
This modification will be submitted to the Ethics Committee for approval as amendment to this protocol.

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APPENDIX A
AML Trials reviewed for the discussion
of the thesis

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Summary

According to current guidelines, patients with thrombocytopenia due to myelosuppression are supported with platelet concentrates in order to prevent and treat bleeding complications using algorithms which include the level of thrombocytopenia as well as varying clinical parameters, e.g. concomitant infection, the use of anticoagulant drugs, specific interventions. In the last three decades, mainly driven by safety issues, several platelet product changes were made with leukoreduction in the eighties of the previous century, plasma reduction and the use of additive solution in the nineties and the use of pathogen reduction in the first decade of this century (chapter 1). Pre-transfusion in-vitro quality testing, considered essential by the FDA draft guidance, shows several significant differences during storage, however the used tests do not predict clinical efficacy.
It is hypothesized that a combination of tests using a rating score could be a better alternative for the prediction of clinical efficacy (chapter 2). This thesis is mainly based on two randomised controlled trials testing the clinical efficacy of the use of additive solutions and pathogen reduction, essentially showing a decreased clinical efficacy as well as a decrease in adverse transfusion events (chapter 3-5). Platelet transfusion refractoriness occurred very frequent, but more importantly it was mainly if not solely caused by clinical factors (chapter 3) and associated with bleeding and a decreased patient survival (chapter 6). The second trial emphasising the difficulty of measuring and grading bleeding complications, nowadays considered as an essential endpoint in platelet transfusion trials, resulted in the performance of a pilot study showing that despite platelet transfusion support bleeding occurred in the vast majority (87%!) of patients (chapter 7). Similar studies from other investigators as well as these observations are leading to an era of "rethinking" the pathophysiology of bleeding and the role of platelet transfusion support: endothelial damage as a common pathway (chapter 8). As the development of novel platelet products continues recently a randomised controlled trial started, comparing conventional plasma stored platelets with riboflavin-UVB treated platelets using bleeding as primary outcome. In addition, this trial allows for several side studies, including HLA-immunisation as well as testing patient and product parameters in relation to clinical efficacy and the occurrence of bleeding (chapter 9). A better understanding of the pathophysiology of bleeding, thrombocytopenia and platelet transfusion refractoriness will lead to improvements in supportive care as well as patient survival, the common goal of all physicians.

Samenvatting

Volgens de huidige richtlijnen worden patiënten met een trombocytopenie als gevolg van beenmergsuppressie ondersteund met plaatjes concentraten ter preventie en behandeling van bloedingen, gebruikmakend van algoritmes welke rekening houden met de mate van trombocytopenie als ook een variërend aantal klinische parameters, b.v. infectie, het gebruik van bloedverdunnende medicatie, specifieke interventies. In de laatste drie decennia, vooral gedreven door veiligheidsaspecten, hebben plaatjes concentraten veranderingen ondergaan met leukoreductie in de tachtiger jaren van de vorige eeuw, plasma reductie en de toepassing van synthetische bewaarmedia in de negentiger jaren en het gebruik van pathogeen reductie in het eerste decennium van deze eeuw (hoofdstuk 1). Pre transfusie in-vitro kwaliteitstesten, als essentieel gezien door de FDA richtlijn, toont verschillende significante verschillen gedurende de bewaarperiode, echter geen van deze testen voorspellen de klinische effectiviteit. Er wordt een hypothese geponeerd dat wellicht een combinatie van testen resulterend in een punten schaal een alternatief vormt om klinische effectiviteit te voorspellen (hoofdstuk 2). Dit proefschrift is vooral gebaseerd op twee gerandomiseerde studie welke de klinische effectiviteit onderzochten van bewaarmedia en pathogeen reductie, en in essentie een afname van klinische effectiviteit maar ook een afname van nadelige transfusiereacties laten zien (hoofdstuk 3-5). Transfusiefalen bleek zeer frequent voor te komen en belangrijker nog vooral zoniet volledig te worden verklaard door klinische factoren (hoofdstuk 3) en geassocieerd te zijn met bloeden en een verminderde overleving van patienten (hoofdstuk 6). De tweede studie, de moeilijkheid van het meten en graderen van bloedingen, tegenwoordig als essentieel eindpunt van plaatjestransfusie studies beschouwt, benadrukkend, resulteerde in het verrichten van een voorstudie die liet zien dat, ondanks plaatjestransfusies, bloedingen in de overgrote meerderheid (87%!) van de patienten optraden (hoofdstuk 7). Vergelijkbare studies van andere onderzoekers als ook deze observaties leiden tot het "overdenken" van het mechanisme van bloeden en de rol van plaatjestransfusies: Endotheelschade als "common pathway" (hoofdstuk 8). Omdat de ontwikkeling van nieuwe plaatjes producten verder gaat, werd in 2011 met een derde gerandomiseerde studie gestart, welke conventionele in plasma bewaarde plaatjes vergelijkt met riboflavine-UVB behandelde plaatjes met bloeden als primaire uitkomstmaat. Daarnaast fungeert deze studie als platvorm voor verschillende zijstudies, waaronder onderzoek naar HLA-immunisatie als ook het testen van patient- en productfactoren in relatie tot het optreden van bloedingen (hoofdstuk 9). Een beter begrip over de achtergrond van bloedingen, trombocytopenie en het optreden van transfusiefalen zal uiteindelijk leiden tot verbeteringen in de ondersteunende zorg en overleving van de patiënt, het gemeenschappelijke doel voor alle behandelaren.

Dankwoord

Na een gevoelsmatig lange weg is er dan nu toch een proefschrift. Hieraan werd ik telkens herinnerd door de jaarlijks terugkerende kerstboodschap van Christl Vermeij-Keers. Eigenlijk staat zij aan de basis van mijn interesse voor het doen van wetenschappelijk onderzoek. Ik zie mij zelf nog staan in de kelder van het oude Anatomiegebouw van de Leidse Universiteit. Vreugdevol was ik dan ook dat ik haar op de kerstkaart kon schrijven dat de promotie in 2012 echt zal plaatsvinden.

Het tweede bepalende moment is mijn kennismaking met Anneke Brand, die mij op een bank naast het transfusielaboratorium enthousiast maakte voor klinisch transfusieonderzoek. Dankbaar ben ik voor de drempelloze begeleiding, die zij mij al die jaren gaf en nog geeft. Mijn eerste studie had ik niet kunnen doen zonder de medewerking van het transfusielaboratorium in Leiden, de verpleegkundigen en niet in het minst de secretaresses van de hematologie afdeling van het LUMC. Zonder enige kennis van GCP werd er nog gerandomiseerd met envelopjes en de afdeling gedecoreerd met oranje stickers. In die tijd leerde ik ook Rinie kennen als een trouwe steun en toeverlaat. De studie toen, maar ook de daaropvolgende studies had ik niet kunnen volbrengen zonder haar toewijding. Zij is in de afgelopen jaren gegroeid als datamanager en bovenal als vriendin.

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Hoewel het zeker niet al tijd 'koek en ei' is, zorgen zij er al jaren voor dat ik op een topklinische hematologieafdeling kan werken en onderzoek kan doen. Een andere hematoloog die ik dankbaar ben, is Peter Huijgens. Hij stond voor en naast me toen het recht om wetenschappelijk onderzoek te publiceren werd bedreigd.

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De laatste alinea van dit proefschrift (hoewel het ook de eerste had kunnen zijn) wil ik gebruiken om mijn maatje Corine te bedanken. Zij is al meer dan 20 jaar mijn steun en toeverlaat. Zonder haar had ik dit alles niet kunnen bereiken. Zij was, is en zal altijd zijn: een klankbord en een knuffel. Amo ergo sum!

Curriculum Vitae

Jean-Louis Kerkhoffs werd op 4 maart 1967 geboren in Roermond. Na het behalen van het eindexamen op het Bisschoppelijk College Schöndeln in 1985 ging hij geneeskunde studeren aan de Rijksuniversiteit Leiden, waar hij zijn doctoraal afrondde in 1991.
Na de co-schappen ging hij werken als artsassistent heelkunde in het Groene Hart Ziekenhuis te Gouda. In 1994 huwde hij Corine Hamer. Hij heeft drie kinderen, Marijn (1996), Bram (1997) en Julius (2000). In 1997 startte hij met de opleiding inwendige geneeskunde in het Leyenburg ziekenhuis te Den Haag. Na de laatste 2 jaar van zijn opleiding in het Academisch Ziekenhuis in Leiden te hebben volbracht, volgde de registratie in de Inwendige Geneeskunde in 2003 en in de Hematologie in 2004.
In datzelfde jaar startte hij met zijn opleiding tot transfusiespecialist en de werkzaamheden, die geleid hebben tot dit proefschrift, bij Sanquin. Sinds 2004 is hij in deeltijd werkzaam in het HagaZiekenhuis als hematoloog, met als aandachtsgebied sikkelcelziekte en thalassemie, en tevens parttime werkzaam bij Sanquin, waar hij klinisch transfusie onderzoek verricht en consulten verricht voor de klinische consultatieve dienst.

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- **PREPAReS study:** Clinical effectiveness of standard versus pathogen-reduced buffy coat-derived platelet concentrates in plasma in hemato-oncological patients. A multicenter randomised controlled trial.
- **AML study:** a retrospective cohort study investigating the association between bleeding and survival in 160 AML patients treated from 2000 – 2004 in two large hospitals.
- **Albumen study:** a prospective pilot study testing albuminuria and CRP as markers for endothelial damage in AML patients (n = 10) undergoing cytotoxic treatment.
- **ITP-TTP study:** a case-control study testing the occurrence of thromboembolic and cardiovascular complications during the follow-up of patients with TTP in remission