Unexpected effects of transfusion in the critically ill

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Citation for published version (APA):
Since the first recorded successful blood transfusion was performed in 1665 and the first Intensive Care Unit (ICU) was founded in 1952, transfusion has gradually become a common medical procedure in the ICU. However, recent studies have shown an association between transfusion and adverse outcome.

This thesis contains both clinical and pre-clinical investigations of the effects of transfusion in the critically ill. The studies focus on the underlying mechanisms of the adverse outcome associated with transfusion and are clustered in three parts that each focuses on one of the different blood components and has its own aims:

Red blood cells (I)
To investigate the role of storage red blood cells as well as the presence of an inflammatory condition on the association between RBC transfusion and host response

Fresh frozen plasma (II)
To assess the effect of a FFP transfusion on host response and hemostatic balance in critically ill patients

Platelets (III)
To assess the effect of platelet transfusion on the development of infections in critically ill patients
Unexpected Effects of Transfusion in the Critically Ill

Marleen Straat
Unexpected Effects of Transfusion in the Critically Ill

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof. dr. van den Boom
ten overstaan van een door het College voor Promoties ingestelde commissie,
in het openbaar te verdedigen in de Aula der Universiteit
op vrijdag 8 juli 2016, te 13:00 uur
door Marleen Straat
deboren te Amsterdam
PROMOTIECOMMISSIE:

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Chapter 1

General introduction and outline of this thesis
INTRODUCTION

The history of blood transfusion

It was in 1628 that the English physician William Harvey discovered the circulation of blood and soon after the first known blood transfusion was attempted. However, only in 1665 was the first recorded successful blood transfusion performed: physician Richard Lower kept dogs alive by transfusion of blood from other dogs. It was only in 1818 that the first successful human to human blood transfusion saved the life of a woman suffering from profound postpartum hemorrhage. The caring obstetrician, James Blundell from the United Kingdom, performed 10 transfusions of which only 5 were of benefit to the receiving patients.

In 1900, Karl Landsteiner, an Austrian physician, discovered the human blood groups for which he received the Nobel prize for Medicine and this led to the discovery of the importance of crossmatching in 1907. The subsequently implemented crossmatching before transfusion prevented a substantial amount of acute hemolytic transfusion reactions. In 1914 were the first long-term anticoagulants developed and one of these, sodium citrate, was used a year later by Richard Lewishohn from Mt. Sinai Hospital in New York to change the practice of transfusion from direct (patient-to-patient) to indirect (patient-blood bank-patient). After the second World War, Edwin Cohn from Harvard Medical School developed a system that enabled blood component therapy. The different blood components are described below.

Red blood cells (erythrocytes)

In the late 1970s, both the new anticoagulant CPDA-1 as well as the first red blood cell (RBC) additive solution, saline-adrenaline-glucose (SAG) are developed. In 1981, the same researchers that developed SAG added mannitol to help protect the RBC membrane and reduce hemolysis. This new solution was named SAGM and nowadays SAGM is the most widely used RBC additive solution. Of note, SAGM has not been licensed by the Food and Drug Administration (FDA), and hence is not used in the United States of America (USA). After having observed that a lower concentration of leukocytes led to less adverse effects, leukoreduction by means of filtration after collection is common practice in The Netherlands now. RBC volume is approximately 280 ml and the hematocrit ranges from 50% to 65%. One unit of RBCs transfused to an adult should in theory raise the haemoglobin concentration by 0.5 mmol/L. In the Netherlands, a bag of RBCs can be stored for up to 35 days at 2-6 °C.

Fresh frozen plasma

The first plasma transfusion in animals took place in the 1870s, when Bowditch and Luciani injected serum from sheep into frogs in their search for a blood substitute.
Chapter 1

The first plasma transfusion in humans was to treat the Spanish flu in 1918\textsuperscript{14}. By the late 1920s and early 1930s, plasma became routinely used in hospitals\textsuperscript{1,3,5}. Initially, fresh plasma was used, but researchers proved that both dried and frozen plasma were just as effective and easier to store\textsuperscript{1,3,5,6}. During World War II, the United States Army used dried plasma as a blood substitute because of its easy storage\textsuperscript{1,7}. However, it soon proved that trauma victims needed oxygen-carrying capacity and whole blood quickly replaced dried plasma\textsuperscript{3,7}. After World War II, the indications for a Fresh Frozen Plasma (FFP) transfusion was gradually extended to sepsis, burns, nutritional deficiencies, nephrotic syndrome, sickle cell anemia and childhood acute lymphoblastic leukemia (ALL)\textsuperscript{1,8-12}. In 1964, the first randomized controlled trial on FFP transfusion was published\textsuperscript{1,13} and approximately 70 followed. The only indications for which an FFP transfusion is indicated according to the current guidelines nowadays are single coagulation factor deficiencies for which no virus-safe fractionated product is available, multiple coagulation factor deficiencies (e.g. diffuse intravascular clotting, DIC) in the presence of severe bleeding, thrombotic thrombocytopenic purpura (TTP), reversal of warfarin effect in the presence of severe bleeding and surgical bleeding and massive transfusion\textsuperscript{14}.

Nowadays, Omniplasma is used instead of FFP in the Netherlands. Omniplasma is manufactured by thawing and pooling multiple units of single donor FFP. The advantage of Omniplasma is that it is a pooled product of 600-1200 donations which ensures adequate levels of clotting factors and the dilution of the possible presence of antibodies, thereby reducing the risk of transfusion-related acute lung injury. One bag of Omniplasma contains approximately 310 ml and can be stored for up to 4 years at -18 °C in the dark.

Platelets (thrombocytes)

The first use of platelet transfusions was described in the 1950s to reduce mortality from haemorrhage in patients with acute leukaemia\textsuperscript{15-17}. The use of platelets has steadily grown since then. The change from glass bottles to the disposable plastic bag sets for the collection of blood we still use nowadays was an important development, since it made collection and preparation of platelets within a closed system possible. This reduced the risk of bacterial contamination and facilitated the implementation of a simple, two-step centrifugation platelet preparation protocol. In the 1970s, investigators began removing leucocyte-rich and platelet-rich buffy coats from red-cell concentrates, to use the white cells for interferon production and to reduce leucocyte-related transfusion side effects\textsuperscript{17,18}. The regular use of this procedure led to the development of a novel whole-blood procedure for the preparation of platelet concentrates, named the buffy coat method, which we still use in the Netherlands. First, whole blood is spun which leads to the sedimentation of all cells. The thin layer of white blood cells and platelets is called the buffy coat and is removed from the other blood cells. Subsequently, five buffy coats of the same ABO/Rh group are collected, pooled, and diluted in autologous
plasma or in a crystalloid solution. The pooled buffy coats are gently centrifuged and the platelet-rich supernatant is collected. One bag of platelets contains approximately 330 ml, the amount of platelets is at least 250 x 10^9 /ml and the storage solution is Platelet Additive Solution type III (PAS-III). A bag of platelets can be stored for up to 7 days, when kept at 20–24 °C on a shaking device.

**Transfusion in the critically ill – two sides of the medal**

*Transfusion of red blood cells*

Anaemia is a frequently encountered problem in the critically ill patient. As a consequence, critically ill patients are frequently transfused, with up to 44% of patients receiving a blood transfusion. In sepsis, transfusion rates can even reach 73%. The obvious causes of anaemia in critically ill patients are a decreased production of red blood cells (e.g. by a poor nutritional status, the existence of co-morbidities, a reduced production of erythropoietin and a reduced iron availability) and an increased loss of red blood cells (e.g. increased blood loss due to surgical procedures or repeated phlebotomy or bleeding). In addition to that, there are indications an increased clearance of RBCs also plays an important role that in inflammatory states. It is commonly thought that the clearance of red blood cells in inflammation is mediated in the spleen. This has, however, never been proven and other mechanisms of clearance may be implicated. These include an increased PS-exposure on the red blood cell membrane as a consequence of increased plasma concentrations of sphingomyelinase induced by tumor necrosis factor-α or direct production by bacteria, which then can act as an “eat-me” signal to circulating macrophages. Also, RBCs can be phagocytosed directly by increased expression of Band 3 on their membrane, an increase that is present in septic mice but absent in healthy mice. Inflammatory conditions induced by LPS or sepsis can also lead to a decreased deformability of the red blood cell, which may eventually lead to the trapping of RBCs in the microvasculature. Also, trapping of RBCs in the microvasculature during inflammation may be mediated by adhesion of RBCs to the endothelium in vitro.

Since RBCs transport oxygen from the lungs to the tissues, it has since long been common practice to supplement red blood cells liberally in order to ensure an adequate supply of oxygen to the tissues. However, the Transfusion Requirements in Critical Care (TRICC) trial demonstrated that lower Hb levels were well tolerated in the critically ill. Also, younger patients and the less severely ill transfused according to the restrictive transfusion strategy had an improved 30-day mortality rate compared with the patients in the liberal strategy group. Hereby, is an association between RBC transfusion and adverse outcome. In line with this, several observational studies have reported an association between RBC transfusion and adverse outcome in the critically ill.
In particular, an association between RBC transfusion and the development of acute lung injury and acute kidney injury is repeatedly found. TRALI (Transfusion-Related Acute Lung Injury) is a clinical diagnosis and defined as the onset of acute lung injury within 6 hours of blood transfusion without an additional risk factor for acute lung injury. Also possible TRALI (which is TRALI in the presence of another risk factor) and delayed TRALI (which is TRALI within 6-72 hours after the blood transfusion) have been described as clinical entities. TRALI incidence varies between 0.08–15.1% per patient transfused and 0.01–1.12% per product transfused. TRALI occurs more frequently in the critically ill patient population.

Although the finding of an association between transfusion and organ injury has led to the adoption of a lower transfusion trigger in most Intensive Care Units (ICUs), a large variance in transfusion practice remains. As anemia is also associated with adverse outcome and the currently used low Hb transfusion trigger may already be too low for some patient populations on the ICU, this poses a challenge to both the treating physician as well as to the scientific community, calling for interventions which improve RBC transfusions and reduce the risk of associated organ failure. Of note, the mechanisms of the observed association between RBC transfusion and adverse outcome still remain unknown.

In animal studies, prolonged storage of the transfused red blood cells is clearly related with the development of lung injury. Clinical data, however, are conflicting. There are observational studies showing association between transfusion with stored red blood cells and adverse outcome and increased mortality, whereas other studies not found an association between age of blood and outcome. These differences may be explained by the heterogenous study populations and the fact that most patients received both fresh and stored red blood cell products. Recently, two large randomized controlled trials found no difference in clinically relevant outcome parameters between critically ill patients receiving fresh and those receiving stored red blood cell products. However, these findings do not rule out that storage lesion exists. Given that the association between transfusion and organ failure exists, the need for improved preparation and storage conditions remains.

Organ injury is thought to be caused by bioactive substances which accumulate during storage of cellular blood products. Bioactive lipids and sCD40L have been implicated as soluble mediators in TRALI, but other studies have not confirmed this association. Recently, extracellular vesicles (EVs) have been proposed as the responsible mediators. EVs are small phospholipid vesicles released from most cell types. EVs facilitate intercellular exchange of receptors, ligands, signaling molecules, genetic information, etc. without direct cell-to-cell contact. High concentrations of RBC-derived EVs are present in the supernatant of RBC transfusion bags. These EVs initiate an
propagate thrombin generation and shorten clotting time in vitro\textsuperscript{80-82}. These effects of EVs likely depend on storage time of the blood bags, because the concentration and thrombin-generating ability of EVs increases with storage duration\textsuperscript{81,82}.

Alternatively to a causative soluble factor, the RBC itself also undergoes changes during storage. For example, it loses Duffy antigen expression and thereby chemokine scavenging function. The Duffy antigen is a minor blood group antigen that binds a variety of inflammatory chemokines, thereby rendering these red blood cell bound chemokines inaccessible to circulating neutrophils\textsuperscript{83}. Furthermore, when erythrocytes age they lose their deformability, which impedes their passage through the microcirculation of the organs\textsuperscript{84}. The subsequent adherence may even be further augmented in inflammatory conditions, due to activated endothelium\textsuperscript{38,39}.

In conclusion, despite the obvious benefits and optimalization of the storage process which has taken place, an association between RBC transfusion and adverse effects remains, which warrants further research into the mechanisms of this association in an effort to optimize RBC storage conditions.

**Transfusion of fresh frozen plasma**

Substantial units of FFP are utilized in the ICU\textsuperscript{85,86}. In practice, FFP is transfused to correct abnormal coagulation tests to prevent bleeding. Studies show that the prevalence of coagulation abnormalities in critically ill patients is high: 30-66% of patients have an International Normalized Ratio (INR) of $>1.5$ or a prothrombin (PT) ratio of $>1.5$\textsuperscript{87,88} and 8 to 45% has a thrombocytopenia\textsuperscript{89-92} at some point during their ICU stay\textsuperscript{93}. The most common causes of a deranged coagulation are sepsis, multiple trauma, brain injury, major blood loss, liver disease, disseminated intravascular coagulation, use of vitamin K antagonists before ICU admission, vitamin K deficiency, renal failure, cardiac surgery and thrombotic micro-angiopathies\textsuperscript{93,94}.

Fresh frozen plasma effectively corrects multiple clotting factor deficiencies and guidelines recommend its use in severe bleeding\textsuperscript{14}, but also patients who have a coagulopathy, but lack signs of active bleeding receive a substantial amount of FFP\textsuperscript{85,86,95}. Even when evidence that prophylactic administration prevents bleeding complications is absent\textsuperscript{96,97} inappropriate use of FFP is widespread. In the Netherlands, 80,000 FFP units are issued annually and most of these are transfused in the ICU\textsuperscript{85,86}. Three misapprehensions are deemed responsible for this inappropriate use of FFP: physicians assume that an elevated PT or INR predicts an enhanced bleeding risk in patients undergoing an invasive procedure, that the pre-procedural FFP administration improves PT/INR values and that prophylactic FFP transfusion results in fewer bleeding complications\textsuperscript{1,93,98-100}.

But does an elevated PT/INR predict an enhanced bleeding risk? The coagulation system consists of three main components. The pro-coagulant elements include the endothe-
lium, thrombocytes, individual coagulation factors and fibrinogen. The anti-coagulant system includes proteins C and S and antithrombin. The third component of coagulation is the fibrinolytic system. Most standard coagulation tests (platelet count, aPTT, PT, fibrinogen and d-dimer) only reflect a part of this complex system, and therefore fail to reflect the contemporary result of the balance between the three separate components. Thereby, standard coagulation tests cannot reliably predict potential bleeding risk.

In contrast to these conventional coagulation tests, rotational thromboelastography assesses both clot formation and degradation. The resulting thromboelastogram represents initiation of clot formation, fibrin formation and clot degradation. In critically ill patients suspected of a coagulopathy, thromboelastography may improve identification of patients who have an increased bleeding risk and are in need of FFP transfusion. However, threshold values indicative of coagulopathy have not been validated for the critically ill.

Furthermore, does pre-procedural FFP administration improve PT/INR values and in general, restore coagulation ability? Only a few clinical trials have studied the efficacy of transfusion of FFP in critically ill patients with a coagulopathy, but these used different doses, did not assess the effect of FFP administration on occurrence of bleeding complications and included both bleeding and non-bleeding patients. In assessment of efficacy of FFP, the dose is of importance. An adequate dose of FFP will correct the PT/INR, since it suppletes all clotting factors. However, since the PT/INR only reflects part of the coagulation system, this does not mean that an FFP transfusion effectively corrects coagulopathy. Altogether, evidence supporting the efficacy of FFP to correct coagulopathy in critically ill patients is scarce. Furthermore, no evidence exists that prophylactic transfusion of FFP to critically ill patients with a coagulopathy can reduce bleeding complication. Of interest, epidemiological studies in trauma patients suggest that in trauma patients requiring a massive transfusion, resuscitation with a higher ratio of FFP to red blood cell units was associated with decreased mortality. Of note, this association was independent of the effect of FFP on correction of coagulopathy. As FFP is supposedly beneficial in bleeding because it corrects a deficiency in coagulation factors, this observation suggests another mechanism of action of FFP.

Comparable to a RBC transfusion, there is an association between FFP transfusion and adverse outcome in the critically ill, such as multi-organ failure, transfusion-related circulatory overload and an increased risk of infections. But most importantly, FFP has been linked to TRALI. More specifically, antibody-mediated TRALI, which is caused by the passive infusion of human leucocyte antigen (HLA) and human neutrophil antigen (HNA) antibodies in donor blood. These antibodies are found predominantly in blood from multiparous women as they have become sensitized during pregnancy by becoming exposed to the antigens of their fetus.
The risk of female plasma donation was confirmed in two studies in critically ill patients\(^1\). The United Kingdom implemented a male donor fresh-frozen plasma transfusion policy and many countries followed. TRALI incidence has decreased significantly since then\(^2\) and two recent meta-analyses reported that excluding female donors reduces plasma-related TRALI incidence by 73\%\(^3,4\). However, the policy of use of male plasma only has decreased TRALI, but has not completely abrogated this risk.

In conclusion, despite its widespread use in the ICU we still lack knowledge on all the effects of an FFP transfusion. Knowledge on these effects is needed to weigh the benefits and risks of plasma transfusion in the critically ill.

Transfusion of platelets

Trombocytopenia is a frequent finding in the critically ill. Approximately 40 percent of adult patients admitted to an ICU have a trombocytopenia (platelet count less than 150 \(\times 10^9\) per L)\(^1\). There is a wide variety of causes of thrombocytopenia in the critically ill, of which sepsis, disseminated intravascular coagulation (DIC), massive blood loss, thrombotic microangiopathy, heparin-induced thrombocytopenia, immune thrombocytopenia and drug-induced thrombocytopenia are the most important\(^1\).

Transfusion trigger for platelets in the critically ill has not been formulated. Transfusion triggers have been established based on evidence from randomized trials\(^5\), and resulting guidelines, although not specifically designed for the critically ill patient population, recommend different transfusion triggers, ranging from 10 \(\times 10^9\) to 50 \(\times 10^9\) per L for prophylactic transfusions for different indications\(^6,7\).

Although thrombocytopenia is a risk factor for adverse outcome, such as major bleeding\(^8,9\), increased length of ICU-stay and death\(^10\), studies show conflicting results on whether platelet transfusions improve\(^11,12\) or worsen survival\(^13,14\). Arguably, worsening of survival may be a confounder to disease severity. However, there may also be adverse effects of a platelet transfusion, which may outweigh the supposed benefits. These include transmission of infection\(^1\), allergic reactions\(^2\), TRALI\(^4,9,53,68\), transfusion-related immunomodulation (TRIM)\(^4,5\) and venous thromboembolism\(^6\). TRIM has been associated with delayed graft rejection, increased cancer recurrence and higher susceptibility to nosocomial bacterial infections\(^14,15\). Indeed, studies showed that platelet transfusion is associated with nosocomial infections in a variety of critically ill patient populations\(^14,16,17\). Therefore, the benefit of a platelet transfusion should be weighed against the risks and a transfusion trigger should be carefully determined.

Whereas naive platelets have a natural life span of 8–12 days\(^1\), those prepared for transfusion can be stored only for 2–7 days at 20–24°C with agitation. This primarily reduces the risk of bacterial growth and secondarily minimizes the “platelet storage lesion” (correlates with reduced in vivo recovery and survival as well as haemostatic activity after
transfusion\textsuperscript{151,152}. Although aged platelets are clearly associated with the development of TRALI in animal models\textsuperscript{54,61,63,73,75,153}, clinical studies show conflicting results\textsuperscript{49,54,68-70,154}.

In conclusion, the advantages and disadvantages of platelet transfusion in the ICU remain surprisingly indefinite.

**AIMS OF THIS THESIS**

This thesis contains both clinical and pre-clinical investigations of effects of a blood component transfusion in the critically ill. The studies focus on investigations of mechanisms of the association between transfusion of blood components and adverse outcome.

**Red blood cells:** To investigate the role of storage red blood cells as well as the presence of an inflammatory condition on the association between RBC transfusion and host response (part I)

**Fresh frozen plasma:** To assess the effect of a FFP transfusion on host response and haemostatic balance in critically ill patients (part II)

**Platelets:** To assess the effect of platelet transfusion on the development of infections in critically ill patients (part III)

**OUTLINE OF THIS THESIS**

**Chapter 2** provides a summary of the knowledge of mechanisms of the clearance of red blood cells in patients with an inflammatory condition.

**Chapter 3** investigates the routing of donor RBCs following transfusion in a rat model using fresh and stored human RBCs.

**Chapter 4** shows the results of a study performed in endotoxemic rats receiving a transfusion with either fresh or stored labeled rat RBCs. It was hypothesized that endotoxemia contributes to increased clearance of donor RBCs from the circulation, with concomitant trapping of RBCs in organs, thereby accounting for transfusion-related organ failure. Furthermore, we hypothesized that stored donor RBCs will be more rapidly cleared from the circulation than fresh donor RBCs.

**Chapter 5** constitutes the results of an in vitro experiment to investigate the hypothesis that EVs accumulating in RBC products during storage contribute to a pro-inflammatory host response in recipients and that this is related to both amount as well as to storage duration.
Chapter 6 constitutes the results of an *in vitro* experiment to investigate the hypothesis that RBC-derived EVs can activate endothelial cells through activation of host immune cells and that this effect is a function of storage time.

Chapter 7 shows the results of a prospective pre-defined substudy of a randomized trial in coagulopathic non-bleeding critically ill patients receiving a prophylactic transfusion of FFP (12 ml/kg) prior to an invasive procedure are reported. We investigated whether INR prolongation correlates with viscoelastic assays, and evaluated the effect of a fixed dose of FFP on the haemostatic balance in these patients.

Chapter 8 constitutes the results of a prospective pre-defined substudy of a randomized trial in coagulopathic non-bleeding critically ill patients receiving a prophylactic transfusion of FFP (12 ml/kg). It was hypothesized that an FFP transfusion elicits a pro-inflammatory host-response.

Chapter 9 explores the effect of transfusion products on the occurrence of nosocomial infection in a prospective multicenter observational study in the critically ill. We hypothesized that the number of blood products was independently associated with an increased risk of nosocomial infection.

This thesis ends with a summary of the studies mentioned above and a general discussion in Chapter 10, of which a Dutch translation is provided in Chapter 11.
LITERATURE


84. Barshtein G, Manny N, Yedgar S. Circulatory risk in the transfusion of red blood cells with impaired flow properties induced by storage. Transfusion medicine reviews 2011;25:24-35.


| PART | Red Blood Cells | Unexpected Effects of Transfusion in the Critically Ill |
Chapter 2

Red blood cell clearance in inflammation

Straat M, van Bruggen R, de Korte D, Juffermans NP

ABSTRACT

Anaemia is a frequently encountered problem in the critically ill patient. The inability to compensate for anaemia includes several mechanisms, collectively referred to as anaemia of inflammation: reduced production of erythropoietin, impaired bone marrow response to erythropoietin, reduced iron availability and increased red blood cell (RBC) clearance. This review focusses on mechanisms of RBC clearance during inflammation. We state that PS expression in inflammation is mainly enhanced due to an increase in ceramide, caused by an increase in sphingomyelinase activity due to either Platelet Activating Factor, tumor necrosis factor-α or direct production by bacteria. Phagocytosis of RBCs during inflammation is mediated via RBC membrane protein Band 3. Reduced deformability of RBCs seems an important feature in inflammation, also mediated by Band 3, as well as by nitric oxide, reactive oxygen species and sialic acid residues. Also, adherence of RBCs to the endothelium is increased during inflammation, most likely due to increased expression of endothelial adhesion molecules as well as phosphatidylserine on the RBC membrane, in combination with decreased capillary blood flow. Thereby, clearance of RBCs during inflammation shows similarities to clearance of senescent RBCs, but also has distinct entities, including increased adhesion to the endothelium.
INTRODUCTION

It is commonly known that anaemia is a frequently encountered problem in the critically ill patient. Almost 95% of patients admitted to the intensive care unit (ICU) has a haemoglobin level below normal after three days of ICU admission. As a consequence, critically ill patients are frequently transfused. The CRIT study showed that 44% of the patients received at least one or more units of red blood cells (RBC). In sepsis patients, transfusion rates even reach 73%. The association between transfusion and adverse outcome found in a number of observational studies in several critically ill patient populations calls for a thorough understanding of the causes of anaemia. In this review, we discuss the causes of anaemia in sepsis patients, focussing on anaemia of inflammation. We discuss mechanisms of anaemia of inflammation, with a special emphasis on increased red blood cell clearance.

CAUSES OF ANAEMIA IN SEPSIS

In sepsis, pre-existent factors which contribute to chronic anaemia before admission to the ICU are often present, including a poor nutritional status, the existence of co-morbidities such as renal failure, or intensive treatment for malignancies. These factors not only to anaemia, but at the same time pose these patients at increased risk of acquiring a sepsis.

Causes of anaemia in sepsis are multifactorial and summarized in Table 1. A cause of anaemia which stands somewhat separate is blood loss. Increased blood loss can occur via the gastrointestinal tract, from surgical procedures or through repeated phlebotomy. In total, a median blood loss of 128 ml per day has been calculated in critically ill patients. Healthy individuals donating blood can compensate a loss of about 10 ml of red blood cells per day. Critically ill patients however, are impaired in their ability to regenerate these losses. Causes of this inability to correct for deficiencies are the consequence of inflammatory processes, collectively referred to as anaemia of inflammation.

This type of anaemia was once known as anaemia of chronic diseases. However, it turned out not to be restricted to chronic diseases, but also to occur in patients with acute inflammation. Moreover, some of the chronic disorders that were covered by the term led to anaemia through a different mechanism, for example chronic kidney failure. Therefore, already in 1983, it was advocated to change the term anaemia of chronic diseases to anaemia of inflammation.
Chapter 2

Pathogenesis of Anaemia of Inflammation

The pathogenesis of anaemia of inflammation comprises four different mechanisms: a reduced production of erythropoietin (EPO), an impaired bone marrow response to erythropoietin, a reduced iron availability and an increase in RBC clearance.

Decreased production of erythropoietin

Reduced erythropoiesis is a result of reduced maturation of erythroid precursors. When haemoglobin levels drop, the normal response is an increase in EPO production. In sepsis however, the rise in EPO levels in response to anaemia is blunted, corresponding with low hematocrit. The blunted endogenous EPO response to anaemia in critically ill patients is irrespective of the presence of renal failure. As low levels of EPO are found in several subsets of patients, including trauma and sepsis, the common denominator most likely is the presence of an inflammatory condition.

Table 1. Causes of anaemia in sepsis

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<td>· Repeated phlebotomy</td>
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<th>Anaemia of inflammation</th>
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<td>· Reduced production of erythropoietin</td>
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<td>· Impaired bone marrow response to erythropoietin</td>
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| Diffuse Intravascular Coagulation |

PATHOGENESIS OF ANAEMIA OF INFLAMMATION
Impaired bone marrow response to erythropoietin

Critically ill patients are able to respond to EPO administered exogenously, in terms of an increase in reticulocyte count\(^1\)\(^2\) and in terms of a decreased need for blood transfusion\(^1\). However, the erythropoietic response to exogenously administered EPO is also blunted, as doses of EPO given to generate a response are higher when compared to doses administered to patients with renal failure. At present, a recent meta-analysis of the efficacy of EPO has not led to the recommendation to supply EPO in the critically ill\(^1\).

The cause of bone marrow hypo-responsiveness to EPO is not known, but is likely to include inflammatory pathways. EPO binds to a specific receptor, resulting in proliferation and differentiation of erythroid progenitors. This process may be counterbalanced by death signals, resulting in impaired red cell production. In line with this, increased apoptosis of bone marrow erythroid precursors was demonstrated in sepsis patients. Also, erythroid progenitors can be inhibited by incubation with serum of septic patients\(^1\). This suggests that inflammation directly suppresses erythroid precursors.

Alternatively, as the amplification of erythropoiesis that results from the administration of EPO increases the need for iron, a lack of iron may underlie the blunted response to EPO. In anaemia of chronic kidney disease, it is well recognized that response to EPO is greatly enhanced by giving intravenous iron. It should be noted that only one study in the meta-analysis administered intravenous iron in association with EPO. In that study, the effect of EPO treatment was greater in terms of reduction in blood transfusion and increase in haemoglobin concentrations\(^1\).\(^8\)

Reduced iron availability

Iron for erythropoiesis comes from dietary sources through absorption in the gut. However, this accounts for only a small part of our daily iron need. The majority of iron used for erythropoiesis comes from lysis of aged red blood cells (RBCs), which generates free heme, which is then degraded to iron and recycled towards the circulation or stored in ferritin molecules.

Hepcidin is a major regulator of iron metabolism, which acts by binding to the iron exporter ferroportin, causing internalization of iron and inhibiting the release of iron from tissue macrophages. Thus, hepcidin reduces the concentration of iron in the blood. The production of hepcidin is up regulated in response to elevated serum iron levels. Inflammation also induces hepcidin, which is a fast response, resulting in a drop in iron levels within hours\(^1\). The induction of hepcidin synthesis by inflammation is not fully understood but depends on interleukin-6 (IL-6), as infusion of IL-6 into human volunteers induces increased hepcidin synthesis, with a decrease in plasma iron levels\(^2\). Indeed, hepcidin levels have been found to be elevated in critically ill trauma patients, correlating with the duration of the anaemia\(^2\), as well as in critically ill patients not sus-
pected for iron deficiency. Thereby, it is reasonable to assume that hepcidin is elevated in inflammatory conditions, contributing to anaemia. However, given the complexity of separating iron deficiency form anaemia of inflammation, more research is needed in this area.

**Increased red blood cell clearance**

Although some microbial agents can elicit severe haemolytic reactions in the course of sepsis, increased hemolysis in general is thought to play a minor role in anaemia of inflammation, as parameters of hemolysis are usually not disturbed.

The decrease in life span of RBCs in inflammation is rather thought to be due to altered morphology of RBCs, resulting in increased adherence to the endothelium and clearance from the circulation. The following chapters describe these changes in morphology in detail. As changes occurring during inflammation to some extend are similar to those observed during ageing of RBCs, a short description of changes during ageing is given first.

**SENESCENCE OF RED BLOOD CELLS**

**PS exposure**

Apoptosis is the term used for the suicidal cell death of nucleated cells and is characterized by loss of cellular K+ with subsequent cell shrinkage, nuclear condensation, DNA fragmentation, mitochondrial depolarization, cell membrane blebbing and phosphatidylserine (PS) exposure at the cell surface. However, since RBCs are devoid of nuclei and mitochondria, the term eryptosis was introduced to describe the process of apoptosis in these cells.

Eryptosis is triggered by an increased cytosolic Ca²⁺ concentration due to activation of cation channels by a number of different causes. In an *in vivo* experiment, an increase in PS-expression with increasing age of RBCs has been found, which correlated with the rate of removal of RBCs from the circulation. It was long believed that after increased PS-expression, macrophages eliminate these apoptotic RBCs by recognizing them through their specific PS receptor. However, various receptors have been identified recently, such as Tim1, Tim4 and Stabilin-2, that can mediate binding and phagocytosis of apoptotic cells by the recognition of PS on these cells. In addition, several plasma proteins, such as lactadherin, GAS6 and Protein S, have been described to bind to PS and act as bridging molecules to direct PS to receptors on phagocytes, α₄β₃/5 integrins and receptors of the Axl family respectively and mediate clearance of PS positive cells.
**Erythrocyte phagocytosis – Band 3 and CD47**

In contrast to eryptosis, RBCs can also be phagocytosed directly, without being apoptotic first. There are two distinct mechanisms of erythrophagocytosis due to ageing.

Naturally occurring antibodies (NAb) that bind to band 3 are implicated in the clearance of senescent RBCs. Band 3 is an abundant RBC integral membrane protein which has two different domains: the membrane spanning domain catalyzes anion exchange and is recognised by NAb, whereas the cytoplasmic domain binds different proteins and thereby regulates structure and function of the RBC. Band 3 undergoes a conformational change in senescent RBCs, although no consensus exists over the exact mechanism leading to this change. It is thought that oxidative damage to haemoglobin, which occurs during senescence, and the subsequent formation of hemichromes which bind to band 3, can eventually lead to clustering of band 3 into large aggregates. These clusters show enhanced affinity for NAb. Indeed, a mutual correlation has been shown to exist between the amount of membrane-bound hemichromes, percentage of aggregated Band 3 and phagocytosis intensity. Another hypothesis is that proteolytic degradation of band 3 is required to form the band 3 epitope(s) recognised by NAb.

NAb are not efficient opsonins, due to their low affinity and low circulating numbers. Their efficiency is increased by the activation of the classical complement pathway, which significantly lowers the amount of NAb needed for the induction of phagocytosis. For example, an in vitro experiment showed that phagocytosis of sheep RBCs (SRBCs) was at least 10-fold more effective when opsonized with C3b-IgG compared to opsonization with IgG alone. NAb form complexes with C3b, which are more resistant to inactivation by factors H and I than free C3b. Furthermore, the activation of C3 convertase is more potent by these complexes than by immobilized C3b. However, the exact mechanism how opsonization with C3b-IgG increases phagocytosis compared to opsonization with IgG or C3B alone remains unclear.

An alternative mechanism of erythrophagocytosis involves the regulation of expression of ‘eat me’ and ‘don’t eat me’ signals. CD47, one of the membrane proteins expressed by RBCs, has an inhibitory effect on erythrocyte phagocytosis by macrophages. CD47 binds to SIRPα on the macrophage, eventually leading to inhibition of phagocytosis. However, CD47-SIRPα can also, through an unknown mechanism, promote phagocytosis of apoptotic cells. Recently, it was shown that SIRPα plays a role in the removal of aged erythrocytes through CD47 binding. CD47 undergoes a conformational change in response to oxidative damage due to ageing after which CD47 binds thrombospondin-1 and is subsequently recognized as an “eat me” signal by SIRPα (see review by R. van Bruggen and D. de Korte, this issue of THM).
Reduced deformability

To be able to pass through capillaries with a diameter 2-3 μm, while their own diameter is 8 μm, RBCs are highly deformable. The ability to deform relies on different characteristics of the RBC, including membrane composition, cellular geometry and cytoplasmic viscosity. Reduced deformability is not only observed in senescence, but also occurs in a wide variety of pathological conditions, such as malaria, hereditary spherocytosis, sickle cell disease and sepsis. In the following chapter, the effects of inflammation on RBC clearance are discussed, by comparing mechanisms in ageing with inflammatory pathways (Table 2).

Table 2. Identified mechanisms of erythrocyte clearance in physiologic senescence and inflammation

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<tr>
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<th>Physiologic senescence</th>
<th>Inflammation</th>
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<tr>
<td>PS exposure</td>
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<td>+</td>
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<tr>
<td>Erythrocyte phagocytosis</td>
<td>+</td>
<td>+/-</td>
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<td>Reduced deformability</td>
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<td>Adherence to endothelium</td>
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EFFECTS OF INFLAMMATION ON CLEARANCE OF RED BLOOD CELLS

PS-exposure in inflammation

PS exposure has been claimed to be involved in accelerated RBC clearance during inflammation. This is thought to be due to an increase in the plasma concentrations of sphingomyelinase, an enzyme that converts sphingomyelin into ceramide. Ceramide enhances the sensitivity of erythrocytes to an already increased intracellular Ca^2+ concentration, and thereby enhances PS exposure. Sphingomyelinase normally resides in the lysosomes of macrophages, but can be secreted into plasma. Many factors can lead to an increase in plasma levels of sphingomyelinase and some of these are also implicated in sepsis, such as tumor necrosis factor-alpha (TNF-α) and Platelet Activat-
RBC clearance in inflammation

Inflammation Factor (PAF)\textsuperscript{78}. Furthermore, bacteria such as \textit{Staphylococcus aureus} can also produce sphingomyelinase\textsuperscript{79}. 

\textit{In vitro} experiments show that PS exposure is induced by incubation of RBCs with plasma of sepsis patients, but not by incubation with plasma of healthy volunteers\textsuperscript{79}. Furthermore, PS exposure is induced after treatment with supernatant from cultured \textit{S. aureus} with sphingomyelinase activity, but not after exposure to supernatant from mutated \textit{S. aureus} lacking sphingomyelinase activity\textsuperscript{79}.

**Erythrocyte phagocytosis in inflammation – Band 3 and CD47**

As in ageing, band 3 may be involved in mediating erythrocyte phagocytosis in inflammation. It is shown that the RBC band 3/α-spectrin ratio increased in septic mice compared to non-septic mice in a cecal ligation and puncture (CLP) model\textsuperscript{80}. In a different experiment, but using the same \textit{in vivo} model, the same group found an increase in band 3 phosphorylation in septic mice compared to non septic mice\textsuperscript{81}. However, no evidence exists linking these changes in band 3 directly to an increased RBC clearance in a sepsis-model. No evidence has been published concerning the CD47-thrombospondin-1 combination that is recognized as an “eat me” signal by SIRPα. However, since this is a recently elucidated mechanism, additional research is needed.

**Reduced deformability in inflammation**

\textit{In vitro}, LPS induced a change in deformability in RBCs after whole blood stimulation, but not in isolated RBCs\textsuperscript{68}. Also in patients with sepsis due to both Gram-negative and Gram-positive bacteria, a reduced red blood cell deformability has been shown when compared to healthy controls\textsuperscript{69-73}. This may implicate that other factors beside the erythrocyte itself may be needed to induce a reduced deformability. Several factors are involved in the decrease of RBC deformability during sepsis, which we will discuss here.

As in aging, Band 3 may be involved in reduced deformability in inflammation. It was found in an \textit{in vivo} CLP mouse model that a higher RBC Band 3/α-spectrin ratio was associated with a decreased RBC deformability\textsuperscript{80}. Also, \textit{in vitro}, RBC deformability was found to depend on Band 3 phosphorylation state\textsuperscript{82,83}.

Reactive oxygen species (ROS) influence RBC deformability in inflammation. ROS can lead to protein degradation in RBCs \textit{in vitro}\textsuperscript{84}, in particular membrane proteins such as Band 3 and spectrin\textsuperscript{85}. A clear link between ROS and loss of deformability was established in an \textit{in vitro} experiment showing that human RBCs undergo loss of deformability after exposure to H\textsubscript{2}O\textsubscript{2}\textsuperscript{86}. Also, in an \textit{in vivo} CLP rat model, decreased RBC deformability was found in septic rats when compared to rats that underwent sham-surgery. Decreased RBC deformability was prevented by pretreating the rats with ROS scavenger α-tocopherol\textsuperscript{73}.
Another mechanism that is implicated in the reduced deformability encountered in inflammation is nitric oxide (NO). NO is a mediator that is released by vascular endothelial cells and acts mainly as a vasodilator\textsuperscript{87-89}. Small amounts of NO are present in the blood under physiological conditions, but during inflammation and infection, its concentration may increase 10-fold\textsuperscript{90}. Several \textit{in vitro} experiments showed that NO causes a decrease in red cell deformability\textsuperscript{91,92} and this was also shown in an \textit{in vitro} sepsis model in which this loss of red cell deformability was attenuated by the NO-inhibitor N-monomethyl arginine\textsuperscript{93}. Furthermore, a selective inhibitor of NO synthase prevented overproduction of NOS, accumulation of NO within the RBC as well as a decrease in RBC deformability in murine sepsis\textsuperscript{94}.

Another factor of importance in clearance of RBCs during inflammation are sialic acid residues (SA), which are bound to glycophorin and account for the negative force of the RBC membrane\textsuperscript{95}. Due to this negative force, RBCs have repellent properties. When the SA content is cleaved from glycophorin after treatment with neuraminidase, RBCs have a reduced mean curvature\textsuperscript{96}. Also, a reduction in sialic acid content was found in RBCs from critically ill patients when compared to RBCs from healthy volunteers, associated with a decrease in RBC deformability\textsuperscript{97}.

\textbf{Increased adherence of red blood cells to endothelium in inflammation}

In sepsis, there are profound disturbances of the microcirculation, occurring already in the early phase\textsuperscript{98-100} and clearly contributing to adverse outcome\textsuperscript{99-101}. The resistance of the microcirculatory disorders to vasodilators\textsuperscript{102} and the apparent independence of the mean arterial blood pressure and cardiac output\textsuperscript{98,99,103} raises the hypothesis that these disorders may be caused by an obstruction due to adherence of cells to the endothelium and may not be solely due to a low flow state. Indeed, in diffuse intravascular coagulation, which occurs in 25\% of patients with sepsis\textsuperscript{104}, increased aggregation of cells with formation of micro thrombi are apparent. However, also RBC adherence to the endothelium may play a role. Although not extensively investigated, there is some evidence for this phenomenon. Incubation of both endothelial cells and red blood cells with endotoxin increased adherence of RBCs to endothelial monolayers\textsuperscript{105}. This effect was also observed after stimulation with TNF-\alpha\textsuperscript{106}.

The mechanisms mediating RBC adhesiveness to endothelium during inflammation are not well characterized. Most knowledge comes from specific diseases characterized by the presence of vascular pathology, including sickle cell anaemia, diabetes mellitus and malaria. Specific ligand-receptor interactions have been identified in enhanced RBC adherence to endothelium in sickle cell disease\textsuperscript{107}. In diabetes, advanced glycation endproduct (AGE) expressed on diabetic RBCs ligate with the receptor for AGE (RAGE) expressed on endothelial cells. Also, AGE-RAGE interactions have been found in other
inflammatory states, including trauma. Of interest, AGE-RAGE interactions may play a role in adverse effects of blood transfusion, as AGE formed in stored red blood cells was found to ligate to endothelial bound RAGE, resulting in endothelial damage.

Whether specific ligand-receptor interactions play a role in other inflammatory states is not known. However, there is evidence that non-receptor cytoadhesion is mediated by exposure of PS on the RBC membrane. RBCs expressing PS on their outer membrane are more prone to adhere to endothelial cells, irrespective of the cause of the Band 3/α-spectrin ratio PS-exposure. A definite role for PS in adherence of RBCs to endothelium has been shown by a reversal of adhesion following blocking of PS by PS liposomes. As discussed before, inflammatory conditions are able to induce PS exposure on RBCs. Besides specific RBC-endothelial interactions, low flow may contribute to increased RBC adhesiveness. Flow in the microcirculation in sepsis is diminished, with a decrease in the number of perfused capillaries, which display intermittent flow and differences in RBC velocities. This led to the hypothesis that flow may have an effect on adhesion of RBCs to vascular endothelium. Indeed, it was found that higher flow rates reduced and lower flow rates increased RBC adherence to endothelium.

Taken together, similar to the Virchow’s triad in thrombosis, both alterations in the microcirculatory flow as well as activation of endothelial adhesion markers may contribute to increased RBC adherence to the endothelium in sepsis.

**CONCLUSIONS**

Anaemia is a common feature in sepsis, due to several inflammatory pathways, collectively referred to as anaemia of inflammation. Red blood cell clearance is likely to contribute significantly to anaemia of inflammation. We postulate that besides mechanisms that also play a role in clearance of RBCs during senescence, RBC-endothelial interactions are important features underlying clearance of RBCs from the circulation during inflammation.
LITERATURE


Chapter 3

Accelerated clearance of human red blood cells in a rat transfusion model

Straat M, Klei T, de Korte D, van Bruggen R, Juffermans NP

Intensive care medicine experimental 2015, 3(1):27.
ABSTRACT

**Background** Animal models are valuable in transfusion research. Use of human red blood cells (RBCs) in animal models facilitates extrapolation of the impact of storage conditions to the human condition but may be hampered by the use of cross species.

**Methods** Investigation of clearance and post transfusion recovery in a rat model using fresh and stored human RBCs.

**Results** Directly following transfusion, human RBCs could be detected in the circulation of all recipients, with higher recovery rates for stored RBCs then for fresh RBCs. After 24 hours following transfusion, no donor RBCs could be detected in the circulation, but donor RBCs could be detected in all organs of all recipients.

**Conclusion** The use of human donor RBCs in a rat transfusion model resulted in clearance from cells from the circulation. Donor cells were found in different organs of the recipients. Rat transfusion models are thus not appropriate to study efficacy of human RBC transfusion.
BACKGROUND

The growing awareness of transfusion-associated morbidity and mortality has initiated a surge of investigations into the underlying mechanisms, which may be related to storage. Storage lesion of red blood cells (RBCs) includes alteration of the plasma composition, together with changes in morphology of RBCs resulting in reduced deformability, which together may reduce tissue oxygen availability and have pro-inflammatory and immunomodulatory effects \(^1\). These changes may all reduce post-transfusion viability of red blood cells. For decades, one of the standards of blood conservation as set by the Food and Drug Administration (FDA) is more than 75% recovery measured at 24 hours post transfusion \(^2\).

Optimizing storage conditions may negate some of the changes occurring during storage of RBCs. To test hypotheses of storage lesion as well as interventions aimed at improving storage conditions, an appropriate animal model of RBC transfusion would be of great value. However, the biology of stored murine RBCs may not accurately reflect that of human RBCs. Indeed, RBC storage lesion of rodents appears to occur much faster than of human RBCs. Rodent RBCs stored for 15 days in a commonly used storage solution were reported to rapidly deteriorate, with only a 26% recovery at 24 hours following transfusion \(^3\). However, not all reports using rodent RBCs are consistent, with also reports of normal 24-hour posttransfusion survival of stored rodent RBCs \(^4\). A model in which functional effects of storage can be studied using human RBCs would overcome these problems with translation to the human condition.

To that end, cross species models with transfusion of human RBCs into rodents have been used to study the role of RBC storage in transfusion-related acute lung injury, in recruitment of the microcirculation, in mechanisms of storage lesion and in rejuvenation of storage solutions \(^5-7\).

However, given the differences in size of the RBCs as well as the capillaries, it is conceivable that the use of cross species may lead to enhanced clearance of human donor RBCs from the rodent circulation. In this study, we investigated the role of storage on routing of human RBCs following transfusion into rodents.

METHODS

The Institutional Animal Care and Use Committee of the Academic Medical Center and the Medical Ethical Committee of Sanquin Blood Bank Foundation approved all experiments. All animals were handled in accordance with the guidelines prescribed by the Dutch legislation and the International Guidelines on protection, care, and handling of laboratory animals.
Preparation of human RBC products

Blood of healthy volunteers was collected using a needle connected to a syringe containing 1.25 ml citrate-phosphate-dextrose. Blood was handled and stored according to national standards for human blood (Sanquin Blood Supply Foundation), by overnight storage at room temperature, followed by centrifugation for 10 min at 1,892 g and 20 °C. Plasma was removed and the buffy coat was separated from the packed red blood cells. Saline-adenine-glucose-mannitol was added to the red blood cells up to a hematocrit of 55-60%. The final products were at 4 °C. Samples of the RBC products were analyzed for pH, potassium, sodium, glucose and lactate with a Rapidlab 865 blood gas analyzer (Siemens Medical Solutions Diagnostics; Breda, The Netherlands). ATP, 2,3 diphosphoglycerate (2,3 DPG) and hemolysis were analyzed as previously described.

RBC transfusion model

Male Sprague-Dawley rats (275 g) raised on a regular diet were weighed and anaesthetized with isoflurane 2%. Animals were randomized to receive fresh (1 day storage) or stored RBCs (35 days storage, n=6 per group). The tail vein was cannulated with a 24 gauge venflon (Vasofix Certo; B.Braun, Meisungen, Germany) and blood was aspirated to verify intravascular placement. A 10% circulating volume transfusion was administered. One hour after transfusion, a blood sample was taken. Rats were placed back in their cages to recover. 24 hours after transfusion, rats were sedated with ketamin and medetomidin as described and bled via the inferior caval vein in citrated (0.109 M) vacutainer tubes for analysis and blood culture.

Assays

Blood taken 1 and 24 hour after transfusion were used for cell counts on the Advia 2120 hematology system. Analysis of the human RBC from the circulation was done by spinning down 1x10^8 RBCs for 15 minutes at 1500 RPM and subsequently removing the top layer containing the white blood cells. 5x10^5 RBCs were washed twice in HEPES and stained with anti-human CD235a-PE (Sanquin Pelicluster Ref M1732) or control antibody (control IgG1PE Ref M1628) and analyzed by flow cytometry (Becton Dickinson LSRII). Data analysis was performed with FACSDiva software (Becton Dickinson).

We were not able to collect organs and analyze them from all animals, because time on flow cytometry and personnel were limited. We measured organs from 3 animals per group. After transfusion, kidney, lung, liver and spleen were collected. The organs were cut into 1-5 mm³ cubes, resuspended in HEPES buffer (132mM NaCl, 20mM HEPES, 6mM KCl, 1mM MgSO4, 1.2mM K2HPO4) supplemented with 2mM CaCl2 and 10mM glucose. and homogenized using GentleMACS C-Tubes (Miltenyi Biotec) and the GentleMACS Dissociator (Miltenyi Biotec). Samples were filtered through a 40 um nylon mesh (Becton...
Dickinson Cell strainer). Afterwards, $5 \times 10^5$ cells were stained with anti-CD235a or control antibody as above.

**Statistical analysis**

Results are expressed as mean ± SD. Results of donor RBCs are expressed as percentage human RBCs of the total amount of stained RBCs. Comparison between groups at the same time period or in the same organ was done using Student’s t-test. A P-value of $< 0.05$ was considered statistically significant.

**RESULTS**

Parameters of storage lesion of the human RBC products are shown in the Table. Directly following transfusion, human RBCs could be detected in the circulation of all recipients (figure). Hemoglobin levels were similar to baseline. Of note, the percentage of human RBCs per circulating rat RBCs was higher for stored RBCs then for fresh RBCs.

In contrast, 24 hours following transfusion, no donor RBCs could be detected in the circulation, regardless of storage time. Donor RBCs were detected in different organs of all recipients (figure). The presence of fresh donor RBCs in the liver appeared to be higher compared to stored donor RBCs. Following transfusion, hemoglobin levels were similar to baseline.

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<tr>
<td>Potassium (mmol/L)</td>
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<tr>
<td>Glucose (mmol/L)</td>
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</tr>
<tr>
<td>ATP (µmol/g Hb)</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>2,3DPG (µmol/g Hb)</td>
<td>14.9</td>
<td>0</td>
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<tr>
<td>Hemolysis (%)</td>
<td>0.1</td>
<td>0.124</td>
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**DISCUSSION**

The use of human donor RBCs in a rat transfusion model resulted in clearance from cells from the circulation, associated with the presence of donor cells in all organs. A possible explanation may be the size of human RBCs, which are somewhat larger than rat RBCs (7 - 8 versus 6 µm), and may get stuck in the capillaries of organs.
Of note, 1 hour following transfusion we found that the percentage of fresh donor RBCs in the circulation was lower compared to the percentage of stored RBCs, suggesting that fresh RBCs are cleared more rapid than stored human RBCs. In line with this, the percentage of fresh RBCs stuck in the liver following transfusion was higher compared to stored RBCs. However, this did not hold true for all organs, also not for the lungs, in which the capillaries have the smallest diameter. Besides size, there may be a immunologic mechanism of clearance of human RBCs from the rat circulation. We were unable to define a precise mechanism. Whether fresh RBCs may elicit a stronger immune response than stored RBCs is not known. Alternatively, hemolysis may have played a role. Although we did not measure markers of hemolysis, visual inspection of the samples did not suggest hemolysis.

This study is limited by small sample size, in particular of the organ measurements, because we were not able to analyze organs from all recipients due to logistic reasons. Although the mechanism is undefined, this study clearly shows that human RBCs get stuck in organs in a rat transfusion model. Given that the standard of appropriate
transfusion requires more than 75% post-transfusion survival, the use of human RBCs in rodent recipients are not appropriate to study efficacy of RBC transfusion.

**CONCLUSION**

The use of human donor RBCs in a rat transfusion model is not appropriate to study efficacy of RBC transfusion. Transfusion models should preferably be syngeneic. Thereby, translation of animal storage lesion to the human condition remains a challenge.
LITERATURE

Endotoxemia results in trapping of transfused red blood cells in organs: an explanatory mechanism of transfusion-related organ failure


Submitted
ABSTRACT

**Background** Red blood cell (RBC) transfusion is associated with organ failure. We hypothesized that endotoxemia contributes to increased trapping of RBCs in organs. Furthermore, we hypothesized that this effect is more pronounced following transfusion of stored RBCs compared to fresh RBCs.

**Materials and Methods** Rats were pretreated with lipopolysaccharide from E. coli (7.5 mg/kg), controls received vehicle. Subsequently, animals were transfused with fresh or stored biotinylated RBCs. After 24 hours, animals were sacrificed. The amount of biotinylated RBCs in organs was measured by flow cytometry, as well as 24-hour posttransfusion recovery. Markers of organ injury and histopathology of organs were assessed.

**Results** Endotoxemia resulted in systemic inflammation and organ injury. RBC transfusion resulted in an increased presence of donor RBCs in the lung and in the kidney in endotoxemic recipients (1.2 [0.8-1.6] % and 2.2 [0.4-4.4] % of donor RBCs respectively) compared to healthy recipients (0.0 [0.0-0.0] % and 0.3 [0.0-1.0] % of donor RBCs respectively). This was associated with increased lung injury and with increased creatinine levels when compared to healthy recipients, but not with kidney injury. Stored RBCs induced organ injury in the spleen and yielded a lower 24-hour posttransfusion recovery, but other effects of storage time were limited.

**Conclusion** Endotoxemia results in an increased percentage of donor RBCs trapped in the lung and kidney, associated with lung injury following transfusion. Trapping of donor RBCs in the microvasculature of the lungs during an inflammatory state may explain the association between lung injury and RBC transfusion in the critically ill.
INTRODUCTION

A red blood cell (RBC) transfusion is frequently administered in the Intensive Care Unit (ICU), with up to 44% of patients receiving a blood transfusion\(^1\). In sepsis, transfusion rates can even reach 73%\(^1\). Observational studies suggest that RBC transfusion is associated with organ failure, of which acute lung injury is the most striking\(^2,9\), but also acute kidney injury is often described\(^5,10\). These associations are most prominent in the critically ill and injured patient populations\(^3,4,8,9\).

There is a paucity in the knowledge of the mechanisms underlying the association between RBC transfusion and organ failure during critical illness, but activation of endothelium has been implicated\(^11,12\). Inflammation can induce upregulation of endothelial adhesion markers, resulting in ‘sticky’ endothelium\(^12,15\). Under these conditions, transfused RBCs may adhere to the vasculature. In line with this, RBCs were found to adhere to the endothelium in the presence of inflammation in vitro, induced by both endotoxin and TNF\(^16,17\).

A much debated determinant of the effects of RBC transfusion is storage time. Although recent trials in critically ill patient populations suggest that transfusion of stored RBCs is not associated with adverse outcome\(^18,19\), not all trials have been consistent\(^20-22\). In addition, a large number of experimental animal studies suggest that stored RBCs can induce organ injury\(^23-27\), possibly via an effect on the vasculature, including diminished vasoreactivity and increased adherence to the vasculature\(^28\).

We hypothesized that endotoxemia contributes to increased clearance of donor RBCs from the circulation, with concomitant adherence of RBCs to the endothelium in organs, thereby accounting for transfusion-related organ failure. Furthermore, we hypothesized that stored RBCs would further enhance clearance of donor RBCs compared to fresh RBCs. These hypotheses were investigated in a rat model of endotoxemia combined with transfusion of biotinylated red blood cells.

MATERIALS AND METHODS

Animal study design

The animal care and use committee of the Academic Medical Center, University of Amsterdam, Netherlands approved this study. Animal procedures were carried out in compliance with Institutional Standards for Use of Animal Laboratory Animals.

Preparation of biotinylated rat RBC products

Male Sprague–Dawley rats (350 g; Charles River, Leiden, The Netherlands were anesthetized with 4% isoflurane (Abbott Lab Ltd, Queensborough, Kent, UK) in a 0.4-L/ min
flow of 1:1 \(O_2\):medical air. Blood was collected from the inferior vena cava in a syringe containing 1.25 ml citrate–phosphate–dextrose (Fresenius HemoCare GmbH, Bad Homburg, Germany). Approximately 8–10 ml blood could be obtained from a single rat. Blood of five rats was pooled for component preparation. Blood was handled and stored according to national standards for human blood (Sanquin Blood Supply Foundation, Amsterdam, The Netherlands), with minor changes to adapt for the smaller volumes. After overnight storage at room temperature, blood was centrifuged for 10 min at 1,892g and 20°C. Plasma was removed and the buffy coat was separated from the packed erythrocytes. Saline–adenine–glucose–mannitol (Fresenius HemoCare GmbH, Bad Homburg, Germany) was added to the erythrocytes up to a hematocrit of 55–60%. The final products were stored in 50 ml Falcon's at 4°C for either 2 (fresh RBCs) or 14 days (stored RBCs). On the day of the experiment, the RBCs were labeled with N-hydroxysulfosuccinimide biotin (EZ-Link Sulfo-NHS-Biotin, Thermo Fisher Scientific, Rockford, IL).

**Experimental protocol**

Male Sprague–Dawley rats (350 g) were anesthetized with 4% isoflurane in a 0.4-L/min flow of 1:1 \(O_2\):medical air. The tail vein was cannulated with a 24-gauge venflon and blood was aspirated to verify intravascular placement and to remove 0.5 ml blood for baseline measurements. A 10% circulating volume transfusion was administered in 5 minutes using a syringe. Two hours prior to transfusion, animals received 7.5 mg/kg lipopolysaccharide (LPS from E. coli 0111:B4; Sigma; St. Louis, MO) intravenously, whereas controls received NaCl 0.9% in an equal volume. Animals received RBCs stored for 14 days or RBCs stored for 2 days. A storage time of 14 days was chosen because previous experiments showed that rat RBCs stored for 14 days showed storage-related changes that are comparable to human RBCs stored for 28–35 days.

Animals were randomized into 4 groups (n=6 per group): 1. LPS with fresh RBCs, 2. LPS with stored RBCs, 3. saline with fresh RBCs and 4. saline with stored RBCs.

**Blood and tissue sampling**

24 hours after transfusion, rats were anesthetized by administering 90 mg/kg ketamine (Pfizer, New York, NY, USA), 0.25 mg/kg dexmedetomidine (Orion Pharma, Espoo, Finland) and 0.5 mg/kg atropine (Centrafarm, Etten-Leur, The Netherlands) intraperitoneally and bled from the inferior caval vein in 10 ml syringes coated with heparin (LEO Pharma B.V., Amsterdam, The Netherlands). The lungs were removed en bloc and the right upper lobe was fixed in 1% buffered formaldehyde and embedded in paraffin for histopathology examination. The right middle lobes were collected in PBS for FACS-analysis. The right kidneys, upper halves of the spleen, right lobes of the liver and right upper lobes of the lung were collected in PBS for FACS-analysis, whereas the left kidney, lower halves of
Endotoxemia results in trapping of transfused RBCs in organs

the spleen, middle lobes of the liver and the right middle lobes of the lung were fixed in 1% buffered formaldehyde and embedded in paraffin for histopathology examination.

Assays

Levels of tumor necrosis factor (TNF)-α, interleukin-1b (IL-1b), interleukin-6 (IL-6) and cytokine-induced neutrophil chemoattractant (CINC)-3 in plasma were measured using ratspecific ELISAs according to the manufacturer’s instructions (all: R&D Systems, Abingdon, UK). ASAT was measured in plasma by spectrophotometry with α-ketoglutaric acid as substrate and pyridoxal phosphate as activator and creatinin was measured in plasma using a colorimetric test with the use of creatininase, both on the cobas c702 (Roche, Basel, Switzerland) according to the manufacturer’s instructions and the recommendations of the IFCC (International Federation of Clinical Chemistry and Laboratory Medicine). Free hemoglobin was measured on the UV-2401 PC (Shimadzu, Kyoto, Japan) as described30.

Histological assessment of organ injury

4-µm-thick paraffin sections of lung, kidney, spleen and liver were fixed on glass and stained with hematoxylin and eosin. A pathologist who was blinded to group randomization analyzed the organs with the use of a total histology score. Lung injury was scored on the presence of interstitial inflammation, endothelialitis and edema. Spleen injury was scored on inflammation, necrosis and reactivity of the white pulp. Kidney injury was assessed by scoring necrosis and apoptosis, neutrophil extravasation, proximal tubuli injury and interstitial changes. Liver injury was assessed by scoring necrosis and portal inflammation. Each variable was graded on a scale of 0–3 (0, absent; 1, mild; 2, moderate; 3, severe). The histopathology score was expressed as the sum of the scores for all variables per organ.

FACS-analysis to assess presence of biotinylated donor-RBCs

After transfusion, blood, kidney, lung, liver and spleen were collected. Analysis of the recovery of transfused RBC in the circulation was done by spinning down 1 × 10⁸ RBCs for 15 min at 1500 RPM and subsequently removing the top layer containing the white blood cells. RBCs (5 × 10⁵) were washed twice in HEPES buffer (132 mM NaCl, 20 mM HEPES, 6 mM KCl, 1 mM MgSO₄, 1.2 mM K₂HPO₄) supplemented with 2 mM CaCl₂ and 10 mM glucose. Subsequently, 5 × 10⁵ cells were stained with Streptavidin Alexa 488 (ThermoFisher Scientific), washed three times with HEPES buffer and analysed by flow cytometer (LSRII, Becton Dickinson). We measured organs from 3 animals per group in duplo. The organs were cut into 1–5 mm³ cubes, resuspended in HEPES buffer and homogenized using GentleMACS C-Tubes (Miltenyi Biotec) and the GentleMACS Dissociator (Miltenyi Biotec). Samples were filtered through a 40-µm nylon mesh (Becton Dickinson Cell strainer). Again, 5 × 10⁵ cells were stained with Streptavidin Alexa 488, washed three times with HEPES buffer and analysed by flow cytometry.
Statistical analysis
Data are expressed as median with interquartile range (IQR). Comparisons between the groups were performed using a Mann-Whitney U test. Correlation was assessed using Pearson’s R. A P-value less than 0.05 was considered statistically significant. Statistical analyses were performed with SPSS 22.0 (SPSS, Chicago, IL) and GraphPad Prism 5.0 (Graph-Pad Software, San Diego, CA).

RESULTS
All animals survived the experiment. In the 2 groups who did not receive LPS, 1 animal in each group was excluded due to insufficient amount of transfusion product. During storage, hematocrit increased (Table 1). There was no effect of biotinylation on hematology markers.

Table 1. Storage-related biochemical changes in the various stages of the preparation of the rat transfusion product

<table>
<thead>
<tr>
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<th>Blood after withdrawal</th>
<th>Blood day 2</th>
<th>Transfusion product before labelling</th>
<th>Transfusion product after labelling</th>
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<td>Fresh</td>
<td>Stored</td>
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<td>Stored</td>
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<tr>
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</table>

Endotoxemia resulted in systemic inflammation and organ injury
Animals treated with LPS lost significantly more body weight compared to baseline whereas weight in healthy controls was stable (-38 [-27--43] mg vs 6 [2-10] mg, p<0.0001). LPS induced organ injury, exemplified by increased histology score of the lung, spleen and liver when compared to controls receiving vehicle (figure 1a and b and figure 2). LPS did not induce histologically visible kidney injury. However, creatinin values were significantly higher in animals treated with LPS (figure 3). The histological liver damage in the LPS-treated animals was paralleled with a significant increase in ASAT (figure 3).

Endotoxemia results in trapping of donor RBCs in organs
The post transfusion recovery (PTR) 24 hours after transfusion showed that donor RBCs were in the circulation. There were no statistical differences in PTR between endotoxemic and healthy rats (figure 4). In healthy recipients, no donor RBCs were found in the lungs, whereas in kidney, liver and spleen the percentage of donor RBCs was around 1%. In contrast, in endotoxemic animals, transfusion resulted in trapping of donor RBCs in the
Endotoxemia results in trapping of transfused RBCs in organs

Figure 1. Histology-scores of lung (A), spleen (B), liver (C) and kidney (D) of endotoxemic or healthy animals transfused with fresh or stored RBCs.

Figure 2. Histopathologic changes in endotoxemia and transfusion in the lung (A-D) and spleen (E-G). Shown are representative hematoxylin and eosin–stained photomicrographs (magnification, 10x) of lung tissue from rats receiving saline and fresh RBCs (A), saline and stored RBCs (B), LPS and fresh RBCs (C), LPS and stored RBCs (D) and of spleen tissue from rats receiving saline and fresh RBCs (E), saline and stored RBCs (F), LPS and fresh RBCs (G) and LPS and stored RBCs (H).
Chapter 4

lung and in the kidney (figure 5). The percentage of donor RBCs recovered in the spleen and liver during endotoxemia did not differ from healthy controls (figure 5). The level of free hemoglobin was significantly higher in endotoxemic than in healthy rats (figure 6).

Prolonged storage time decreases post transfusion recovery but does not significantly influence trapping of donor RBCs in organs

Rats transfused with stored RBCs had a significantly lower 24-hour PTR than rats transfused with fresh RBCs (figure 4). This lower PTR was not due to trapping of donor RBCs in organs, as the percentage of donor RBCs recovered in organs did not differ significantly between rats transfused with fresh or stored RBCs (figure 5). Also, there was no correlation between age of blood and trapping in organs. However, there was a significant increase in free hemoglobin in endotoxemic rats transfused with stored blood compared to fresh blood (figure 6).
Endotoxemia results in trapping of transfused RBCs in organs

Prolonged storage time induces spleen injury in healthy recipients but does not contribute to organ injury in endotoxemia

Stored RBCs induced organ injury in the spleen even in the absence of endotoxemia when compared to transfusion with fresh RBCs, as assessed by histology (figure 1). Stored RBCs did not result in other organ injury in healthy recipients (figure 1 and 3). In endotoxemic animals, stored blood did not augment organ injury as assessed by histology (figure 1). However, in endotoxemia, serum creatinine was further increased in animals receiving stored RBCs compared to those receiving fresh RBCs (figure 3).

DISCUSSION

Using a biotinylated RBC transfusion model, we found that endotoxemia induces trapping of transfused RBCs in different organs. More specifically, transfused RBCs were
predominantly trapped in the lung and kidney, which are the two sites most often associated with transfusion-related organ failure\textsuperscript{2-10}. Trapping of donor RBCs was associated with lung injury in this endotoxemia model, but not with kidney injury. Thereby, the lung seems the most vulnerable organ for adverse effects of transfusion during an inflammatory condition. This observation may explain why transfusion-related acute lung injury is most prominent in the critically ill when compared to the general patient population\textsuperscript{9,31,32}. Of note, endotoxemia did not induce trapping of donor RBCs in the spleen and liver. In accordance, there is no association between transfusion and the occurrence of liver injury in clinical studies. An explanation for differential effects on organs remains to be determined. It is tempting to speculate that the diameter of the microcirculation is a determinant of trapping of donor RBCs in organs. Alternatively, since the lungs are the first line of defense against micro-organisms, the pulmonary endothelium may be more susceptible to activation and subsequent upregulation of adherence molecules than endothelia of other organs, which may result in enhanced trapping of donor RBCs\textsuperscript{12}. Unfortunately, we did not measure adhesion markers on the endothelium in organs in this study. Another explanation for the absence of kidney injury on histology despite trapping of donor RBCs may be that kidney injury was not very outspoken in this endotoxemia model. However, creatinine levels were further increased following transfusion. It can be argued that the observed lung injury in this study was due to LPS and not to the subsequent effects of transfusion, as no control group receiving only LPS was investigated. This control group was not included because we have shown previously that during endotoxemia, stored RBCs augment lung injury compared to LPS alone\textsuperscript{26} and we did not intend to repeat those experiments. Instead, the focus in this study was on the impact of endotoxemia on the routing of donor RBCs in a recipient following transfusion.

The increased trapping of donor RBCs in the lungs and kidney of endotoxemic rats was not accompanied by a decreased 24-hour PTR compared to healthy recipients. Indeed,
the amount of transfused RBCs trapped in the organs following LPS was small, not influencing PTR. However, PTR was influenced by storage time, as stored RBCs yielded a significantly lower PTR compared to fresh RBCs, both in healthy and endotoxemic rats. This is in line with previous studies\textsuperscript{33,34}. As an increased storage time did not result in enhanced trapping of donor RBCs in the organs, the question remains what the routing is of these stored donor RBCs. Possibly, stored donor RBCs may be removed from the circulation by the spleen, similar to senescent RBCs\textsuperscript{35-38}. In line with this, stored RBCs induced spleen injury in this study, which may be a result of increased removal of stored donor RBCs. However, of interest, we did not find an increase in trapped the amount of donor RBCs in the spleen. This finding may challenge the currently held view of the spleen being the most important organ in clearance of RBCs. An alternative explanation of lower PTR following transfusion of stored RBCs may be an increased hemolysis, as the hemolysis index was significantly higher in endotoxemic rats transfused with stored RBCs. However, this mechanism may hold during endotoxemia but does not account for low PTR in healthy recipients, as hemolysis was not affected. Further research to the underlying mechanism of a decreased 24-hour PTR of transfused stored RBCs is warranted.

This study has several limitations, the most obvious being the use of rats to assess a mechanism in humans. However, using a syngeneic model, it can be ruled out that donor RBCs are trapped in organs due to cross species effect, as we have found before\textsuperscript{39}. Also, the group sizes were limited. Some of the trends we observed may not have been significant due to underpowering.

**CONCLUSIONS**

Taken together, we have shown that endotoxemia results in an increased percentage of donor RBCs trapped in the lung and kidney, associated with lung injury following transfusion. Trapping of donor RBCs in the microvasculature of the lungs during an inflammatory state may explain the association between lung injury and RBC transfusion in the critically ill.


Endotoxemia results in trapping of transfused RBCs in organs


Chapter 5

Extracellular vesicles from red blood cell products induce a strong pro-inflammatory host response, dependent on both numbers and storage duration

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Transfusion medicine and hemotherapy: offizielles Organ der Deutschen Gesellschaft für Transfusionsmedizin und Immunhamatologie 2015, Epub.
**ABSTRACT**

**Background** Transfusion of red blood cells (RBCs) is associated with adverse outcome, but the causative factor is unknown. Extracellular vesicles (EVs) have pro-inflammatory properties. We hypothesized that EVs released from both fresh and stored RBC products can induce a host inflammatory response in a dose-dependent manner.

**Materials and Methods** Whole blood was incubated with supernatant from RBC units stored for a different time periods, either containing (different numbers of) EVs or depleted from EVs.

**Results** Incubation with both fresh and stored supernatant containing EVs induced a strong host response with production of TNF, Interleukin-6 and Interleukin-8. In supernatant depleted from EVs, this host response was completely abrogated. Interleukin-10 levels were not affected. EV-induced host response was both dependent on the number of EVs as well as on storage time.

**Conclusions** EVs from both fresh and stored RBC units illicit a strong inflammatory host response in recipients and may therefore contribute to adverse outcome of RBC transfusion.
INTRODUCTION

Despite having obvious benefits, red blood cell (RBC) transfusion also carries risks. In several patient populations, RBC transfusions have been associated with inflammatory conditions such as acute lung injury, nosocomial infections and systemic inflammatory response syndrome \(^1\text{-}^3\). The mechanism of transfusion-related adverse effects is unknown. Experimental studies suggest that RBC storage duration is a risk factor for the occurrence of transfusion-related adverse events \(^4\text{-}^5\), although results of clinical studies on the influence of age of RBC on outcome are conflicting \(^5\text{-}^8\). Previously, we found that the supernatant of a RBC product, but not the RBCs themselves, induce pulmonary inflammation in a rodent model, but the causative factor was not identified \(^5\).

Extracellular vesicles (EVs) are small phospholipid vesicles released from a wide variety of cells, including leucocytes, platelets, red blood cells and endothelial cells. EVs have also been referred to as microparticles. EVs facilitate intercellular exchange of receptors, ligands and signaling molecules, without direct cell-to-cell contact \(^9\). High concentrations of RBC-derived EVs are present in the supernatant of RBC transfusion bags \(^10\). These EVs have pro-coagulant properties, including thrombin generation and activation of platelets. During storage, both the concentration of EVs as well as their pro-coagulant properties increases \(^11\text{-}^12\).

We hypothesize that EVs accumulating in RBC products during storage contribute to a pro-inflammatory host response in recipients, which is related both to their amount as well as to their storage duration.

METHODS

Preparation of samples

Samples were collected from RBC units that were transfused to patients. These RBC units were prepared and stored according to Sanquin blood bank standards, including leucoreduction. Directly after transfusion, 3 milliliters of blood product that were left behind in the bag were 1:1 diluted with filtered PBS. The diluted RBC sample was centrifuged for 20 minutes at 1.550 g, 20°C to remove red blood cells. Subsequently, the EV-containing supernatant was isolated and centrifuged again. The EV-containing supernatant was snap frozen and stored at -80°C until further use. Just prior to whole blood stimulation, samples from both fresh (≤ 12 days old) and stored (≥ 35 days old) RBC bags were thawed and ultracentrifuged for 60 minutes at 50.000g, 4°C. Hereafter, the EV-free supernatant was isolated by pipetting of the upper supernatant fraction. The EV-pellet was resuspended in supernatant. EVs were quantified using tunable resistive
pulse sensing (TRPS; qNano; Izon Science Ltd, Christchurch, New Zealand), as described by van der Pol et al. In short, the concentration and size distribution of particles was measured using a NP200A nanopore. This nanopore was suitable for the detection of 100 - 400 nm particles. Samples were measured with 7 mbar pressure, 45 mm stretch, and 0.34 V. Samples were analyzed until 1,000 vesicles were counted. To calibrate size and concentration, carboxylated polystyrene beads (Izon Science Ltd) were sonicated for 10 seconds, diluted in PBS with 0.3 mM sodium dodecyl sulfate, and analyzed immediately after dilution.

**Whole blood stimulation**

The response of blood leucocytes to RBC-derived EVs was determined in a whole blood stimulation system. After written informed consent was obtained, blood was drawn from healthy volunteers in 10 ml Sodium Heparin tubes using the BD Vacutainer system (New Jersey, USA). Immediately after drawing, 250 µl of whole blood was 1:1 diluted with RPMI, to which a fixed amount of 50 ul of supernatant from a RBC product was added. Blood was incubated with supernatant from fresh or stored RBC product, either containing EVs or depleted from EVs. A positive control group was added by stimulation with LPS (from E.coli, Sigma, Missouri, 10 ng/ml), whereas stimulation with PBS served as a negative control. After 6 hours of incubation at 37°C with 5% CO₂, samples were centrifuged at 600 g for 10 minutes at 4°C. Supernatant was stored at −80°C. Levels of TNF, Interleukin-6 (IL-6), Interleukin-8 (IL8) and Interleukin-10 (IL-10) were determined by ELISA according to the manufacturer’s instructions (R&D Systems Minneapolis, MN, USA).

In separate stimulation experiments, the dose response to EVs was investigated. Whole blood from healthy donors (250 µl) was stimulated with 250 µl supernatant samples from fresh and stored RBC bags, to which fixed consecutive concentrations of EVs were added. EVs were isolated as described above and resuspended in supernatant and diluted with supernatant to a fixed volume containing increasing numbers of EVs.

**Statistical analysis**

Variables are expressed as medians and interquartile ranges. For comparisons, Kruskal Wallis was done followed by Dunn’s post test. Statistical significance level of 0.05 was taken. For the analyses, Graphpad Prism 5 (Graphpad software, San Diego, CA, USA) was used.
RESULTS

Whole blood samples
We obtained blood from 12 healthy volunteers, aged 24-45 years. Blood groups were mixed (4 times type A, 6 times type B, 2 times type O). One volunteer was excluded from analysis because of strongly elevated levels of cytokines in the plasma.

Fresh versus stored extracellular vesicles
Supernatant from fresh RBC units (N=12) contained $5.4 \times 10^6 \pm 1.6 \times 10^5/\mu l$ of EVs, which were around $255.4 \ (\pm 45) \ nm$ in size. The amount of EVs in supernatant from stored RBC units (N=12) was higher, namely $7.27 \times 10^7 \pm 1.16 \times 10^7/\mu l$, which were $232.7 (\pm 32) \ nm$ in size. The level of IL-6 in the supernatant did not differ between fresh (4.2±1.4 pg/ml) and stored EV samples (3.5±1.1, NS), nor the level of TNF (14.2±7.2 vs 8.7±1.9 pg/ml, NS) and the level of IL-8 (3.2±0.2 vs 3.0±0.1 pg/ml, NS).

Incubation of whole blood with LPS resulted in increased production of TNF (p<0.001), IL-6 (p<0.001), IL-10 (p=0.002) and IL-8 (p<0.001) compared to the negative control (figure 1). The addition of EV-free supernatant from RBC units did not induce a cytokine response compared to the negative control, regardless of storage time. In contrast, the addition of supernatant containing EVs significantly increased levels of all cytokines compared to incubation with supernatant alone. Of note, incubation with stored EVs induced a significantly higher production of TNF (p<0.05) and IL-6 (p<0.05) compared to incubation with fresh EVs, whereas levels of IL-10 and IL-8 did not differ between fresh and stored EVs (figure 1).

Concentration sequence
After having observed that supernatant with EVs from stored RBC units induced a stronger host response compared to an equal volume of supernatant with EVs from fresh RBC units, we determined whether the increased response was depended on the amount of EVs or on alterations in RBC-derived MPs as a result of storage time. A concentration sequence was performed with supernatant from fresh and stored RBC units with increasing number of EVs. When blood was stimulated with higher numbers of EVs, the production of TNF, IL-6 and IL-8 was more pronounced (P<0.05) (figure 2), indicating a dose-response relationship. In contrast, IL-10 production remained low at all numbers of EV. Furthermore, stored EVs induced a higher production of IL-6 and IL-8, but not of TNF, compared to equal amounts of fresh EVs (figure 2).
**DISCUSSION**

Our study shows that RBC-derived EVs induce a strong pro-inflammatory host response, which in strength equals that of LPS. In vitro, it was found before that RBC-derived EVs have chemokine binding capacity. Here, we extend these findings with functional studies, showing that RBC-derived EVs can induce inflammation. Of note, a recent study found that both supernatant containing RBC-derived EVs as well as supernatant depleted from EVs have neutrophil priming capacity. The explanation for these different results may be related to differences in processing and storage conditions of RBC units. Indeed, the amount of EVs from leucoreduced RBC units decreased during storage in that study, whereas the amount of EVs from leucoreduced RBC units increased during storage in this study. Whether EVs are causal of the adverse effects associated with RBC transfusion in patients is not known, but evidence is emerging that EVs may have an ef-

**Figure 1.** Whole blood stimulated with a fixed volume of supernatant (sup) derived from fresh and stored red blood cell bags either containing extracellular vesicles (EVs) or depleted from EVs (n=11 per group). *=p<0.05, ***=p<0.0001

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**Chapter 5**
EVs from RBCs induce a pro-inflammatory host response

Effect on key players during a transfusion-related acute lung injury, including white blood cells and the endothelium. It was recently found that neutrophil-induced activation of pulmonary endothelial cells depended on the presence of platelet-derived EVs. In experimental murine models, injection of RBC-derived EVs from stored RBC units was shown to exacerbate lung injury. In this study, it is not clear whether EVs have a direct effect on host response or if in addition to EVs derived from blood bags, endogenous circulating EVs also appear to have pro-inflammatory properties. In an animal model of ischemia reperfusion injury, EVs from endothelium and inflammatory cells are shed into the circulation, which can subsequently promote neutrophil migration. Also, in trauma patients, we showed before that endogenously circulating EVs can induce an inflammatory host response. As the EVs in this study are derived from allogenic blood, inflammation may have been induced allogenously, but the EVs may also have caused an endogenous host response. The exact mechanism remains to be determined.

In this study, the pro-inflammatory capacity of RBC-derived EVs is dose-dependent. As numbers of EVs increased during storage, EVs may be considered as a part of the RBC storage lesion. Given that incubation of EVs, but not the stored supernatant, induced inflammation, we think that EVs and not another soluble factor in the supernatant are causative in mediating host response.

Dose-dependent activation of the host response was also present with EVs derived from fresh RBC units, suggesting that EVs are pro-inflammatory regardless of storage time.

Figure 2. Whole blood stimulated with supernatant (sup) derived from fresh and stored red blood cell bags containing a fixed concentration of extracellular vesicles (EVs).
This finding may have clinical relevance. If EVs from RBCs are related to adverse effects of transfusion, interventions aimed at decreasing EVs prior to transfusion, such as use of a cell saver, may improve outcome of RBC transfusion.

In conclusion, this study shows that EVs from both fresh and stored RBC bags illicit a strong inflammatory host response in recipients. This response depends both on the number of EVs as well as on changes of the EVs related to storage.
EVs from RBCs induce a pro-inflammatory host response

LITERATURE


Chapter 6

Monocyte mediated activation of endothelial cells occurs only after binding to extracellular vesicles from red blood cell products, a process mediated by beta integrin


Submitted
ABSTRACT

Background Red blood cell (RBC) transfusion is associated with organ failure. The mechanism remains unknown, but may include adherence of blood cells to the microvasculature. We hypothesized that RBC-derived extracellular vesicles (EVs) interact with monocytes to activate endothelial cells.

Study design and Methods Human umbilical vein endothelial cells (HUVECs) were incubated with supernatant from fresh and stored RBC units either containing EVs or depleted from EVs, with or without the addition of immune cells. We measured expression of adhesion markers by flow cytometry and markers of coagulation and inflammation in the culture medium. We studied phagocytosis of EVs by monocytes by using confocal microscopy and flow cytometry.

Results Incubation of endothelial cells with monocytes alone did not induce upregulation of adhesion markers. The addition of both monocytes and supernatant from RBCs containing EVs resulted in upregulation of endothelial expression of ICAM-1 and E-Selectin when compared to baseline. Upregulation was absent when stimulated with RBC supernatant depleted from EVs. EVs are phagocytosed by monocytes, which was partly abrogated after co-incubation with two different complement receptor 3 (CR3)-blocking antibodies. RBC-derived EVs also directly induce endothelial secretion of vWF. There were no differences between groups related to storage time.

Conclusion EVs from RBC transfusion bags activate monocytes with subsequent upregulation of endothelial cell adhesion markers. EVs are phagocytosed by monocytes through complement receptor 3. Furthermore, these EVs directly induce secretion of vWF. These effects are unrelated to storage time. Thereby, EVs from RBC transfusion bags induce a pro-inflammatory and pro-coagulant endothelial cell response.

Acknowledgements This work was in part supported by a CSL Behring Heimburger award
INTRODUCTION

A red blood cell (RBC) transfusion is frequently administered to patients on the Intensive Care Unit (ICU), with up to 45% of patients receiving a blood transfusion\(^1\). However, the risk-benefit of RBC transfusion is unclear, as observational studies in several patient populations show that RBC transfusion is associated with organ failure, including acute lung injury and acute kidney injury\(^2\)\(^-\)\(^4\). The causative factor in the blood products associated with RBC-related organ failure remains unknown, but includes effects on the vasculature\(^5\). RBCs regulate blood flow by influencing vasomotor tone of blood vessels. This regulation is impaired after prolonged storage by reducing NO bioavailability, both in experimental\(^6\) and clinical settings\(^7\). Furthermore, in an animal model, transfused RBCs adhere to the endothelium in the microcirculation, which is further enhanced after prolonged RBC storage\(^8\). The mechanism of interaction between RBCs and endothelium is unknown. We previously found that washing of RBCs attenuates organ injury in a rat transfusion model\(^9\), suggesting a soluble factor in the blood product. This factor is thus far not identified, but there may be a role for extracellular vesicles (EVs).

EVs are small phospholipid vesicles released from most cell types. EVs facilitate intercellular exchange of information without direct cell-to-cell contact. High concentrations of RBC-derived EVs are present in the supernatant of RBC transfusion bags\(^10\), which were shown to have pro-inflammatory and procoagulant effects\(^11\)\(^-\)\(^13\).

We hypothesize that RBC-derived EVs can activate endothelial cells through activation of host immune cells and that this effect is a function of storage time.

MATERIALS AND METHODS

Isolation of human umbilical vein endothelial cells (HUVECs)

We collected HUVECs from human umbilical veins as described previously\(^14\). Our Ethical Committee waived for informed consent under number W12-167#12.17.096. The HUVECs were cultured in gelatine (0.75%) coated 25 cm\(^2\) flasks (passage 0) and 75 cm\(^2\) flasks (passages 1 and 2). We performed the experiments with cells from the third passage and these cells were grown in a 12-well plate coated with gelatin, containing endothelial cell growth medium with supplement (Promocell, Heidelberg, Germany) to which we added 10% Fetal Bovine Serum (Tico Europe, Amstelveen, The Netherlands), 100 U/ml Penicillin and 0.1 mg/ml Streptomycin (Sigma-Aldrich, St. Louis, MO, USA), 2.5 µg/ml Amphotericin B (Sigma-Aldrich, St. Louis, MO, USA) and 2 mM L-glutamine (Gibco, Paisley, UK). After reaching confluence, the cells were maintained for 1 day until steady state was achieved before the experiments were started.
Preparation of blood samples
We obtained RBC units from Sanquin blood bank (Amsterdam, The Netherlands) after written informed consent from the donors. These RBC units were prepared and stored according to Sanquin blood bank standards, which includes leukoreduction and the use of saline-adenine-glucose-mannitol (150 mM NaCl, 1.25 mM adenine, 50 mM glucose, 29 mM mannitol) (SAGM) (Fresenius Kabi, The Netherlands) as a storage solution. RBC units were sampled at day 4 and day 35 and diluted 1:1 with filtered PBS. The diluted sample was centrifuged for 20 minutes at 1.550 \( g \), 20°C to remove red blood cells. The EV-containing supernatant was isolated and centrifuged again at 1.550 \( g \), 20°C. Finally, the EV-containing supernatant obtained after the second centrifugation step was snap frozen and stored at -80°C until further use. On the day of each experiment, we thawed samples from both fresh (4 days old) and stored (35 days old) RBC bags and centrifuged these for 60 minutes at 150.000 \( g \), 4°C. Hereafter, we isolated the EV-free supernatant. The EV-containing sample was prepared by resuspending the EV-pellet in a fixed volume of supernatant. We quantified EVs using tunable resistive pulse sensing (TRPS; qNano; Izon), which determines the particle size distribution of particles in suspension15.

Cell culture protocol
In some experiments, we added immune cells to the culture assay. We isolated these immune cells by drawing blood from healthy volunteers (after written informed consent was obtained) in 10 ml Sodium Heparin tubes, diluting this blood 1:1 with PBS and isolating granulocytes and monocytes by using Ficoll-Paque (GE Healthcare Life Sciences, Cleveland, USA). After isolation, white blood cells were counted on a hemocytometer (Z2 Coulter Particle Counter; Beckman Coulter Corporation, Hialeah, USA). At the start of the experiment, the culture medium of the HUVECs was replaced with 925 µl fresh culture medium containing either 150.000 monocytes, 3.000.000 neutrophils or no cells. We used Tumour Necrosis Factor alpha (TNF-\( \alpha \)) (10 ng/ml) as a positive control and PBS as a negative control. After one hour, we added 100 µl of RBC supernatant sampled at day 4 (fresh) either containing EVs or depleted from EVs, or RBC supernatant sampled at day 35 (stored) either containing EVs or depleted from EVs. After 5 hours of stimulation with RBC supernatant, cells were detached with 300 µl trypsin and washed in PBS/FCS 1%, after which the supernatant was removed and centrifuged for 10 minutes at 600 \( g \) at 4°C and stored until analysis at -80°C.

We repeated each set of experimental conditions five times in HUVECs derived from umbilical cords from five different donors. Every set of conditions was completely performed in HUVECs from the same donor.
Monocyte experiments

We isolated monocytes from blood from healthy donors using positive selection with CD14 beads on a MACS system (Miltenyi Biotec, Cologne, Germany). RBC supernatant samples containing EVs (1200 µl) were incubated with 1 µl of PKH26 membrane dye (Sigma-Aldrich, St. Louis, MO, USA) in the dark at room temperature during 10 minutes. Subsequently, EVs were centrifuged for 60 min at 150,000 g and the EV-pellet was resuspended in a fixed volume of supernatant. These PKH stained EVs (100,000) were added to 250,000 monocytes in a polystyrene tube in a total volume of 200 µl in HEPES buffer (132 mM NaCl, 20 mM HEPES [N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid], 6 mM KCl, 1 mM MgSO4, 1.2 mM K2HPO4 (all from Sigma-Aldrich, St. Louis, MO, USA) supplemented with 0.5% (v/v) HSA, 1 mM CaCl2 (Sigma-Aldrich, St. Louis, MO, USA) and 10 mM glucose (Sigma-Aldrich, St. Louis, MO, USA) and were placed in a 37°C water bath for 1.5 hours. After incubation, samples were washed with Hepes twice. We analysed the monocytes without and containing PKH stained EVs by confocal microscopy (Zeiss, Oberkochen, Germany).

Flow cytometry

HUVECs were labeled with antibodies against CD54-FITC, IgG1-FITC, IgG1-PE (Beckman Coulter, Brea, USA), CD62E-PE, CD106-FITC, CD138-FITC and CD142-PE (BD Pharmingen, San Jose, USA). After the monocyte experiment, we incubated monocytes with antibodies against CD11b and CD18. CytoB (2.5mg/ml, 1:500) was used as a negative control. We performed flow cytometry with a guava easyCyte™ HT Sampling Flow Cytometer (Merck Millipore, Billerica, USA). Fluorescence and flow rate were calibrated every measuring day. We measured all samples until 5000 events were counted, and we recorded the forward scatter (FSC), sideward scatter (SSC) and the fluorescence-dependent detections. We analysed the obtained data using InCyte™ software (Merck Millipore, Billerica, USA).

Assays

Levels of Interleukin-6 (IL-6), Interleukin-8 (IL8) (R&D Systems Minneapolis, MN, USA) and syndecan-1 (Diaclone, Sanquin, Amsterdam, the Netherlands) were determined by ELISA according to the manufacturer’s instructions. Levels of syndecan-1 and vWF antigen (vWF:Ag) levels were determined with an in-house ELISA using commercially available polyclonal antibodies against vWF (DAKO, Glostrup, Denmark).

Statistical analysis

Results are depicted as median and interquartile range. To study the effect of EVs, comparisons between groups were performed using the percentage of change compared to their negative control (PBS), i.e. the change compared to baseline of a certain parameter when EVs were added versus the change compared to baseline of a certain parameter when EVs were omitted. We initially performed A Kruskal Wallis and when a post-hoc
analysis was warranted, we performed a Mann-Whitney U using SPSS 22 (SPSS inc., Illinois, USA) and Graphpad Prism 5 (Graphpad software, San Diego, CA, USA).

RESULTS

Baseline effect of co-incubation of endothelial cells with immune cells
In the HUVEC model, ICAM-1 is expressed on 40% of cells, VCAM-1 is expressed on 10% of cells and E-selectin is expressed on 2.3% of cells (Table 1). The addition of granulocytes increased expression of all cell adhesion markers significantly, whereas co-incubation with monocytes had no effect on expression of cell adhesion markers (Table 1), nor on secretion of markers of endothelial activation or cytokines into the culture medium (Table 1). TNF-α was used as a positive control and upregulated all adhesion markers tested. Thereby, monocytes alone did not provoke an endothelial cell response in this model.

EVs upregulate expression of endothelial cell adhesion molecules when co-cultured with monocytes.
In the absence of white blood cells, expression of endothelial adhesion markers did not differ between HUVECs incubated with EV-depleted or EV-containing RBC supernatant (Figure 1). However, when HUVECs were incubated with EV-containing RBC supernatant in the presence of monocytes, expression of ICAM-1 and E-selectin significantly increased compared to HUVECs incubated with EV-depleted RBC supernatant. Expression of VCAM-1 also increased

| Table 1. Baseline measurement of the endothelial cell culture condition with and without the addition of immune cells, showing Intercellular Adhesion Molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), E-selectin, Tissue Factor (TF), Von Willebrand factor (vWF), Tumor necrosis factor (TNF-α), Interleukin-6 (IL-6) and Interleukin-8 (IL-8). Data are expressed as median [IQR]. |
| --- | --- | --- |
| **No cells** | **Monocytes** | **Granulocytes** |
| **Adhesion markers** | | | |
| ICAM-1-1 (% positive cells) | 39.9 [26.6-44.2] | 44.6 [29.5-50.8] | 65.8 [38.7-74.4] |
| E-selectin (% positive cells) | 2.8 [2.0-6.1] | 3.0 [2.2-4.9] | 25.1 [3.9-31.6] |
| TF (% positive cells) | 3.9 [3.5-7.1] | 5.0 [3.1-7.4] | 14.7 [5.1-16.6] |
| **Shed endothelial markers** | | | |
| vWF (%) | 0.7 [0.5-0.8] | 0.6 [0.5-0.8] | 0.9 [0.6-1.1] |
| Syndecan-1 (ng/ml) | 3.2 [2.0-7.1] | 2.5 [2.0-6.6] | 3.4 [2.0-7.6] |
| **Shed cytokines (pg/ml)** | | | |
| TNF-α (pg/ml) | 4.7 [3.0-35.5] | 8.1 [3.0-29.5] | 3.0 [3.0-27.3] |
| IL-6 (pg/ml) | 117.4 [25.1-176.5] | 119.5 [28.1-198.7] | 336.1 [311.1-1327.3] |
| IL-8 (pg/ml) | 1523.2 [1248.4-1648.0] | 1426.7 [1111.1-1901.2] | 2090.3 [1298.0-3934.2] |
in response to EV-containing RBC supernatant in the presence of monocytes, but this result was not statistically significant. In contrast to monocytes, the addition of granulocytes to EV-containing supernatant did not influence expression of adhesion markers.

**Figure 1.** The relative change in expression of endothelial adhesion markers (ICAM-1, VCAM-1 and E-Selectin) compared to baseline, in the presence or absence of immune cells and co-incubated with RBC supernatant either containing (+) or depleted (-) of EVs. TNF-α served as a positive control. Data are median [IQR]. * p< 0.05.

**EVs induce shedding of vWF antigen**

Tissue factor (TF) was expressed on a small percentage of HUVECs (Table 1). Incubation with EV-containing RBC supernatant did not upregulate TF expression compared to EV-depleted supernatant, neither in the presence nor the absence of white blood cells (Figure 2). Also, EVs did not result in shedding of syndecan-1. Thereby, EVs did not seem to activate the endothelium. Of interest, EVs increased shedding of vWF antigen into the culture medium, which occurred also without the addition of immune cells. Incubation of endothelial cells with RBC supernatant either with or without EVs, did not induce the secretion of TNF-α, IL-6 or IL-8 into the culture medium (Figure 3).

**EVs are phagocytosed by monocytes via CR3**

Having observed that EVs increase endothelial adhesion markers only in the presence of monocytes, we further delineated the interaction of monocytes with EVs. After incubation of monocytes with stained EVs, confocal microscopy showed that the EVs were
Figure 2. The relative change in expression or level of endothelial activation markers (vWF, syndecan-1 and TF) compared to baseline, in the presence or absence of immune cells and co-incubated with RBC supernatant either containing (+) or depleted (-) of EVs. TNF-α served as a positive control. Data are median [IQR]. * p< 0.05.

Figure 3. The relative change in levels of cytokines (TNF-α, IL-6 and IL-8) in the culture medium compared to baseline, in the presence or absence of immune cells and co-incubated with RBC supernatant either containing (+) or depleted (-) of EVs. TNF-α served as a positive control. Data are median [IQR].
taken up by monocytes (Figure 4). In line with this, monocytes incubated with PKH-stained EVs were positive when analyzed by flow cytometry, demonstrating that EVs bind to monocytes. This process was partially inhibited by incubation with antibodies directed against the $\alpha$ chain of CR3, ($\alpha_{m},$ CD11b) as well as to the beta chain ($\beta_{2},$ CD18). Both anti-CD11b or antiCD18 resulted in a decrease in EV-positive monocytes (Figure 5).

Figure 4. Confocal images of monocytes incubated with (A) and without (B) PKH-stained extracellular vesicles (EVs indicated by the red colour).

Figure 5. Monocytes positive for PKH after incubation with PKH-stained EVs, either untreated, in the presence of the beta integrin antibodies anti-CD18 or anti-CD11b or Cytochalasin B as a negative control. Data are median [IQR]. * $p< 0.05$.

Effects of EVs on the endothelium are independent of storage time

To investigate whether storage time influenced the effects of EV-containing RBC supernatant on endothelial cell activation mediated by monocytes, HUVECs were stimulated in the presence of monocytes to which either fresh or stored RBC supernatant was added containing EVs (Figure 6). There was no difference in inflammatory response of endothelial cells between HUVECs incubated with EVs from fresh RBC supernatant and EVs from stored RBC supernatant, nor was there any difference in upregulation of TF or the shedding of vWF and syndecan-1.
Figure 6. The relative change in expression of endothelial adhesion (A) and activation markers (B) and cytokine levels (C) compared to baseline, in the presence of monocytes and co-incubated with supernatant containing EVs from either fresh or stored RBC products. Data are median [IQR].

DISCUSSION

EVs from RBC products interact with monocytes via a mechanism involving adherence of EVs to monocytes which is at least in part mediated by CR3 on the monocytes,
whereafter these activated monocytes induce upregulation of endothelial cell adhesion molecules. Also, these EV-activated monocytes induce endothelial shedding of vWF, which is involved both as a plasma ligand for cell adhesion as well in the formation of micro-thrombi. Thereby, EVs from RBC can activate a pro-inflammatory and pro-coagulant response from endothelial cells. These processes are unrelated to storage time.

RBC transfusion is associated with immune modulation\textsuperscript{16-20}, demonstrating both pro- and anti-inflammatory effects. In a clinical study in pediatric patients, RBC transfusion resulted in an increase in soluble ICAM-1, which is a marker of activated endothelium. Our data extend these findings by showing that the compound in the blood product resulting in activation of endothelial adhesion markers are RBC-derived EVs, via a process that requires monocytes. These EVs need monocytes in order to upregulate adhesion markers, whereas monocytes alone do not increase expression. We observed that EVs bind to monocytes, a process that is blocked by antibodies to CR3. Thereby, EVs seem to activate monocytes via integrins, which in concert with EVs may then increase (secondary) adhesion of cells to the endothelium. It was shown in vitro, that both LPS and oxidative stress promote adherence of donor RBCs to the endothelium\textsuperscript{21,22}, which may suggest that RBC transfusion may impede blood flow in inflammatory recipients. This observation may be an explanation for the consistent clinical observation of the association of RBC transfusion with organ injury in the critically ill\textsuperscript{2-4}. Alternatively, immune cells may adhere to the endothelium. Whether RBCs or other cells adhere to the endothelium following up-regulation of adhesion markers by donor RBC-derived EVs requires further study.

In this study, we also investigated whether RBC-derived EVs have a pro-coagulant effect on endothelium. RBC-derived EVs have an abundant expression of TF and are also referred to as a ‘blood-borne’ TF source. In addition, RBC-derived EVs express phosphatidyl serine (PS), which was shown to induce thrombin generation in vitro\textsuperscript{21}. In this study, EVs did not stimulate endothelial TF expression. Of note however, RBC-derived EVs induced endothelial shedding of vWF. Thereby, another mechanism of activation of coagulation by EVs unrelated to TF is stimulation of the endothelium to release vWF. This effect occurred also without adding immune cells, suggesting that EVs themselves are pro-coagulant.

The amount of EVs as well as the number of PS-expressing EVs accumulate in RBC products increases during storage\textsuperscript{11,12}, suggesting that EVs from stored blood could induce a hypercoagulable state. We found no effect of storage duration on EV-induced endothelial cell activation. However, some studies show that stored RBCs but not fresh RBCs adhere to endothelial cells in vitro and to the microcirculation in vivo\textsuperscript{8,24}. Apparently, this increased adhesion of stored RBCs does not depend on an increased endothelial cell activation. In line with our findings, another study found that inflammation and not stor-
age time determined the effect of donor RBCs on endothelium\textsuperscript{22}. In accordance, large trials in ICU populations showed that age of blood does not seem to influence clinically relevant outcome parameters\textsuperscript{25,26}. Thereby, the condition of the recipient may influence the efficacy of RBC transfusion. In critically ill patients, RBC transfusion may have more pro-coagulant, pro-inflammatory and pro-adhesive effects than in chronic anemia patients who do not have a hyperinflammatory status. Further studies on mechanisms of the association between RBC transfusion and outcome should turn their focus on the interaction between RBC transfusion and the recipient. Our data suggest that washing or filtering of RBC units to deplete them from EVs prior to transfusion may be a beneficial intervention.

**CONCLUSIONS**

EVs from RBC products activate endothelial cells via a mechanism involving adherence of EVs to monocytes, which is mediated by beta integrin and results in up-regulation of endothelial adhesion markers and shedding of vWF into the medium. Whether depleting of RBC products of EVs prior to transfusion is beneficial on a relevant outcome level, should be further explored.
EVs from RBCs induce monocyte mediated activation of HUVECs

LITERATURE


PART II

Fresh Frozen Plasma
Chapter 7

Fresh frozen plasma transfusion fails to influence the hemostatic balance in critically ill patients with a coagulopathy

Muller MC, Straat M, Meijers JC, Klinkspoor JH, de Jonge E, Arbous MS, Schultz MJ, Vroom MB, Juffermans NP

ABSTRACT

Background Coagulopathy has a high prevalence in critically ill patients. An increased INR is a common trigger to transfuse fresh frozen plasma (FFP), even in the absence of bleeding. Thereby, FFP is frequently administered in these patients. However, efficacy of FFP to correct haemostatic disorders in non-bleeding recipients is questioned.

Objectives To assess whether INR prolongation parallels changes in other tests investigating hemostasis and to evaluate the coagulant effects of a fixed dose of FFP in non-bleeding critically ill patients with a coagulopathy.

Methods Markers of coagulation, individual factor levels and levels of natural anticoagulants were measured. Also thrombin generation and thromboelastometry (ROTEM) assays were performed before and after FFP transfusion (12 ml/kg) to 38 non-bleeding critically ill patients with an increased INR (1.5-3.0).

Results At baseline, levels of factor II, V and VII as well as levels of protein C, S and antithrombin were reduced and thrombin generation was impaired. ROTEM variables were within reference ranges, except for prolonged INTEM CFT. FFP transfusion increased levels of coagulation factors (factor II (34% [26-46] before vs. 44% [38-52] after), factor V (48% [28-76] before vs. 58% [44-90] after) and factor VII (25% [16-38] before vs. 37% [28-55] after)) as well as levels of anticoagulant proteins. Thrombin generation was unaffected by FFP transfusion (endogenous thrombin potential (72% [51-88] before vs. 71% [42-89] after), while ROTEM EXTEM CT and MCF slightly improved in response to FFP.

Conclusion In non-bleeding critically ill patients with a coagulopathy, FFP transfusion does not influence the haemostatic balance.

Trial registration NTR 2262 (www.trialregister.nl) and NCT01143909 (ClinicalTrials.gov).

Funding source The study was funded by ZonMw Netherlands, Organization for Health, Research and Development, The Hague, The Netherlands (Project number 171002206).
BACKGROUND

Coagulopathy occurs in up to 30% of critically ill patients. Seventy percent of Fresh Frozen Plasma (FFP) used by critical care facilities is transfused to patients with prothrombin time (PT) prolongation, mostly in the absence of bleeding. To assess bleeding risk and effectiveness of FFP transfusion, the clinically widely available, International Normalized Ratio (INR) is often used. An increased INR indicates that at least one of the vitamin K dependent coagulation factors is below the haemostatic threshold. Even though the test is designed to monitor therapy with vitamin K antagonists, INR is a common trigger to administer FFP in clinical practice. However, in acquired coagulopathy in critically ill patients, where multiple clotting factors are deficient, the relationship between clotting factor levels and INR prolongation is complex and not well understood. Indeed, INR only represents a part of the coagulation cascade and is insensitive to the activity or concentration of anticoagulant and fibrinolytic proteins. In the critically ill, and particularly in patients with disseminated intravascular coagulation (DIC), levels of anticoagulants are reduced, while fibrinolysis is attenuated.

The haemostatic balance is the net result of the presence of pro-coagulant, anticoagulant and fibrinolytic factors. Disturbance between these components can result in variable in vivo coagulation profiles, ranging from hypocoagulable with increased bleeding tendency to a procoagulant state with (micro-) vascular thrombus formation. Thereby, despite an elevated INR, patients may not necessarily have an increased bleeding tendency. In line with this, INR poorly reflects risk of bleeding in the critically ill.

The efficacy of FFP to correct coagulopathy has only been evaluated in small and heterogeneous studies. Notably, the efficacy of FFP to prevent bleeding in coagulopathic critically ill patients has not been demonstrated. In addition, the net effect of FFP transfusion on anticoagulant proteins and the haemostatic balance in the critically ill is not well known.

In a predefined sub-study of a multicenter randomized controlled trial on the efficacy of prophylactic FFP transfusion to prevent bleeding in critically ill patients with an increased INR who were scheduled to undergo an invasive procedure, we investigated whether INR prolongation parallels changes in other tests investigating hemostasis and evaluated the effect of a fixed dose of FFP on the haemostatic balance of these patients.

MATERIALS AND METHODS

The original study was approved by the medical ethics committee of the Academic Medical Center, Amsterdam, the Netherlands. Before entry in the study, written informed consent was obtained from the patient or legal representative in accordance with the
Declaration of Helsinki. The study protocol was registered with trial identification numbers NTR 2262 (Dutch Trial Register) and NCT01143909 (ClinicalTrials.gov).

**Setting and patients**

The study was performed between May 2010 and June 2013 in four mixed medical-surgical ICUs in the Netherlands. Patients were eligible when INR was 1.5-3.0 and needed to undergo an intervention. Although INR is developed to monitor effects of vitamin K antagonists, in daily practice it is one of the most commonly used tests to assess coagulopathy in critically ill patients and increased values are often a trigger to transfuse FFP. Also, use of INR instead of PT facilitates combining of results obtained in different centers.

Patients younger than 18 years or with a known bleeding diathesis, treated with vitamin K antagonists, activated protein C, abciximab, tirofiban, ticlopidine or prothrombin complex concentrates were excluded. Patients treated with therapeutic doses of heparin or low molecular weight heparin were allowed to participate if medication was discontinued for an appropriate period, which was >2 hours for heparin and >12 hours for low molecular weight heparin. Use of low molecular weight heparin in a prophylactic dose was part of standard care in all patients.

**Design**

A predefined post hoc study of a randomized controlled clinical trial on risk and benefit of FFP transfusion in critically ill patients with a coagulopathy. After inclusion, patients were randomized to a single dose of 12 ml/kg FFP or no FFP transfusion before a scheduled intervention. The FFP was quarantine plasma manufactured by Sanquin, the Dutch National Blood bank. The dose of FFP was based on clinical practice and a previous study with a target of INR reduction to <1.5. Patients were observed until 24 hours after the intervention for bleeding complications.

**Patient data and sample collection**

Patient data were collected from the electronic patient data management system (PDMS) and consisted of medical history, admission diagnosis, use of anticoagulant medication and occurrence of bleeding. Disseminated intravascular coagulation (DIC) was assessed using the ISTH DIC score, which defines overt DIC as a score of ≥ 5 points (table 1 supporting information).

Blood samples were drawn from an indwelling arterial catheter at baseline and the second sample was taken directly after FFP transfusion (but prior to the invasive procedure). Samples were collected in sodium citrate (0.109M 3.2%) tubes. Samples were centrifuged within 30 minutes at 2000 x g for 15 mins at 18°C and subsequently 5 mins at 15000 x g also at 18°C. Supernatant was collected and stored at -80°C until measurements were performed.
Assays
PT, INR, activated partial thromboplastin time (aPTT) and levels of D-dimer and fibrinogen were all determined immediately after the sample was drawn with standard assays on an automated coagulation analyzer (Sysmex CA 7000 and all reagents, Siemens Healthcare Diagnostics, Marburg, Germany) according to manufacturers protocols. After termination of the study the remaining assays were performed collectively in all patients. Levels of factors II, V and VII were determined by a PT based one stage clotting assay (ACL TOP 700, Instrumentation Laboratory, USA), using recombiplastin and factor II, V and VII deficient plasma (Instrumentation Laboratory, USA). Antithrombin was assessed by chromogenic substrate method (Sysmex CA7000) with reagents and protocols of the manufacturer.

Protein C activity was measured by a kinetic assay (Coamatic Protein C, Chromogenix, Mölndal, Sweden). Total protein S levels were determined by enzyme-linked immuno-sorbent assay (ELISA) as described previously. Free protein S was measured by precipitating the C4b-binding protein-bound fraction with polyethylene glycol 8000 and measuring the concentration of free protein S in the supernatant. Thrombin-antithrombin (TATc), prothrombin fragment 1 + 2 (F1+2) and plasmin-α2-antiplasmin complex (PAP) levels were measured using specific commercially available ELISAs according to the instruction of the manufacturer (Siemens Healthcare Diagnostics and DRG, Marburg, Germany).

Thrombin generation assay
The Calibrated Automated Thrombogram® assays the generation of thrombin in clotting plasma using a microtiter plate reading fluorometer (Fluoroskan Ascent, ThermoLab systems, Helsinki, Finland) and Thrombinoscope® software (Thrombinoscope BV, Maastricht, The Netherlands). The assay was carried out as described by Hemker et al. and the Thrombinoscope® manual. Coagulation was triggered by recalcification in the presence of 5 pM recombinant human tissue factor (Innovin®, Siemens Healthcare Diagnostics, Marburg, Germany), 4 μM phospholipids, and 417 μM fluorogenic substrate Z-Gly-Gly-Arg-AMC (Bachem, Bubendorf, Switzerland). Fluorescence was monitored, and the parameters (lag time, peak thrombin, area under the curve or endogenous thrombin potential) were calculated using the Thrombinoscope software.

Thromboelastometry
Using ROTEM (Tem International, Munich, Germany), three separate assays were carried out, including EXTEM to assess tissue factor-initiated coagulation, INTEM to assess the intrinsic pathway and FIBTEM to qualitatively assess fibrinogen status. For EXTEM, 20 μL of 0.2 mol/L CaCl₂ (star-tem®) and 20 μL of recombinant tissue factor (r EXTEM®) were added to a test vial, subsequently 300 μL of the citrated blood sample was added. For the INTEM test 20 μL of 0.2 mol/L CaCl₂ (star-tem®), 20 μL of partial thromboplastin made
of rabbit brain (in-tem®) and 300 μL of blood were added to the test cuvette. FIBTEM test was carried out by adding 20 μL of recombinant human tissue factor (r EXTEM®), 20 μL of platelet inactivating cytochalasin D solution 0.2 mol/L CaCl₂ and 300 μL of the blood sample to the test vial. The electronic pipette program guided all test steps. For INTEM and EXTEM clotting time (CT), clot formation time (CFT), clot firmness (MCF), alpha angle and maximum lysis (ML) were recorded. For the FIBTEM assay CT and MCF were recorded.

Statistics
All variables are expressed as median (interquartile ranges). To compare groups two group t-test or Mann-Whitney test was used. Paired data were compared using the Wilcoxon signed rank test. Fisher’s exact test was used for comparisons in crosstab. A p value of less than 0.05 was considered significant. Statistical analyses were performed with SPSS 20.0 (SPSS, Inc, Chicago, IL, USA) and Prism Version 5.0 (Graphpad Software, San Diego, USA).

RESULTS

Patients
In total 38 patients were randomized to FFP transfusion and all samples before and after transfusion were available for analysis. Patients were critically ill, as reflected by a high disease severity score (e.g. APACHE IV and SOFA score) (Table 1). Half of the patients had sepsis and more than a third had DIC.

Baseline coagulation tests in critically ill with a prolonged INR
Median INR levels at baseline were 1.8 [1.5-2.2]. As expected, median baseline levels of coagulation factors were reduced, with a factor II of 34% [26-46], factor V of 48% [28-76] and VII level of 25% [16-38] (Figure 1). Also, medians of levels of endogenous anticoagulant factors were decreased, including antithrombin (47% [35-78]), protein C activity (33% [21-50]), levels of total protein S (51% [36-70]) and free protein S (53% [32-75]) (Figure 2). Markers of activation of coagulation TATc and F1+2 were above upper reference value at baseline (10 ug/L [5-22] and 370 pMol/L [113-608] respectively). Also, the fibrinolytic marker PAP was above upper reference value at baseline (842 ug/L [322-1267]).

In all patients included in one center (n=16), ROTEM was performed on paired samples in addition. ROTEM variables were within reference ranges, except for prolonged INTEM CFT. (Table 2). In addition, EXTEM CFT was at the upper reference range and both INTEM and EXTEM MCF were at the lower reference range.
Table 1. Patient characteristics

<table>
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<tr>
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<td>Age (years)</td>
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<tr>
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<td>28 day mortality, % (n)</td>
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</table>

Data expressed as median and interquartile ranges.

* therapeutic dose

APACHE = Acute Physiology and Chronic Health Evaluation
DIC = Disseminated Intravascular Coagulation
FFP = Fresh Frozen Plasma
SOFA = Sequential Organ Failure Assessment
RBC = Red blood cells
ICU = Intensive Care Unit

Figure 1. Levels of individual coagulation factors at baseline and after Fresh Frozen Plasma transfusion (12 ml/kg)
Effect of FFP transfusion on coagulation tests, factor levels and anticoagulants

FFP transfusion reduced median INR to 1.4 [1.3-1.6] (Table 2). As expected, FFP transfusion increased median levels of coagulation factors II (44% [38-52]), V (58% [44-90]) and VII (37% [28-55]). However, medians of factor levels remained under the lower limit of reference values after transfusion (Figure 1). Protein C, S and antithrombin levels also increased in response to FFP transfusion (Figure 2). FFP did not increase activation markers of coagulation, but rather reduced levels of F1+2. TATc levels were unaffected by FFP transfusion. We also measured parameters of fibrinolysis. FFP did not affect PAP levels, although D-dimer concentration was reduced after FFP transfusion (Table 3). The response of patients with and without bleeding complications following FFP transfusion did not differ, with no differences in levels of factors II, V and VII and anticoagulants antithrombin, protein C and S (data not shown).

Effect of FFP transfusion on thrombin generation

In 27 patients, paired samples were available to perform thrombin generation tests. At baseline, patients had prolonged lag time, which is the time from start of the assay until detection of the first thrombin and peak values were slightly reduced. Median ETP (endogenous thrombin potential) values were normal before FFP transfusion and did
FFP fails to influence haemostatic balance in coagulopathic ICU patients

Figure 2. Levels of anticoagulant proteins before and after Fresh Frozen Plasma transfusion (12 ml/kg).

Figure 3. Thrombin generation test results before and after Fresh Frozen Plasma transfusion (12 ml/kg). Dotted lines indicate reference ranges.
not change (72% [51-88] before vs. 71 [42-89] after, p=0.27). Of note, at baseline about half of the patients had peak or ETP values below the reference values at baseline (Figure 3). Transfusion of FFP only improved peak values, while the other thrombin generation parameters were unaffected by FFP transfusion, with persistent prolonged lag time and time to peak (Figure 3, supporting information table 2).

### Table 3. Markers of coagulation at baseline and after Fresh Frozen Plasma transfusion (12 mg/kg) ml/kg

<table>
<thead>
<tr>
<th></th>
<th>Reference values</th>
<th>Before FFP transfusion</th>
<th>After FFP transfusion</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=38</td>
<td>N=38</td>
<td>n=38</td>
<td></td>
</tr>
<tr>
<td>INR</td>
<td>1.0</td>
<td>1.8 (1.5-2.2)</td>
<td>1.4 (1.3-1.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>APTT (sec)</td>
<td>22-30</td>
<td>43 (38-52)</td>
<td>39 (32-46)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Platelet count (*10^9/L)</td>
<td>150-400</td>
<td>89 (51-183)</td>
<td>96 (45-158)</td>
<td>0.001</td>
</tr>
<tr>
<td>D dimer (mg/L)</td>
<td>≤0.5</td>
<td>7.5 (2.1-11.3)</td>
<td>6.4 (3.3-11.0)</td>
<td>0.009</td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>1.5-4.0</td>
<td>3.1 (2.1-5.2)</td>
<td>2.8 (2.3-5.4)</td>
<td>0.97</td>
</tr>
<tr>
<td>F1+2 (pMol/L)</td>
<td>53-271</td>
<td>370 (113-608)</td>
<td>323 (162-480)</td>
<td>0.02</td>
</tr>
<tr>
<td>TATc (ug/L)</td>
<td>&lt;4.6</td>
<td>10 (5-22)</td>
<td>11 (6-22)</td>
<td>0.56</td>
</tr>
<tr>
<td>PAP (ug/L)</td>
<td>47-563</td>
<td>842 (322-1267)</td>
<td>833 (411-1151)</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Data expressed as median (IQR)

Wilcoxon Signed Rank test.

FFP = Fresh Frozen Plasma

APTT = Activated Partial Thromboplastin Time

INR = International Normalized Ratio

F1+2 = Prothrombin Fragment 1+2

TATc = Thrombin-antithrombin complex

PAP = Plasmin-α2-antiplasmin complex

Figure 4. ROTEM EXTEM variables before and after Fresh Frozen Plasma transfusion (12 ml/kg). Dotted lines indicate reference ranges.
Table 4. Coagulation parameters after Fresh Frozen Plasma transfusion (12 ml/kg) in patients with and without minor bleeding complications

<table>
<thead>
<tr>
<th></th>
<th>Minor bleeding N=8</th>
<th>No bleeding N=30</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>INR</td>
<td>1.5 (1.3-1.6)</td>
<td>1.4 (1.3-1.6)</td>
<td>0.75</td>
</tr>
<tr>
<td>APTT (sec)</td>
<td>37 (30-48)</td>
<td>43 (36-52)</td>
<td>1.00</td>
</tr>
<tr>
<td>Hemoglobin (mmol/L)</td>
<td>5.7 (5.4-6.1)</td>
<td>5.8 (5.2-6.4)</td>
<td>0.65</td>
</tr>
<tr>
<td>Platelet count (×10^9/L)</td>
<td>64 (40-114)</td>
<td>103 (46-167)</td>
<td>0.27</td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>1.9 (0.9-2.8)</td>
<td>4.0 (2.5-5.5)</td>
<td>0.11</td>
</tr>
<tr>
<td>Factor II (%)</td>
<td>40 (30-52)</td>
<td>46 (40-52)</td>
<td>0.23</td>
</tr>
<tr>
<td>Factor V (%)</td>
<td>48 (38-56)</td>
<td>67 (50-95)</td>
<td>0.05</td>
</tr>
<tr>
<td>Factor VII (%)</td>
<td>44 (30-58)</td>
<td>37 (28-54)</td>
<td>0.56</td>
</tr>
</tbody>
</table>

Data expressed as median (IQR)

FFP = Fresh Frozen Plasma
INR = International Normalized Ratio
APTT = Activated Partial Thromboplastin Time

**Effect of FFP transfusion on thromboelastometry (ROTEM) variables**

Transfusion with 12 ml/kg FFP reduced median EXTEM CT and improved median EXTEM MCF, indicating enhanced coagulation. However, in most patients, variables only changed marginally after FFP transfusion (Figure 4). FIBTEM MCF was unaffected by FFP transfusion and the same applied for the INTEM variables (Table 2).

**Bleeding complications**

In 8 of 38 patients, minor bleeding complications occurred in the first 24 hours after the intervention. Complications consisted of prolonged oozing needing extra compression (n=4) or an extra suture at the insertion site (n=1) or hematoma formation not requiring additional interventions (n=3). None of these events required transfusion of extra FFP or an intervention to cease bleeding. Coagulation parameters of bleeding and non-bleeding patients are depicted in table 4.

**DISCUSSION**

In the current study, we demonstrated that critically ill patients with a coagulopathy as reflected by a prolonged INR have reduced levels of individual coagulation factors with a concurrent decrease in levels of natural occurring anticoagulant factors, associated with delayed thrombin generating capacity and a tendency towards hypocoagulability as measured by whole blood assay. Transfusion of a fixed dose of FFP improved individual factor levels, but also increased levels of natural anticoagulants. Both thrombin generation and thromboelastometry variables only improved marginally in response to FFP.
transfusion. Thereby, FFP transfusion has a marginal effect on the haemostatic balance in non-bleeding coagulopathic patients.

Critically ill patients with an increased INR have reduced levels of individual coagulation factors, as found previously\(^6\). These reduced levels may render them susceptible for bleeding complications. Concurrently, also in line with previous reports\(^6\), we found that levels of natural anticoagulants antithrombin, protein C and S were also reduced. As INR fails to reflect altered levels of anticoagulants, contribution of cellular components to hemostasis and the amount of fibrinolysis, this test poorly reflects \textit{in vivo} coagulation and actual bleeding risk. In patients with liver disease, it has been shown that INR elevation indeed failed to predict bleeding complications\(^20\). In these patients, reduced levels of clotting factors, natural anticoagulants\(^{21}\) and pro- and anti-fibrinolytic factors\(^{22}\) led to the concept of “rebalanced hemostasis”\(^{23}\). A reduction in coagulation factors and natural anticoagulants, accompanied by relatively normal clot formation has also been observed in severe sepsis patients\(^6\). Based on our findings, including near normal viscoelastic test results, it is conceivable that this concept of rebalanced hemostasis also applies to non-bleeding critically ill patients with a coagulopathy.

The present study aimed to establish the effect of a standardized dose of FFP on individual components of the coagulation system, as well as on global tests of coagulation. First, we demonstrated that FFP transfusion indeed reduced INR value, in line with an increase in individual factor levels. However, all individual levels remained under the lower limit of the reference values. Of note, the increase in factor levels was equal in those patients experiencing minor bleeding after the intervention compared to those who did not. Equally important to the concept of the ability of FFP to mitigate the risk of bleeding, is the effect of FFP on levels of anticoagulant factors. Indeed FFP resulted in a concomitant rise of levels of natural anticoagulants antithrombin, protein C and S. In a study in critically ill neonates, prophylactic FFP transfusion resulted in similar results to ours, with increased coagulation factor levels but also levels of anticoagulants\(^{24}\). A frequently referenced study investigating factor levels after FFP transfusion in the critically ill also showed decreased levels of all factors and small increments after FFP transfusion\(^8\). In this study, different doses of FFP were used in small patients groups. We used a fixed dose of 12 ml/kg FFP. In addition, only individual factor levels were measured, hereby rendering the net effect of FFP on haemostatic balance unknown.

In this study, the effect of FFP on hemostasis was assessed with thrombin generation and whole blood assay. Results of thrombin generation tests in our patients demonstrated prolonged lag times and times to peak, although the total amount of thrombin generated (expressed by ETP) was preserved. The profile with delayed thrombin generation most likely results from reduced levels of factors II and VII\(^{6,25}\) and is in line with previous reports\(^6,26\). The concomitant reduction of antithrombin levels in addition to
reduced prothrombin levels is probably responsible for preserved ETP values, as was demonstrated previously \(^{25}\). In our study, FFP transfusion did not affect this pattern of delayed thrombin generation and only marginally improved peak values, without affecting ETP values. Levels of F1+2 were slightly reduced and TATc levels were unaffected by FFP administration, also contradicting enhanced thrombin generation as a result of FFP transfusion. In line with this, in a similar study performed in critically ill neonates, prophylactic FFP transfusion even attenuated thrombin formation, which was thought to be due to increased levels of anticoagulants following FFP \(^{24}\). Altogether, FFP transfusion has limited effect on thrombin generation in critically ill patients with a coagulopathy. In whole blood assays, a fixed dose of FFP reduced EXTEM CT and increased EXTEM MCF, whereas INTEM variables remained unaffected, suggestive of a decrease in time to stable clot formation. Of note however, observed increments were only small and values all remained within reference ranges. Taken together, despite a reduction in INR, administration of FFP fails to alter the haemostatic balance to a more pro-coagulant state. Although the original clinical trial was terminated prematurely \(^{14}\), findings of the current sub-study suggest that the trial would have been negative if completed.

Our study has several limitations. First, our group size was relatively small. However, patient characteristics correspond well with those of patients with a coagulopathy in a large prospective cohort study in mixed medical/surgical ICUs \(^{1}\), supporting the generalizability of our results. Of note, we found almost no differences in haemostatic tests between patients with and without bleeding following an intervention, but assessment of the ability of haemostatic tests to predict bleeding complications requires a larger sample size. Second, ROTEM testing was performed only at 1 center, yielding a small sample size. Test precision of ROTEM is a subject of debate, with reported high coefficients of variance \(^{27}\). In order to limit variation as much as possible, all tests were carried out using the same device by only two experienced researchers. Third, we did not perform thrombin generation assays with the addition of thrombomodulin or activated protein C. In patients undergoing liver transplantation, thrombin generation was preserved after addition of thrombomodulin hereby demonstrating a defective endogenous anticoagulant system. Indeed these patients had reduced levels of antithrombin and protein C and S \(^{21}\). Of note, levels of antithrombin, protein C and S were reduced to a similar extent in our patients. Fourth, one could rightfully debate the use of INR as a test to estimate coagulation status. We choose this test because INR is often measured as part of clinical practice to determine coagulation status \(^{1,28,29}\) and tried to relate effects on INR to other haemostatic tests. We feel that the results are valuable information to the practicing physician. Finally, the dose of FFP was chosen with the aim to reduce INR to <1.5 and not to fully correct INR \(^{13}\). The observed limited increment in coagulation factor levels could have been calculated beforehand and higher dose of FFP than 12 ml/kg would have been required to normalize factor levels. However, transfusion with
higher doses of FFP is not current practice\textsuperscript{2,15,30,31}, moreover audits have revealed that a substantial number of patients is transfused with a dose less than 10 ml/kg\textsuperscript{16,32}. Again, we think that results are relevant to the practicing physician.

**CONCLUSION**

Critically ill patients with increased INR display delayed thrombin generation, but preserved viscoelastic test results. Prophylactic FFP transfusion in a dose of 12 ml/kg increased individual coagulation factors, with a concomitant increase in levels of anticoagulants. In addition, effect of FFP transfusion on thrombin generation and thromboelastometry was highly limited and failed to induce a more procoagulant state. These results contribute to our knowledge on effects of FFP and underline the lack of rationale to administer FFP to non-bleeding ICU patients with a coagulopathy.
LITERATURE


SUPPLEMENTARY APPENDIX TO: FRESH FROZEN PLASMA TRANSFUSION FAILS TO INFLUENCE THE HAEMOSTATIC BALANCE IN CRITICALLY ILL PATIENTS WITH A COAGULOPATHY

SUPPLEMENTAL TABLES

**Supplementary Table 1.** ISTH score for disseminated intravascular coagulation (Taylor FB et al, Thromb Haemost 2001;86:1327-30)

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet count (*10⁹/L)</td>
<td>&gt;100</td>
<td>&lt;100</td>
<td>&lt;50</td>
<td></td>
</tr>
<tr>
<td>Fibrin related marker</td>
<td>No increase</td>
<td>Moderate increase</td>
<td>Strong increase</td>
<td></td>
</tr>
<tr>
<td>Prolonged prothrombin time</td>
<td>&lt;3 sec</td>
<td>&gt;3 but &lt;6 sec</td>
<td>&gt;6 sec</td>
<td></td>
</tr>
<tr>
<td>Fibrinogen level</td>
<td>&gt; 1.0 gram/l</td>
<td>&lt;1.0 gram/l</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Supplementary Table 2.** Thrombin generation values at baseline and after Fresh Frozen Plasma transfusion (12 ml/kg)

<table>
<thead>
<tr>
<th></th>
<th>Reference values</th>
<th>Before FFP transfusion N=27</th>
<th>After FFP transfusion N=27</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag time (min)</td>
<td>1.5-3.2</td>
<td>6.2 (4.6-8.0)</td>
<td>5.3 (4.5-7.2)</td>
<td>0.54</td>
</tr>
<tr>
<td>Peak (%)</td>
<td>63-154</td>
<td>58 (26-72)</td>
<td>64 (34-92)</td>
<td>0.03</td>
</tr>
<tr>
<td>Time to peak (min)</td>
<td>3.3-6.6</td>
<td>9.2 (6.9-11.5)</td>
<td>8.3 (6.8-11.0)</td>
<td>0.93</td>
</tr>
<tr>
<td>ETP (%)</td>
<td>65-146</td>
<td>72 (51-88)</td>
<td>71 (42-89)</td>
<td>0.27</td>
</tr>
</tbody>
</table>

Data expressed as median and interquartile ranges.

*FFP = fresh frozen plasma  ETP = endogenous thrombin potential*
Chapter 8

Effect of transfusion of fresh frozen plasma on parameters of endothelial condition and inflammatory status in non-bleeding critically ill patients: a prospective substudy of a randomized trial

Straat M, Muller MC, Meijers JC, Arbous MS, Spoelstra-de Man AM, Beurskens CJ, Vroom MB, Juffermans NP

ABSTRACT

Introduction Much controversy exists on the effect of a fresh frozen plasma (FFP) transfusion on systemic inflammation and endothelial damage. Adverse effects of FFP have been well described, including acute lung injury. However, it is also suggested that a higher amount of FFP decreases mortality in trauma patients requiring a massive transfusion. Furthermore, FFP has an endothelial stabilizing effect in experimental models. We investigated the effect of fresh frozen plasma transfusion on systemic inflammation and endothelial condition.

Material and Methods A prospective pre-defined sub-study of a randomized trial in coagulopathic non-bleeding critically ill patients receiving a prophylactic transfusion of FFP (12 ml/kg) prior to an invasive procedure. Levels of inflammatory cytokines and markers of endothelial condition were measured in paired samples of 33 patients before and after transfusion. Statistic tests used were paired t-test or the Wilcoxon signed rank test.

Results At baseline, systemic cytokine levels were mildly elevated in critically ill patients. FFP transfusion resulted in a decrease of levels of TNF-α (from 11.3 to 2.3 pg/ml, p=0.01). Other cytokines were not affected. FFP also resulted in a decrease in systemic syndecan-1 levels (from 675 to 565 pg/ml, p=0.01) and a decrease in factor VIII levels (from 246 to 246%, p<0.01), suggestive of an improved endothelial condition. This was associated with an increase in ADAMTS13 levels (from 24 to 32%, p<0.01) and a concomitant decrease in von Willebrand (vWF) levels (from 474 to 423 %, p<0.01).

Conclusions A fixed dose of FFP transfusion in critically ill patients decreases syndecan-1 and factor VIII levels, suggesting a stabilized endothelial condition, possibly by increasing ADAMTS13 which is capable of cleaving vWF.

Trial registration Trialregister.nl NTR2262, registered 26 March 2010 and Clinicaltrials.gov NCT01143909, registered 14 June 2010.

Acknowledgement The study was funded by ZonMw Netherlands, Organization for Health, Research and Development, The Hague, The Netherlands (Project 171002206).
INTRODUCTION

Substantial units of Fresh Frozen Plasma (FFP) are utilized in the intensive care unit (ICU)\(^1,2\). FFP is effective in correcting clotting factor deficiencies\(^3\) and is therefore transfused in patients with active bleeding, but also frequently in patients with abnormal coagulation tests to prevent bleeding\(^2,4\). In sepsis patients, FFP transfusion rates of up to 57% have been reported\(^5\). However, there is an association between FFP transfusion and adverse outcome in the critically ill, including transfusion-related acute lung injury (TRALI)\(^4,6-8\), transfusion-related circulatory overload\(^9,10\), multi-organ failure\(^8,11\) and an increased risk of infections\(^12\). Although not entirely understood, the pathological mechanisms underlying the association between FFP transfusion and lung injury is thought to result from an inflammatory response including a neutrophil influx into the lungs and elevated pulmonary levels of IL-8 and IL-1, as demonstrated in TRALI patients\(^13,14\). In line with this, FFP increased expression of endothelial adhesion molecules in human pulmonary endothelial cells\(^15\). Together, these data suggest that endothelial cell activation and disruption may be an early event following lung injury due to transfusion\(^16\).

On the other hand, FFP also seems to have protective effects. In trauma patients requiring a massive transfusion, resuscitation with a higher ratio of FFP to red blood cell units is associated with decreased mortality\(^17,18\). Interestingly, some studies suggest that this decreased mortality is irrespective of correction of coagulopathy by restoring coagulation factor levels\(^18,19\), although not all studies support this observation\(^20,21\). Instead, a beneficial effect of FFP may be related to the restoration of injured endothelium. Syndecan-1 is a proteoglycan on the luminal surface of endothelial cells which inhibits leukocyte adhesion. During endothelial damage, syndecan-1 is shed, resulting in increased levels of syndecan-1 in the systemic compartment\(^22\). Patients in hemorrhagic shock have a disrupted endothelial integrity and glycocalix layer, with decreased syndecan-1 expression\(^23\). Vascular integrity is also disrupted in various populations of critically ill patients, as demonstrated by increased systemic levels of syndecan-1\(^24,25\). Of interest, in a hemorrhagic shock model, FFP was found to improve endothelial integrity, associated with increased expression of syndecan-1 on endothelial cells\(^26\).

The effect of transfusion of FFP on endothelial and cytokine host response in patients is unknown. In a study investigating the risk-benefit ratio of FFP transfusion in non-bleeding critically ill patients with a coagulopathy, we investigated the inflammatory and endothelial host response to a fixed dose of FFP transfusion.
Chapter 8

METHODS

Study design
This was a predefined post-hoc sub-study of a multicenter trial in which non-bleeding critically ill patients with an increased International Normalized Ratio (INR, 1.5-3.0) were randomized between May 2010 and June 2013 to omitting or administering a prophylactic transfusion of FFP (12 ml/kg) prior to an invasive procedure. Only patients randomized to receive FFP were included in this sub-study. Patients were enrolled at 3 sites in The Netherlands: two university hospitals (Academic Medical Center, Amsterdam and Leiden University Medical Center, Leiden) and one large teaching hospital (Tergooi Ziekenhuizen, Hilversum). The Institutional Review Board of the Academic Medical Center approved the study protocol. Before entry in the study, written informed consent was obtained from the patient or legal representative in accordance with the Declaration of Helsinki. The study protocol was registered with trial identification numbers NTR2262 and NCT01143909.

Exclusion criteria were clinically overt bleeding, thrombocytopenia of < 30 x 10^9/L, treatment with vitamin K antagonists, activated protein C, abciximab, tirofiban, ticlopidine or prothrombin complex concentrates and a history of congenital or acquired coagulation factor deficiency or bleeding diathesis. Patients treated with low molecular weight heparin (LMWH) or heparin in therapeutic dose were eligible if medication was discontinued for an appropriate period. Sepsis was defined by the Bone criteria. Disseminated intravascular coagulation (DIC) was defined by an ISTH score of ≥ 5. The FFP was quarantine plasma manufactured by Sanquin, the Dutch National Bloodbank. As of 2007, women are deferred for donation for preparation of FFP in the Netherlands.

Sample collection
Citrated blood samples were drawn from an indwelling arterial catheter before and within 10 minutes after FFP transfusion. During transfusion, respiratory settings were kept constant. Samples were collected in sodium citrate (0.109M 3.2%) tubes and were centrifuged twice within 30 minutes: first 15 minutes at 2000 x g and then 5 minutes at 15000 x g, both at 18°C. Supernatant was collected and stored at -80°C.

Assays
Tumor necrosis factor-α (TNF-α) levels were measured by enzyme-linked immunosorbent assay (ELISA), according to instructions of the manufacturer (R&D Systems Inc., Minneapolis, MN, USA). Serum levels of IL-1β, IL-1RA, IL-8, IL-10, Macrophage Inflammatory Proteins (MIP)-1A, Monocyte Chemotactic Protein (MCP)–1 and soluble CD40 ligand (sCD40L) were determined by Luminex, according to instructions of the manufacturer (Merck Millipore Chemicals BV; Amsterdam; the Netherlands). When less than 50 beads were measured by the Luminex assay, samples were excluded from further analysis. VWF antigen (VWF:Ag) levels were de-
Effect of FFP on endothelial condition and inflammation in ICU patients
determined with an in-house ELISA using commercially available polyclonal antibodies against VWF (DAKO, Glostrup, Denmark). ADAMTS13 activity was determined as described earlier. Factor VIII activity was determined on a Behring XP coagulation analyzer using reagents and protocols from the manufacturer (Siemens Healthcare Diagnostics, Marburg, Germany).

**Statistical analysis**
Variables are expressed as medians and interquartile ranges or means and standard deviations. For comparisons, a paired t-test was used, or the Wilcoxon signed rank test in case of not normally distributed data. For the analyses, we used SPSS 21 (SPSS inc., Illinois, USA) and Graphpad Prism 5 (Graphpad software, San Diego, CA, USA).

**RESULTS**

**Patients**
From 38 patients receiving FFP, paired samples from 33 patients were available for analysis before and after FFP transfusion. Patients were ill, as reflected by a high disease severity score and half of the patients had sepsis (table 1). Patients received a mean dosage of 11.2 (2.8) ml/kg FFP, which was transfused in 121±43 minutes.

**Table 1. Patient characteristics.**

<table>
<thead>
<tr>
<th>General characteristics</th>
<th>FFP transfusion N=33</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender, male, n (%)</td>
<td>21 (64)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>61 (50-70)</td>
</tr>
<tr>
<td>APACHE IV score</td>
<td>96 (79-128)</td>
</tr>
<tr>
<td>SOFA score</td>
<td>11 (10-14)</td>
</tr>
<tr>
<td>Medical history</td>
<td></td>
</tr>
<tr>
<td>Pulmonary disease, n (%)</td>
<td>3 (9)</td>
</tr>
<tr>
<td>Liver disease, n (%)</td>
<td>6 (18)</td>
</tr>
<tr>
<td>Cardiac failure, n (%)</td>
<td>6 (18)</td>
</tr>
<tr>
<td>Medical condition 24 hours before transfusion</td>
<td></td>
</tr>
<tr>
<td>Mechanical ventilation, n (%)</td>
<td>27 (82)</td>
</tr>
<tr>
<td>Sepsis, n (%)</td>
<td>15 (45)</td>
</tr>
<tr>
<td>Disseminated intravascular coagulation, n (%)</td>
<td>16 (49)</td>
</tr>
<tr>
<td>Clinical outcomes</td>
<td></td>
</tr>
<tr>
<td>ICU length of stay</td>
<td>12 (6-23)</td>
</tr>
<tr>
<td>Mortality</td>
<td>21 (64)</td>
</tr>
</tbody>
</table>

Data expressed as median and interquartile ranges.
Inflammatory cytokine and chemokine levels before and after transfusion of 12 ml/kg FFP

At baseline, levels of cytokines were mildly elevated in this cohort. After FFP transfusion, median TNF-α decreased (p=0.01, table 2). Levels of all other cytokines were not affected by FFP transfusion. Chemokine levels IL-8 and MCP1 were elevated at baseline but also not influenced by FFP transfusion. Levels of sCD40L, which has been implicated as a mediator in TRALI 31, were also not significantly altered by FFP transfusion.

Parameters of endothelial condition before and after transfusion of 12 ml/kg FFP

After FFP transfusion, levels of ADAMTS13 increased (p<0.01, figure 1 and table 3). This increase was accompanied by a decrease in von Willebrand Factor (p<0.001) and in systemic levels of syndecan-1 (p=0.01). Factor VIII levels were slightly decreased following FFP transfusion (p=0.02).

Table 2. Inflammatory cytokines in critically ill patients before and after a transfusion of fresh frozen plasma (12 ml/kg).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Before FFP</th>
<th>After FFP</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pro-inflammatory parameters (pg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>11.3 (2.3 – 52.3)</td>
<td>2.3 (2.3 – 41.0)</td>
<td>0.01</td>
</tr>
<tr>
<td>IL1-β</td>
<td>15.0 (11.7 – 18.8)</td>
<td>14.4 (13.1 – 23.3)</td>
<td>0.97</td>
</tr>
<tr>
<td>IL-8</td>
<td>178 (124 – 418)</td>
<td>187 (113 – 412)</td>
<td>0.23</td>
</tr>
<tr>
<td>MCP1</td>
<td>1255 (503 – 3376)</td>
<td>1101 (434 – 5802)</td>
<td>0.89</td>
</tr>
<tr>
<td>MIP1A</td>
<td>19.6 (15.7 – 33.6)</td>
<td>19.1 (13.3 – 34.4)</td>
<td>0.12</td>
</tr>
<tr>
<td>sCD40L</td>
<td>409 (257 – 614)</td>
<td>324 (216 – 537)</td>
<td>0.08</td>
</tr>
<tr>
<td>anti-inflammatory parameters (pg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1RA</td>
<td>69.3 (52.1 – 110.6)</td>
<td>73.5 (47.8 – 104.9)</td>
<td>0.11</td>
</tr>
<tr>
<td>IL-10</td>
<td>36.1 (15.5 – 100.1)</td>
<td>31.5 (14.8 – 279.6)</td>
<td>0.62</td>
</tr>
</tbody>
</table>

Data expressed as median (IQR).

Table 3. Parameters of endothelial condition in critically ill patients before and after a transfusion of fresh frozen plasma (12 ml/kg).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Before FFP</th>
<th>After FFP</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>vWF-ag</td>
<td>474.0 (331.5 – 639.5)</td>
<td>423.0 (313.5 – 539.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Factor VIII</td>
<td>246.4 (203.6 – 364.4)</td>
<td>246.40 (321.2)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ADAMTS13</td>
<td>23.9 (15.8)</td>
<td>31.7 (17.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Syndecan-1</td>
<td>674.6 (132.2 – 1689.8)</td>
<td>565.1 (126.8 – 1175.7)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Data expressed as median (IQR) or mean (SD).

FFP = Fresh Frozen Plasma.
Our patients had mildly elevated levels of inflammatory cytokines at baseline, which corresponds to levels measured before in critically ill patients\textsuperscript{32}. We observed no aggravation of this inflammatory response after FFP transfusion. Rather, there was a decrease in TNF-α level. This is not in line with a study in which FFP elicited an inflammatory response in endothelial cells\textsuperscript{33}, nor with an \textit{in vitro} model of transfusion, in which whole blood incubated with FFP induced TNF-α production\textsuperscript{34}.

Of the cytokines we measured, only TNF-α changed after FFP transfusion. As TNF-α is known to be the quickest responder among all cytokines, we may have timed our measurement too early after FFP transfusion to note an effect of FFP on other cytokine levels.

**Figure 1.** Markers of endothelial condition in critically ill patients before and after a transfusion of fresh frozen plasma (12 ml/kg): ADAMTS13, Von Willebrandfactor, Factor VIII and Syndecan-1

**DISCUSSION**

Our patients had mildly elevated levels of inflammatory cytokines at baseline, which corresponds to levels measured before in critically ill patients\textsuperscript{32}. We observed no aggravation of this inflammatory response after FFP transfusion. Rather, there was a decrease in TNF-α level. This is not in line with a study in which FFP elicited an inflammatory response in endothelial cells\textsuperscript{33}, nor with an \textit{in vitro} model of transfusion, in which whole blood incubated with FFP induced TNF-α production\textsuperscript{34}.

Of the cytokines we measured, only TNF-α changed after FFP transfusion. As TNF-α is known to be the quickest responder among all cytokines, we may have timed our measurement too early after FFP transfusion to note an effect of FFP on other cytokine levels.
However, lung injury following transfusion is thought to be an early event. Also, we choose this early time point to minimize confounding by other factors. Taken together, FFP does not appear to elicit an early inflammatory response.

Of interest, recent in vitro studies support an endothelial stabilizing role of FFP, as FFP reduced vascular endothelial cell permeability and decreased expression of endothelial adhesion markers and endothelial white blood cell binding. Effects of FFP were investigated in a rat hemorrhagic shock model, characterized by systemic shedding of syndecan-1, decreased syndecan-1 expression on pulmonary cells and increased pulmonary vascular permeability. Resuscitation with FFP abrogated these effects, whereas resuscitation with crystalloids did not, and was associated with preservation of the endothelial glycocalyx and improvement of lung injury.

In trauma patients with hemorrhagic shock, syndecan-1 levels are also increased. Studies of the effect of FFP on endothelial condition in patients are however lacking. Of note, recent evidence in trauma patients requiring a massive transfusion suggests that higher dose and earlier administration of FFP decreases mortality. This effect was not associated with improved coagulation ability, as the reduction in mortality in their study was irrespective of the admission INR and coagulopathy does not seem to improve with higher amounts of FFP. Given that FFP restores coagulation factors but also anti-coagulant proteins and that the net effect on hemostasis is unclear, FFP may exert protection via other mechanisms. We found that FFP decreased levels of syndecan-1, associated with decreased levels of FVIII, which both reflect improved endothelial condition. These results support earlier experimental work indicating that FFP preserves endothelial integrity.

The mechanism underlying this beneficial effect of FFP has not yet been described. We found that FFP transfusion was associated with an increase in ADAMTS13 and a decrease in vWF. Thereby, ADAMTS13 may have increased the ability to cleave large vWF multimers present on the activated endothelium. As large vWF multimers damage the endothelium, this effect may have preserved endothelial condition. This is also the rationale behind the treatment of thrombotic thrombocytopenic purpura by therapeutic plasma exchange.

A protective effect of FFP on the endothelium is in apparent contrast with studies that have linked FFP to the occurrence of TRALI. In an effort to reconcile these findings, we suggest that FFP associated with TRALI occurs as a result of an antibody-mediated pathogenesis. Indeed, efforts to reduce antibody positive blood products by male only policies, are associated with a significant reduction in TRALI. In patients in whom transfusion is associated with lung injury in the absence of antibodies, other products such as red blood cells and platelets may be more important in inducing lung injury. Although
dissecting these effects in multiple transfused patients is a challenge, future research should focus on differential effects of the various blood products.

This study is limited by a small and heterogeneous patient population. Thereby, some of the effects may be caused by chance or by regression to the mean. Findings need to be confirmed in a larger sample. Strengths of this study are the use of a fixed dose of FFP and the timing of blood draws both prior and after transfusion, limiting a possible effect of confounders on findings. Thereby, long term effects of FFP were not investigated in this design.

**CONCLUSION**

In conclusion, this study is the first to describe the effect of a fixed dose of FFP transfusion in critically ill patients. Results suggest that FFP stabilizes endothelial condition.
Chapter 8

LITERATURE


35. Pati S, Matijevic N, Doursout MF, et al. Protective effects of fresh frozen plasma on vascular endothelial permeability, coagulation, and resuscitation after hemorrhagic shock are time dependent and diminish between days 0 and 5 after thaw. J Trauma 2011;69 Suppl 1:S55-63.


Chapter 9

Transfusion of platelets, but not of red blood cells, is independently associated with nosocomial bacterial infections in the critically ill

Engele LJ, Straat M, van Rooijen IHM, de Vooght KMK, Cremer OL, Schultz MJ, Bos LDJ, Juffermans NP; on behalf of the MARS Consortium

Submitted
ABSTRACT

Purpose Red blood cell (RBC) transfusion has been associated with nosocomial infection in the critically. However, this association may be confounded by length of stay, as prolonged Intensive Care Unit (ICU) stay increases both risk of infection as well as risk of transfusion. Also, it is not known whether specific blood products have differential risks.

Methods In this prospective multi-center cohort study, the risk of nosocomial bacterial infections associated with transfusion products in critically ill (ICU) patients was determined in an integrated statistical model, using cox proportional hazard analysis to account for attrition bias. In all acutely admitted patients with a length of stay of > 48 hours between January 1st 2011 and December 31st 2012, the occurrence of bacterial nosocomial infections in the ICU was prospectively monitored using CDC criteria.

Results Of 3,502 screened patients, 476 (13.6%) developed a nosocomial bacterial infection. These patients had higher APACHE IV scores, longer ICU length of stay and were more frequently transfused compared to patients without an infection. Logistic regression showed that RBC transfusion was a risk factor for nosocomial infection (odds ratio [OR]: 1.98, 95% confidence interval [CI]: 1.54–2.55, p<0.001), as well the number of RBC units transfused (OR: 1.04, 95%CI: 1.03–1.06, p<0.001). However, these associations disappeared in the cox proportional hazard analysis. In contrast, we found an association between plasma transfusion and infection (hazard ratio [HR]:1.36, 95%CI: 1.10-1.69, p=0.004) and between platelet transfusion and infection (HR: 1.46, 95%CI: 1.18-1.81, p<0.001). However, only platelet transfusion was associated with infection independently from other transfusion products (HR: 1.40, 95%CI: 1.03-1.90, p=0.03).

Conclusions In critically ill patients, transfusion of platelets, but not of RBCs and plasma, is an independent risk factor for acquiring a nosocomial bacterial infection.
INTRODUCTION

In medical and surgical critically ill patient populations, an increased risk of nosocomial bacterial infections following red blood cell (RBC) transfusion has repeatedly been demonstrated in observational studies [1-8]. The risk of infection following RBC transfusion has been related to the amount of transfused blood [1,2,7] as well as to RBC storage lesion, although studies show conflicting results on the association between storage duration and infection [2,4-6,8,9]. Given that the chance of receiving stored blood increases when the amount of transfusion is higher, it is a challenge to determine whether increased risk of infection following transfusion is due to storage or due to the number of transfused units. Regardless of the mechanism, the association between transfusion and infection can be confounded by length of stay in the Intensive Care Unit (ICU), because both the risk of receiving a (stored) blood transfusion as well as the risk of developing an infection increase during prolonged ICU stay. This is called attrition bias. Previous studies have not always accounted for this type of bias.

As RBCs are often administered together with plasma and platelets, another challenge may be to dissect whether these other blood product influence the risk of infection. This is relevant, as also platelet transfusions have been associated with postoperative infection in cardiac surgery patients [10] and in critically ill patients recovering from sepsis [11]. These immunosuppressive effects of transfused platelets have been related to alterations in their expression of MHC class I molecules [12]. Also plasma has been related to infectious complications. An observational study found that plasma increased the risk of infection in critically ill surgical patients, which was not due to concomitant RBC transfusion [13]. Also in cardiac surgery, FFP was associated with nosocomial infection [14].

The purpose of this prospective cohort study was to investigate the risk of nosocomial infections following transfusion in critically ill patients. We hypothesized that the number of blood products was independently associated with an increased risk of nosocomial infection. Cox regression was used to adjust for the effect of attrition bias. An integrated model was used to study the independent effect of the different blood products.

METHODS

Design and subjects
This study was performed within the framework of the Molecular Diagnosis and Risk stratification of Sepsis (MARS) project [15], a prospective observational cohort study performed in the mixed surgical-medical ICU of two university hospitals in the Netherlands, in which all admissions between the period of January 1st 2011 and December 31st 2012
were included, with the exception of cardiac surgery patients. The presence of bacterial nosocomial infection was the primary outcome of this study. The Medical Ethics Committees of the participating study centres gave approval for an opt-out consent method (IRB number 10-056C). Patients with an ICU stay of less than 48 hours were excluded.

Outcomes

All data were gathered prospectively by a team of trained research fellows, using an electronic format. Scoring of nosocomial infection was performed daily based on criteria adapted from the Centre of Disease Control that were published previously. These criteria included the source of infection, the causative pathogen and the plausibility of infection (none, possible, probable and definite). Detailed definitions of infection are given in a supplemental file. In this study, patients with a possible, probable or definite bacterial infection were included. The overall inter-observer variability for all sites of infection was 89%, as previously reported. A cleaning algorithm was used, that alarmed the researchers of inconsistency of data, after which a hand-check of the data was done. Use of this algorithm resulted in an increase in inter–observer agreement to 85% for ventilator-associated pneumonia (VAP), which accounted for 26% of the nosocomial infections in this study.

Risk factors for infection

Potential confounders were selected based on previous data and included mechanical ventilation, sepsis at ICU admission, Acute Physiology and Chronic Health Evaluation (APACHE) predicted length of stay, admission type, cancer, immunosuppressive medication, immunosuppressive condition and trauma.

As part of standard care, patients with an anticipated ICU stay of more than 2 days received either selective digestive tract decontamination (SDD, consisting of a non-absorbable solution of tobramycin, colistin and amphotericin B administered in the buccal cavity and as a suspension through the nasogastric tube, given for the length of ICU stay, combined with cefotaxime (given for 4 days) or selective oropharyngeal decontamination (SOD, consisting only of administration of the non-absorbable solution in the oropharynx). Transfusion data (date of infusion, type and amount of blood product) of all blood transfusions which were given during ICU stay and 1 week prior to ICU admission were prospectively registered in a patient digital monitoring system. The storage time of blood products was obtained from the hospital blood transfusion service. In patients who developed a nosocomial infection, only transfusions given before the onset of infection were evaluated. Per blood product, a qualitative variable (transfusion yes or no) and a quantitative variable (amount of product in units) were generated. Stratification for storage time was done dichotomous (>14 days and >21 days for RBC, >4 days for platelets). RBCs were stored in SAGM. Platelet products were pooled from 5
Platelets are associated with nosocomial bacterial infections in the ICU

Donors and stored in plasma. All blood products were prestorage leuko-reduced. Fresh frozen plasma (FFP) was prepared from male donors only.

Transfusion protocols were similar in both institutions, holding that one RBC unit is transfused to correct for anaemia at a general haemoglobin trigger of 7 g/dL. A unit of pooled platelets from 5 donors is transfused prophylactically at a platelet count of 10 x 10^9/L or of 50 x 10^9/L in case of use of antiplatelet medication. FFPs, RBCs and platelets are liberally transfused at a 1:1:1 ratio (i.e. five units erythrocytes: five units FFP: one 5-donor unit platelets) during haemorrhage.

**Statistical analysis**

We analysed the association between nosocomial infection and RBCs, FFPs or platelets separately. We corrected for potential confounders, the risk of receiving a transfusion (using propensity scores) and attrition bias (ICU observation period) using a five step approach described below and visualized in figure S1. First, we evaluated which features of the blood transfusions (qualitative; transfusion yes/no, quantitative; total number and/or the age of blood products) were associated with infection (details in figure S1, online supplement). A logistic regression model with nosocomial infection as the dependent variable was used and non-informative variables were removed using backward selection by Akaike Information Criterion (AIC) \(^30\). Second, the effect of transfusion was corrected for confounding effects (see online supplement). As in step one, we used backward selection to identify confounders from a group of predefined variables (see risk factors for infection). Third, propensity scores (for explanation; see online supplement) were calculated to estimate the influence of the a-priori likelihood of receiving a blood transfusion on the development of a nosocomial infection using a logistic regression model with transfusion as dependent variable. The propensity score was added as a co-variate in the logistic regression model with nosocomial infection as dependent variable. Fourth, we adjusted for the effect of attrition bias (ICU observation period) by means of Cox-regression. Cox-regression was performed with the same independent variables (transfusion features, confounders and propensity score) as in step 3. A Kaplan-Meijer curve was used to visualize the hazard of nosocomial infection per transfusion product. A sensitivity analysis with a mixed-effect Cox proportional hazards model was performed to evaluate differences in effect size between the two hospitals. The impact of missing data was studied by repeating the previous analyses after multivariate imputation by chained equations and comparing the proportional hazard between the model with and without missing data \(^31\). Finally, we studied the independent association of the three transfusion products with nosocomial infection by inclusion of all variables that were selected in the three previous models into one Cox proportional hazard model. Only features of transfusion that remained significantly associated with infection in this step were considered to be independently associated with nosocomial infection.
Differences between the groups were compared using the Student’s T-test (normally distributed variables) or Mann-Whitney test (non-normally distributed variables) for continuous variables. The Chi–square test was used for categorical variables. Data was summarized using the mean and standard deviation (SD) for normally distributed variables and the median and interquartile range (IQR) for non-normally distributed variables. Categorical variables were expressed in absolute numbers and percentages. All analyses were performed in R statistics using the R–studio interface (www.rstudio.com, version 0.98.501). P–values below 0.05 were considered significant.

RESULTS

Of 3,502 ICU patients, 37% were transfused with any blood product. Patients were most frequently transfused on the first day of ICU admission. With regard to the total cohort, 476 patients (13.6%) developed a nosocomial bacterial infection during their ICU stay. Characteristics summarized for the patients with infection and without an infection are shown in table 1. The median ICU stay of patients who developed a nosocomial bacterial infection was longer compared to patients who did not develop an infection (14 [IQR 8-26] days versus 4 [IQR 2-7] days, respectively). Furthermore, patients with an infection had a significant higher APACHE IV score compared to patients without an infection. The

<table>
<thead>
<tr>
<th>Table 1. Baseline characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Mean age, years ± SD</td>
</tr>
<tr>
<td>Male gender, n (%)</td>
</tr>
<tr>
<td>APACHE IV score, median (IQR)</td>
</tr>
<tr>
<td>Readmission n (%)</td>
</tr>
<tr>
<td>Admission type n (%)</td>
</tr>
<tr>
<td>Medical n (%)</td>
</tr>
<tr>
<td>Surgical elective n (%)</td>
</tr>
<tr>
<td>Surgical emergency n (%)</td>
</tr>
<tr>
<td>Any transfusion, n (%)</td>
</tr>
<tr>
<td>HIV infection, n (%)</td>
</tr>
<tr>
<td>Immunosuppressive medication, n (%)</td>
</tr>
<tr>
<td>Trauma n (%)</td>
</tr>
<tr>
<td>Mechanical ventilation n (%)</td>
</tr>
<tr>
<td>Sepsis n (%)</td>
</tr>
<tr>
<td>Malignancy n (%)</td>
</tr>
</tbody>
</table>

APACHE: Acute Physiology and Chronic Health Evaluation, HIV: human immunodeficiency virus, IQR: inter quartile range, SD: standard deviation
most frequent site of infection was the respiratory tract (51%). Gram-positive pathogens were the most frequent organisms found in nosocomial bacterial infections (table 2).

Overall, RBCs were given in 35% of admitted patients, FFP in 17% and platelets in 18% of patients. Most patients (46%) were transfused on the day of ICU admission. Transfusion descriptives are shown in table 3 and S1. Patients receiving transfusion had a higher APACHE IV score then non-transfused patients. There were no apparent differences between patients receiving RBCs and those receiving other blood products. The patients with infection more often received transfusion compared to the patients without infection, including more units stored for prolonged time.

Table 2. Site of infection and causative organisms in infected patients

<table>
<thead>
<tr>
<th>Infection site</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiratory tract (HAP, VAP, tracheobronchitis, empyema)</td>
<td>242 (42.0)</td>
</tr>
<tr>
<td>Intra-abdominal (peritonitis, biliary tract infection, IAI)</td>
<td>42 (8.8)</td>
</tr>
<tr>
<td>Cardiovascular (BSI, endo-, peri-, myocarditis, mediastinitis)</td>
<td>88 (18.5)</td>
</tr>
<tr>
<td>Soft tissue (erysipelas, phlebitis, abscess, decubitus infection)</td>
<td>12 (2.5)</td>
</tr>
<tr>
<td>Postoperative wound (superficial, deep)</td>
<td>11 (2.3)</td>
</tr>
<tr>
<td>Renal/urinary tract (urosepsis, upper urinary tract infection)</td>
<td>9 (1.9)</td>
</tr>
<tr>
<td>Central nervous system (brain abscess, meningitis, encefalitis)</td>
<td>44 (9.2)</td>
</tr>
<tr>
<td>Others (bones/joints, reproductive system, eye infections)</td>
<td>14 (2.9)</td>
</tr>
<tr>
<td>Unknown</td>
<td>10 (2.1)</td>
</tr>
</tbody>
</table>

Microorganisms

<table>
<thead>
<tr>
<th>Gram-positive</th>
<th>148 (31.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus</td>
<td>38 (8.0)</td>
</tr>
<tr>
<td>Staphylococcus epidermis</td>
<td>26 (5.5)</td>
</tr>
<tr>
<td>Streptococcus species</td>
<td>10 (2.1)</td>
</tr>
<tr>
<td>Enterococcus species</td>
<td>65 (13.7)</td>
</tr>
<tr>
<td>Other</td>
<td>9 (1.9)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gram-negative</th>
<th>139 (28.6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterobacteriaceae species</td>
<td>68 (14.3)</td>
</tr>
<tr>
<td>Haemophilus influenza</td>
<td>18 (3.8)</td>
</tr>
<tr>
<td>Pseudomonas species</td>
<td>40 (8.4)</td>
</tr>
<tr>
<td>Other</td>
<td>13 (2.7)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Other</th>
<th>10 (2.1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anaerobes</td>
<td>6 (1.3)</td>
</tr>
<tr>
<td>Unknown</td>
<td>173 (36.3)</td>
</tr>
</tbody>
</table>
Chapter 9

**Table 3. Transfusion characteristics**

<table>
<thead>
<tr>
<th>Patients</th>
<th>Overall (n=3502)</th>
<th>Infected (N=476)</th>
<th>Non-infected (N=3026)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC n (%)</td>
<td>1235 (35)</td>
<td>272 (57)</td>
<td>963 (32)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>RBC &gt; 14 days n (%)</td>
<td>1053 (30)</td>
<td>224 (47)</td>
<td>829 (27)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>RBC &gt; 21 days n (%)</td>
<td>762 (22)</td>
<td>161 (34)</td>
<td>601 (20)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>FFP n (%)</td>
<td>602 (17)</td>
<td>159 (33)</td>
<td>443 (15)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Platelets n(%)</td>
<td>621 (18)</td>
<td>157 (33)</td>
<td>464 (15)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Platelets &gt; 4 days n (%)</td>
<td>297 (8)</td>
<td>79 (17)</td>
<td>218 (7)</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time between (first) transfusion and infection (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC, median (IQR)</td>
</tr>
<tr>
<td>FFP, median (IQR)</td>
</tr>
<tr>
<td>Platelets, median (IQR)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Blood products in transfused patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall (n=1304)</td>
</tr>
<tr>
<td>RBC</td>
</tr>
<tr>
<td>n. of units, median (IQR)</td>
</tr>
<tr>
<td>n. of units &gt; 14 days, median (IQR)</td>
</tr>
<tr>
<td>n. of units &gt; 21 days, median (IQR)</td>
</tr>
<tr>
<td>FFP</td>
</tr>
<tr>
<td>n. of units, median (IQR)</td>
</tr>
<tr>
<td>Platelets</td>
</tr>
<tr>
<td>n. of units, median (IQR)</td>
</tr>
<tr>
<td>n. of units &gt; 4 days, median (IQR)</td>
</tr>
</tbody>
</table>

**Red Blood Cells**

The median storage time of RBC units in this study was 20 days (IQR: 16-23). 47% percent of the patients with infection received at least one RBC unit older than 14 days and 34% received at least one RBC unit older than 21 days. The patients with infection received a larger amount of RBC units and a larger amount of older RBC units compared to patients without infection (table 3).

Logistic regression identified both transfusion of RBC as a qualitative, dichotomous variable (yes or no) as well as amount of transfusion as independent predictors for nosocomial infection. This association remained significant after adjustment for potential confounders and exposure bias to transfusion (the risk of receiving a transfusion). However, the proportional hazard model showed no significant association between nosocomial infection and both RBC transfusion and amount of transfusion (table 4). We did not observe an independent association between the storage time (RBC units >14 and RBC units >21 days) of RBCs and onset of nosocomial infection (table S2).
Platelets are associated with nosocomial bacterial infections in the ICU

The logistic regression model identified FFP transfusion as a risk factor for nosocomial infection. This association persisted in the proportional hazard model (table 4). We found no association between the amount of FFP transfusion and nosocomial infection.

In the sub-analysis with platelets, both logistic regression model and cox regression showed that transfusion of platelets was an independent risk factor for the onset of nosocomial infection (figure 1) A hazard of 1.40 means that a subject’s hazard at any given time is increased relative to the baseline hazard with 40%, while the baseline hazard may vary.

In the combined analyses for the three different types of blood transfusions, only platelet transfusion remained as an independent risk factor for the development of nosocomial infection, i.e. independent from the effect of RBCs and plasma (table 5).

To evaluate differences in effect size between the two hospitals, mixed effect cox regressions was done, which showed similar effect sizes, suggesting stable models. The Cox models with imputed missing data showed a significant effect for FFP transfusion

### Table 4. Analysis of association between transfusion products and nosocomial infection in separate models

<table>
<thead>
<tr>
<th>Blood product</th>
<th>Logistic regression</th>
<th>Cox regression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Odds ratio</td>
<td>95% CI</td>
</tr>
<tr>
<td><strong>Red blood cells</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RBC transfusion*</td>
<td>1.977</td>
<td>1.535 - 2.547</td>
</tr>
<tr>
<td>RBC Units*</td>
<td>1.044</td>
<td>1.026 - 1.063</td>
</tr>
<tr>
<td><strong>Fresh Frozen Plasma</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FFP transfusion*</td>
<td>2.510</td>
<td>1.978 - 3.186</td>
</tr>
<tr>
<td><strong>Platelets</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelet transfusion*</td>
<td>2.530</td>
<td>1.998 - 3.205</td>
</tr>
</tbody>
</table>

* Corrected for exposure bias (for RBC transfusion) summarized in propensity score including trauma, malignancy, admission type, APACHE IV score and sepsis. Also corrected for confounders including APACHE predicted length of stay and mechanical ventilation.

* Corrected for exposure bias (for FFP transfusion) summarized in propensity score including admission type, APACHE IV score and sepsis. Also corrected for confounders including APACHE predicted length of stay and mechanical ventilation.

* Corrected for exposure bias (for Platelet transfusion) summarized in propensity score including admission type, malignancy, APACHE IV score and sepsis. Also corrected for confounders including APACHE predicted length of stay and mechanical ventilation.

Abbreviations: FFP: fresh frozen plasma, RBC: red blood cells

**FFP**

The logistic regression model identified FFP transfusion as a risk factor for nosocomial infection. This association persisted in the proportional hazard model (table 4). We found no association between the amount of FFP transfusion and nosocomial infection.

**Platelets**

In the sub-analysis with platelets, both logistic regression model and cox regression showed that transfusion of platelets was an independent risk factor for the onset of nosocomial infection (figure 1) A hazard of 1.40 means that a subject’s hazard at any given time is increased relative to the baseline hazard with 40%, while the baseline hazard may vary.

**Combined analysis**

In the combined analyses for the three different types of blood transfusions, only platelet transfusion remained as an independent risk factor for the development of nosocomial infection, i.e. independent from the effect of RBCs and plasma (table 5).

**Sensitivity analysis**

To evaluate differences in effect size between the two hospitals, mixed effect cox regressions was done, which showed similar effect sizes, suggesting stable models. The Cox models with imputed missing data showed a significant effect for FFP transfusion
Chapter 9

and platelets transfusion, suggesting that missing data did not influence results. The influence of pre-ICU transfusions was also investigated, as these may influence risk of infection while on ICU. Table S5 and S6 shows that the different outcomes were not sensitive to exclusion of the pre-ICU transfusion period.

**DISCUSSION**

This study did not demonstrate an association between RBC transfusion and nosocomial bacterial infection in ICU patients after adjusting for ICU length of stay. However, we found that transfusion of platelets was independently associated with nosocomial infection.
Differences between our results and previous studies \(^1,2,7,17\), which demonstrated an association between RBC transfusion and infection, may be explained by the use of different statistical analysis methods. Our results are based on both multivariate logistic regression and cox regression, whereas in previous studies, cox regression is often not used, thereby not accounting for exposure time. Correcting for exposure time in this study may have negated the finding of an association of RBC and infection, suggesting that both transfusion and infection are conditions that occur as a result of ICU stay but are not causally related. Alternatively, differences may be explained by using different models. Of note, plasma or platelets are mostly transfused concomitantly with RBCs. In this study, risks of blood products were analysed in one model instead of analysing effects of products separately, which is more comparable to real life. Explanations other than different statistical methods include differences in preparation methods and use of storage solutions. Also, differences in case mix may explain different findings, as most studies on transfusion and infection were done in trauma patients.

We did not find an association between RBC storage time and nosocomial infection. This is in contrast to other studies \(^3,6\), but in line with recent large trials in which ICU patients or cardiac surgery patients were randomized to fresh or stored blood \(^4,18\).

We found that the hazard ratio of infection was higher in patients receiving platelets, an effect that was independent of the other transfusion products. Besides their haemostatic potential, platelets are recognized to play a role in innate immunity, including activation and homing of leukocytes and production of pro-inflammatory cytokines \(^19\). In line with this, transfusion of platelets can induce TRIM in a mouse model, associated with the expression of platelets MHC Class I antigens \(^12\). Clinical studies also underline an association between platelet transfusion and infection \(^10,11,14\). Given that RBCs were not associated with occurrence of infection in the large group of patients that received only RBCs in this study, it is unlikely that RBCs contributed to the risk of infections when administered together with platelets. Alternatively, one may argue that patients receiving platelets are mostly hematological patients who are at risk of nosocomial infections. However, patient characteristics did not differ between those receiving platelets and those receiving other blood products. Thereby, this study suggests that platelet transfusions are the most important blood products associated with increased risk of infection in the critically ill.

Does this finding have clinical relevance? Arguably, if indicated, platelet transfusions cannot be omitted. However, clear indications for platelet transfusion in ICU patients are lacking. Prophylactic platelet transfusion prevents bleeding in hematology patients \(^20\), but it is not known at which count platelets should be transfused in ICU patients to prevent bleeding. Protocols at both centers pragmatically suggest a count of 10,000 as a trigger for transfusion in ICU patients and a trigger of 50,000 when the patient needs
treatment with anticoagulant medication. These suggestions are however not substantiated with data. The lack of data on clear indications together with the suggestion of possible harm in this study, calls for studies on the risk-benefit of platelet transfusion in the critically ill.

This study has some important limitations. A part of the patients complying to the definition of possible or probable infection may have been misclassified. However, the use of clear definitions limited subjectivity of classification, as suggested by a low inter-observer variation in this study. Statistical modelling using only patients with definite infection was not possible in this study due to a lack of power, even in this relatively large cohort. However, even if this analysis was possible, the use of a very strict definition (e.g. culture positive sepsis) may lead to selection bias, as the amount of culture negative patients is more then 1/3 of all sepsis patients.

Other limitations of this study include the limitation inherent to an observational study, including confounders not accounted for. In addition, as effects of transfusion may depend on preparation method, our results may not be translated to other settings where these methods differ. Furthermore, among ICU centres there is variability in ICU-specific factors, which can lead to heterogeneity in nosocomial infection hazard rates. Lastly, although we accounted for exposure bias which is a well-known confounder in studies investigating risk factors for nosocomial infection, standard Cox regression analysis is limited in correcting for time-dependent bias and competing risks. However, this study also has strong aspects. We investigated the association between transfusion products and nosocomial infection with a prospective study design, using a structured evaluation of all infectious events, to minimize potential sources of bias and confounding. In addition, our study is characterized by a large sample size. Furthermore, the independent effect on nosocomial infections was studied for each type of transfusion product separately.

In conclusion, we investigated the association between different transfusion products and the onset of nosocomial infection in critically ill patients. We suggest that transfusion of platelets, but not of RBCs, is associated with infection. Further studies on the mechanisms of this association and on possible interventions which may modulate this risk are warranted.
Platelets are associated with nosocomial bacterial infections in the ICU

LITERATURE


SUPPLEMENTARY APPENDIX TO: TRANSFUSION OF PLATELETS, BUT NOT OF RED BLOOD CELLS, IS INDEPENDENTLY ASSOCIATED WITH NOSOCOMIAL BACTERIAL INFECTIONS IN THE CRITICALLY ILL”

SUPPLEMENTAL METHODS

Propensity score
The a-priori likelihood of receiving a blood transfusion differs between ICU patients. In this study, in which we investigate the association between transfusion and infection, we corrected for all confounders of infection and also for the a-priori likelihood of transfusion. Variables which may influence the risk of receiving a blood transfusion were added to a logistic regression model for all blood products (with blood transfusion as dependent variable) to calculate a propensity score. This results in a likelihood, expressed as a probability between 0 and 1, for receiving the specific type of blood transfusion. This likelihood can be transformed into a continuous variable by transformation, as done commonly in logistic regression. This variable can now be used as a co-variate in the following models and expresses the risk of patient of receiving a certain type of transfusion, based on clinical data.

SUPPLEMENTAL TABLES

General

Table S1.1 Comparison of the combination of blood transfusion products in all transfused patients (n=1304)

<table>
<thead>
<tr>
<th>Transfusion products</th>
<th>RBC</th>
<th>Plasma</th>
<th>Platelets</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC</td>
<td>536 (41%)</td>
<td>132 (10.1%)</td>
<td>125 (9.5%)</td>
</tr>
<tr>
<td>Plasma</td>
<td>132 (10.1%)</td>
<td>14 (1.1%)</td>
<td>16 (1.2%)</td>
</tr>
<tr>
<td>Platelets</td>
<td>125 (9.5%)</td>
<td>16 (1.2%)</td>
<td>39 (3%)</td>
</tr>
<tr>
<td>Combination all three</td>
<td>440 (34%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table S1.2 Comparison of the combination of blood transfusion products in transfused patients with an infection (n=288)

<table>
<thead>
<tr>
<th>Transfusion products</th>
<th>RBC</th>
<th>Plasma</th>
<th>Platelets</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC</td>
<td>97 (33.7%)</td>
<td>29 (10.1%)</td>
<td>24 (8.3%)</td>
</tr>
<tr>
<td>Plasma</td>
<td>29 (10.1%)</td>
<td>5 (1.7%)</td>
<td>3 (1%)</td>
</tr>
<tr>
<td>Platelets</td>
<td>24 (8.3%)</td>
<td>3 (1%)</td>
<td>8 (2.8%)</td>
</tr>
<tr>
<td>Combination all three</td>
<td>122 (42.4%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table S1.3  *Patient characteristics according to transfusion product.*

<table>
<thead>
<tr>
<th>Transfusion product</th>
<th>any RBC (n=1233)</th>
<th>any Plasma (n=602)</th>
<th>any Platelets (n=620)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age, years ± SD</td>
<td>60.1 ± 16.2</td>
<td>60.6 ± 16.8</td>
<td>59.2 ± 16.3</td>
</tr>
<tr>
<td>APACHE IV score, median (IQR)</td>
<td>81 (64-103)</td>
<td>80 (62-103)</td>
<td>81 (63-105)</td>
</tr>
<tr>
<td>Immunosuppressive medication, n(%)</td>
<td>157 (13)</td>
<td>61 (10)</td>
<td>87 (14)</td>
</tr>
<tr>
<td>Trauma, n(%)</td>
<td>98 (8)</td>
<td>62 (10)</td>
<td>50 (8)</td>
</tr>
<tr>
<td>Malignancy, n(%)</td>
<td>238 (19)</td>
<td>94 (16)</td>
<td>124 (20)</td>
</tr>
<tr>
<td>Sepsis, n(%)</td>
<td>606 (49)</td>
<td>232 (39)</td>
<td>256 (41)</td>
</tr>
<tr>
<td>Mechanical ventilation, n(%)</td>
<td>1146 (93)</td>
<td>575 (96)</td>
<td>587 (95)</td>
</tr>
</tbody>
</table>

Red blood cell transfusion

Table S2.1  *Logistic regression with selection of most relevant transfusion characteristics*

<table>
<thead>
<tr>
<th>Variables removed</th>
<th>AIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC &gt;14 days</td>
<td>-0.56</td>
</tr>
<tr>
<td>RBC &gt;21 days</td>
<td>-1.99</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variables in model</th>
<th>Regression coefficient</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC transfusion</td>
<td>0.717</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RBC units</td>
<td>0.048</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Table S2.2  *Logistic regression with correction for confounders. Selection of most relevant clinical characteristics*

<table>
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<th>AIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Admission type</td>
<td>-1.85</td>
</tr>
<tr>
<td>Immunosuppressive condition</td>
<td>-3.17</td>
</tr>
<tr>
<td>Immunosuppressive medication</td>
<td>-4.98</td>
</tr>
<tr>
<td>Trauma</td>
<td>-5.37</td>
</tr>
<tr>
<td>Malignancy</td>
<td>-5.13</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variables in model</th>
<th>Regression coefficient</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC transfusion</td>
<td>0.729</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RBC units</td>
<td>0.045</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mechanical ventilation</td>
<td>0.746</td>
<td>0.002</td>
</tr>
<tr>
<td>APACHE IV predicted length of stay</td>
<td>0.138</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Table S2.3 Calculation of propensity score for RBC transfusion and addition of the propensity score to the model

<table>
<thead>
<tr>
<th>Variables removed from propensity score</th>
<th>AIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variables in propensity score</th>
<th>Regression coefficient</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Admission type</td>
<td>0.445</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Trauma</td>
<td>0.148</td>
<td>0.071</td>
</tr>
<tr>
<td>Malignancy</td>
<td>0.347</td>
<td>0.001</td>
</tr>
<tr>
<td>APACHE IV score</td>
<td>0.020</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sepsis</td>
<td>0.227</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variables</th>
<th>Odds ratio</th>
<th>95% Confidence interval</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propensity score</td>
<td>1.157</td>
<td>0.993 - 1.378</td>
<td>0.061</td>
</tr>
<tr>
<td>RBC transfusion</td>
<td>1.977</td>
<td>1.535 - 2.547</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>RBC units</td>
<td>1.044</td>
<td>1.026 - 1.063</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Mechanical ventilation</td>
<td>2.034</td>
<td>1.266 - 3.270</td>
<td>0.003</td>
</tr>
<tr>
<td>APACHE IV Predicted length of stay</td>
<td>1.141</td>
<td>1.077 - 1.210</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Table S2.4 Cox proportional hazard model with the previously selected variables

<table>
<thead>
<tr>
<th>Variables</th>
<th>Hazard</th>
<th>95% confidence interval</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propensity score</td>
<td>1.162</td>
<td>1.004 - 1.345</td>
<td>0.044</td>
</tr>
<tr>
<td>RBC transfusion</td>
<td>1.143</td>
<td>0.906 - 1.442</td>
<td>0.259</td>
</tr>
<tr>
<td>RBC units</td>
<td>1.014</td>
<td>1.000 - 1.028</td>
<td>0.053</td>
</tr>
<tr>
<td>Mechanical ventilation</td>
<td>1.281</td>
<td>0.811 - 2.022</td>
<td>0.288</td>
</tr>
<tr>
<td>APACHE IV Predicted length of stay</td>
<td>0.982</td>
<td>0.931 - 1.036</td>
<td>0.504</td>
</tr>
</tbody>
</table>

Fresh Frozen Plasma transfusion

Table S3.1 Logistic regression with selection of most relevant transfusion characteristics

<table>
<thead>
<tr>
<th>Variables removed</th>
<th>AIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variables in model</th>
<th>Regression coefficient</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FFP transfusion</td>
<td>0.822</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FFP units</td>
<td>0.031</td>
<td>0.005</td>
</tr>
</tbody>
</table>
### Table S3.2 Logistic regression with correction for confounders. Selection of most relevant clinical characteristics

<table>
<thead>
<tr>
<th>Variables removed</th>
<th>AIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunosuppressive condition</td>
<td>-2.00</td>
</tr>
<tr>
<td>Admission type</td>
<td>-3.97</td>
</tr>
<tr>
<td>Malignancy</td>
<td>-5.45</td>
</tr>
<tr>
<td>Immunosuppressive medication</td>
<td>-6.93</td>
</tr>
<tr>
<td>Trauma</td>
<td>-7.38</td>
</tr>
<tr>
<td>FFP units</td>
<td>-4.34</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variables in model</th>
<th>Regression coefficient</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FFP transfusion</td>
<td>1.053</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mechanical ventilation</td>
<td>0.765</td>
<td>0.001</td>
</tr>
<tr>
<td>APACHE IV predicted length of stay</td>
<td>0.161</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

### Table S3.3 Calculation of propensity score for FFP transfusion and addition of the propensity score to the model

<table>
<thead>
<tr>
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<th>AIC</th>
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</thead>
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<tr>
<td>Malignancy</td>
<td>-1.58</td>
</tr>
<tr>
<td>Trauma</td>
<td>-0.65</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variables in propensity score</th>
<th>Regression coefficient</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Admission type</td>
<td>0.707</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>APACHE IV score</td>
<td>0.017</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sepsis</td>
<td>-0.263</td>
<td>0.011</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variables</th>
<th>Odds ratio</th>
<th>95% confidence interval</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propensity score</td>
<td>1.294</td>
<td>1.120 - 1.494</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>FFP transfusion</td>
<td>2.510</td>
<td>1.978 - 3.186</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Mechanical ventilation</td>
<td>1.950</td>
<td>1.214 - 3.132</td>
<td>0.006</td>
</tr>
<tr>
<td>APACHE IV Predicted length of stay</td>
<td>1.171</td>
<td>1.106 - 1.240</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

### Table S3.4 Cox proportional hazard model with the previously selected variables

<table>
<thead>
<tr>
<th>Variables</th>
<th>Hazard</th>
<th>95% confidence interval</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propensity score</td>
<td>1.347</td>
<td>1.180 - 1.537</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>FFP transfusion</td>
<td>1.362</td>
<td>1.101 - 1.685</td>
<td>0.004</td>
</tr>
<tr>
<td>Mechanical ventilation</td>
<td>1.154</td>
<td>0.730 - 1.824</td>
<td>0.541</td>
</tr>
<tr>
<td>APACHE IV Predicted length of stay</td>
<td>0.996</td>
<td>0.945 - 1.050</td>
<td>0.881</td>
</tr>
</tbody>
</table>

Platelet transfusion
Platelets are associated with nosocomial bacterial infections in the ICU

**Platelet transfusion**

**Table S4.1 Logistic regression with selection of most relevant transfusion characteristics**

<table>
<thead>
<tr>
<th>Variables removed</th>
<th>AIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLT &gt;3 days</td>
<td>-1.66</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variables in model</th>
<th>Regression coefficient</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLT transfusion</td>
<td>0.829</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PLT units</td>
<td>0.045</td>
<td>0.026</td>
</tr>
</tbody>
</table>

**Table S4.2 Logistic regression with correction for confounders. Selection of most relevant clinical characteristics**

<table>
<thead>
<tr>
<th>Variables removed</th>
<th>AIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Admission type</td>
<td>-1.71</td>
</tr>
<tr>
<td>Immunosuppressive medication</td>
<td>-3.26</td>
</tr>
<tr>
<td>Immunosuppressive condition</td>
<td>-4.94</td>
</tr>
<tr>
<td>Malignancy</td>
<td>-4.73</td>
</tr>
<tr>
<td>PLT units</td>
<td>-4.83</td>
</tr>
<tr>
<td>Trauma</td>
<td>-4.22</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variables in model</th>
<th>Regression coefficient</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLT transfusion</td>
<td>1.054</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mechanical ventilation</td>
<td>0.786</td>
<td>0.001</td>
</tr>
<tr>
<td>APACHE IV predicted length of stay</td>
<td>0.163</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

**Table S4.3 Calculation of propensity score for platelet transfusion and addition of the propensity score to the model**

<table>
<thead>
<tr>
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<th>AIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trauma</td>
<td>-1.92</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variables in propensity score</th>
<th>Regression coefficient</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malignancy</td>
<td>0.433</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Admission type</td>
<td>0.593</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>APACHE IV score</td>
<td>0.019</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sepsis</td>
<td>-0.248</td>
<td>0.015</td>
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</table>

<table>
<thead>
<tr>
<th>Variables</th>
<th>Odds ratio</th>
<th>95% confidence interval</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propensity score</td>
<td>1.308</td>
<td>1.126 - 1.520</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>PLT transfusion</td>
<td>2.530</td>
<td>1.998 - 3.205</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Mechanical ventilation</td>
<td>1.984</td>
<td>1.235 - 3.187</td>
<td>0.005</td>
</tr>
<tr>
<td>APACHE IV Predicted length of stay</td>
<td>1.174</td>
<td>1.109 - 1.244</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>
**Table S4.4. Cox proportional hazard model with the previously selected variables**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Hazard</th>
<th>95% confidence interval</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propensity score</td>
<td>1.297</td>
<td>1.130 - 1.489</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>PLT transfusion</td>
<td>1.463</td>
<td>1.184 - 1.806</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Mechanical ventilation</td>
<td>1.205</td>
<td>0.763 - 1.904</td>
<td>0.423</td>
</tr>
<tr>
<td>APACHE IV Predicted length of stay</td>
<td>1.000</td>
<td>0.948 - 1.054</td>
<td>0.989</td>
</tr>
</tbody>
</table>

Influence of pre-ICU transfusions

**Table S5. Logistic and cox regression model without pre-ICU blood transfusions for each type of transfusion separately.**

<table>
<thead>
<tr>
<th>Blood products</th>
<th>Logistic regression</th>
<th>Cox regression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Odds ratio</td>
<td>95% CI</td>
</tr>
<tr>
<td><strong>Red blood cells</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RBC transfusiona</td>
<td>2.080</td>
<td>1.620 - 2.670</td>
</tr>
<tr>
<td>RBC Unitsa</td>
<td>1.041</td>
<td>1.022 - 1.060</td>
</tr>
<tr>
<td><strong>Fresh Frozen Plasma</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FFP transfusionb</td>
<td>2.542</td>
<td>1.990 - 3.191</td>
</tr>
<tr>
<td><strong>Platelets</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelet transfusionc</td>
<td>1.610</td>
<td>2.131 - 3.168</td>
</tr>
</tbody>
</table>

*a Corrected for exposure bias summarized in propensity score including trauma, admission type, sepsis, malignancy and APACHE IV score. And corrected for confounders including APACHE predicted length of stay and mechanical ventilation.

*b Corrected for exposure bias summarized in propensity score including admission type, APACHE IV score and sepsis. And corrected for confounders including APACHE predicted length of stay and mechanical ventilation.

*c Corrected for exposure bias summarized in propensity score including admission type, sepsis, malignancy and APACHE IV score. And corrected for confounders including APACHE predicted length of stay and mechanical ventilation.

Abbreviations: FFP: fresh frozen plasma, RBC: red blood cells
Table S6. Analysis of independent transfusion risk factors for nosocomial infection without pre-ICU blood transfusions

<table>
<thead>
<tr>
<th>Blood products</th>
<th>Cox regression</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hazard ratio</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red blood cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RBC transfusion</td>
<td>1.114</td>
<td>0.867 - 1.431</td>
<td>0.398</td>
</tr>
<tr>
<td>RBC Units</td>
<td>0.991</td>
<td>0.973 - 1.010</td>
<td>0.340</td>
</tr>
<tr>
<td>Fresh Frozen Plasma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FFP transfusion</td>
<td>1.092</td>
<td>0.806 – 1.480</td>
<td>0.569</td>
</tr>
<tr>
<td>Platelets</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelet transfusion</td>
<td>1.482</td>
<td>1.098 – 2.002</td>
<td>0.030</td>
</tr>
</tbody>
</table>

Corrected for exposure bias summarized in propensity scores of the individual transfusion products and corrected for confounders including APACHE predicted length of stay and mechanical ventilation.
Abbreviations: FFP: fresh frozen plasma, RBC: red blood cells

SUPPLEMENTAL FIGURES

Figure S1. Schematic representation of the statistical analyses
SUPPLEMENTAL DEFINITIONS OF INFECTION

Contents

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Hospital Acquired Pneumonia
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Lung abscess and empyema

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Sinusitis
Community acquired faryngitis, laryngitis, epiglottitis, retrofaryngeal abscess

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Bloodstream infection (BSI): Secondary BSI (CRBI- catheter related bloodstream infec-
tion)
Endocarditis
Myocarditis and pericarditis
Mediastinitis

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Secundaire meningitis
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Urosepsis in catheterized patients
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retroperitoneal or perinephric spaces)

Skin and soft tissue
Skin infection, erysipelas, phlebitis
Surgical site infections: Superficial wounds
Surgical site infections: Deep wounds
Soft tissue infections
Decubitus infections
Mastitis

_Infections of the GI tract_
Intra-abdominal infection/abscess (IAI
Pancreatic infection, infected necrotizing pancreatitis
Biliary tract infection
Primary peritonitis (spontaneous bacterial peritonitis)
Secondary peritonitis
Tertiary peritonitis
Peritoneal dialysis-related peritonitis
Gastroenteritis
Viral hepatitis
Necrotizing enterocolitis (typhilitis)

_Infections of the reproductive system_
Endometritis, epididymitis, prostatitis

_Bones and joints_
Osteomyelitis
Discitis
Bursitis

_Eye_
Conjunctivitis
Endophtalmitis

_Ear_
Ear

_Infections of mouth, tongue or gums_
Infections of mouth, tongue or gums

_Other definitions_
CPIS
### Lower respiratory tract

**Community Acquired Pneumonia**

**Clinical setting** Patients presenting with respiratory symptoms within 48 hours of hospital admission

<table>
<thead>
<tr>
<th>Possible</th>
<th>Abnormal chest radiograph of uncertain cause and low clinical suspicion of pneumonia with at least one of the following symptoms/signs:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a) cough</td>
</tr>
<tr>
<td></td>
<td>b) new onset of purulent sputum or change in character of sputum</td>
</tr>
<tr>
<td></td>
<td>c) fever or hypothermia</td>
</tr>
<tr>
<td></td>
<td>d) leukocytosis</td>
</tr>
<tr>
<td></td>
<td>e) elevated CRP (&gt;30 mg/l)</td>
</tr>
<tr>
<td></td>
<td>f) hypoxemia (pO2&lt;60 mmHg)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Probable</th>
<th>Evident new or progressive radiographic infiltrate, consolidation, cavitation, or pleural effusion and high clinical suspicion of pneumonia with at least two of the criteria at possible and one or more of the following:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a) rales or dullness to percussion on physical examination of the chest</td>
</tr>
<tr>
<td></td>
<td>b) positive rapid diagnostic tests such as Legionella or pneumococcal</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Definite</th>
<th>Evident new or progressive radiographic infiltrate, consolidation, cavitation, or pleural effusion and high clinical suspicion of pneumonia and at least two of the criteria at probable and isolation of a likely pulmonary pathogen, with at least one of the following symptoms/signs:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a) pathogen cultured from blood</td>
</tr>
<tr>
<td></td>
<td>b) pathogen in high concentration from a quantitative lower respiratory tract sample</td>
</tr>
<tr>
<td></td>
<td>c) isolation of virus from or detection of viral antigen in respiratory secretions</td>
</tr>
<tr>
<td></td>
<td>d) diagnostic single antibody titer (IgM) or fourfold increase in paired sera (IgG) for pathogen</td>
</tr>
<tr>
<td></td>
<td>e) histopathologic evidence of pneumonia</td>
</tr>
</tbody>
</table>

**Comment** Sputum cultures are not useful in the diagnosis of pneumonia but may help to identify the etiologic agent and provide antimicrobial susceptibility data
<table>
<thead>
<tr>
<th>Hospital Acquired Pneumonia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical setting</td>
</tr>
</tbody>
</table>
| Possible                    | 1. Abnormal chest radiograph of uncertain cause and low clinical suspicion of pneumonia with at least one of the following symptoms/signs:  
  a) cough  
  b) new onset of purulent sputum or change in character of sputum  
  c) fever or hypothermia  
  d) leukocytosis  
  e) elevated CRP (>30 mg/l)  
  f) hypoxemia (pO2<60 mmHg) |
| Probable                    | 1. Evident new or progressive radiographic infiltrate, consolidation, cavitation, or pleural effusion and high clinical suspicion of pneumonia with at least two of the criteria at possible and one or more of the following:  
  a) isolation of an etiologic agent from a specimen obtained by transtracheal aspirate, bronchial brushing, or biopsy  
  b) likely/possible respiratory pathogen in concentrations below threshold (104 in BAL and 103 in protected specimen brush) using quantitative cultures of a lower respiratory tract sample (endotracheal aspirate, BAL, or protected specimen brush) |
| Definite                    | 1. Evident new or progressive radiographic infiltrate, consolidation, cavitation, or pleural effusion and high clinical suspicion of pneumonia with at least two of criteria at probable and at least one of the following:  
  a) likely/possible respiratory pathogen cultured from blood;  
  b) likely/possible respiratory pathogen in concentrations above threshold (104 in BAL and 103 in protected specimen brush) using quantitative cultures of a lower respiratory tract sample  
  c) isolation of virus from or detection of viral antigen in respiratory secretions  
  d) diagnostic single antibody titer (IgM) or fourfold increase in paired sera (IgG) for pathogen  
  e) histopathologic evidence of pneumonia |
| Comment                     | Sputum cultures are not useful in the diagnosis of pneumonia but may help identify the etiologic agent and provide antimicrobial susceptibility data |
### Ventilator-Associated Pneumonia

<table>
<thead>
<tr>
<th>Clinical setting</th>
<th>Possible</th>
<th>Probable</th>
<th>Definite</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical setting</strong></td>
<td>Pneumonia in patients who had a device to assist or control respiration continuously through a tracheostomy or by endotracheal intubation within the 48-hour period before the onset of infection, inclusive of the weaning period</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Possible</strong></td>
<td>1. Abnormal chest radiograph of uncertain cause and high clinical suspicion of pneumonia with CPIS &gt; 6 and isolation of an etiologic agent from a specimen obtained by transtracheal aspirate or bronchial brushing</td>
<td>1. Evident new or progressive radiographic infiltrate, consolidation, cavitation, or pleural effusion and high clinical suspicion of pneumonia with CPIS &gt; 6 and at least one of the following: a) likely/possible respiratory pathogen in concentrations above threshold (10⁴ in BAL and 10³ in protected specimen brush) using quantitative cultures of a lower respiratory tract sample b) likely/possible respiratory pathogen cultured from blood and lower respiratory tract sample</td>
<td>1. Evident new or progressive radiographic infiltrate, consolidation, cavitation, or pleural effusion and high clinical suspicion of pneumonia with CPIS &gt; 6 and at least one of the following: a) histopathologic evidence of pneumonia b) radiographic evidence of lung abscess or pleural empyema and isolation of an etiologic agent from a specimen</td>
</tr>
<tr>
<td><strong>Probable</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Definite</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

### Tracheobronchitis, bronchitis, tracheitis

<table>
<thead>
<tr>
<th>Clinical setting</th>
<th>Possible</th>
<th>Probable</th>
<th>Definite</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical setting</strong></td>
<td>Patients presenting with respiratory symptoms but without clinical or radiographic evidence of pneumonia</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Possible</strong></td>
<td>1. At least two of the following signs or symptoms with no other recognized cause: a) fever (&gt;38°C) b) leukocytosis or leucopenia c) elevated CRP (&gt;30 mg/L) d) new onset or increased purulent sputum and absence of radiographic infiltrate on X-chest</td>
<td>N.A.</td>
<td></td>
</tr>
<tr>
<td><strong>Probable</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Definite</strong></td>
<td>1. See criteria at possible and isolation of an etiologic agent from a specimen obtained by transtracheal aspirate, bronchial brushing, or biopsy</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Platelets are associated with nosocomial bacterial infections in the ICU

**Lung abscess and empyema**

**Clinical setting**: Patients presenting with respiratory symptoms and clinical or radiographic evidence of abscess.

**Possible**

1. Low clinical suspicion, with at least one of the following signs or symptoms with no other recognized cause:
   a) fever
   b) cough, sputum production

   **and** low suspicion for abscess on radiographic examination

**Probable**

1. See criteria at possible

   **and** high suspicion for abscess on radiographic examination

**Definite**

1. See criteria at probable

   **and** drainage of pus from suspected lung abscess or empyema by puncture or surgical operation. The pus has to be clinically evident or confirmed with biochemical or histopathologic procedures.

   **and** confirmed etiologic agent visible in gram staining or pathogen isolated from pus culture

**Upper Respiratory Tract**

**Sinusitis**

**Possible**

1. Patient with clinical suspicion for sinusitis with at least one of the following signs or symptoms with no other recognized cause:
   a) fever (>38°C)
   b) leukocytosis

**Probable**

1. See criteria at possible

   **and** at least one of the following criteria:
   a) positive transillumination with air-fluid level;
   b) radiologically suspected for sinusitis (CT, ultrasound)

**Definite**

1. See criteria at probable

   **and**

   a) positive culture (>1000 colonies/ml) of purulent discharge from sinus cavity plus > 5 PMN per oil immersion field

   b) in case antibiotics are prescribed: > 5 PMN per oil immersion field
Faryngitis, laryngitis, epiglottitis, retrofaryngeal abscess

**Possible**
1. Patient with clinical suspicion for the above infections with at least one of the following signs or symptoms with no other recognized cause:
   a) fever (>38°C)
   b) red, sore throat
   c) cough
   d) hoarseness
   e) purulent exudate in throat

**Probable**
1. See criteria at possible
   and trismus
   and positive blood culture

**Definite**
1. Abscess observed directly or histopathologically, during surgery or histopathologic examination
2. See criteria at probable
   and positive culture of affected body part

Cardiovascular

**Bloodstream infection (BSI): Primary BSI**

**Clinical setting**
Bloodstream infection in a patient without an evident focus

**Possible**
N.A.

**Probable**
N.A.

**Definite**
1. At least one of the following:
   a) patient has a recognized pathogen (defined as a microorganism not usually regarded as a common skin contaminant, i.e., diphtheroids, Bacillus species, Propionibacterium species, coagulase-negative staphylococci, or micrococci) cultured from one or more blood cultures
   b) a common skin contaminant (e.g., diphtheroids, Bacillus species, Propionibacterium species, coagulase-negative staphylococci, or micrococci) cultured from two or more blood cultures drawn on separate occasions (including one drawn by venipuncture)
   and the organism cultured from blood is not related to an infection at another site, including intravascular-access devices
### Bloodstream infection (BSI): Secondary BSI (CRBI- catheter related bloodstream infection)

<table>
<thead>
<tr>
<th>Clinical setting</th>
<th>Bloodstream infection in a patient with one or more intravascular-access devices for more than 72 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Possible</strong></td>
<td>1. Negative blood culture: culture of blood obtained by venapuncture or other catheter line is negative</td>
</tr>
<tr>
<td></td>
<td>and suspected catheter line with one or more of the following criteria:</td>
</tr>
<tr>
<td></td>
<td>• In case catheter is removed:</td>
</tr>
<tr>
<td></td>
<td>a) catheter tip is positive</td>
</tr>
<tr>
<td></td>
<td>b) culture of catheter not performed because fever disappears within 48 hours after catheter line removal</td>
</tr>
<tr>
<td></td>
<td>• In case a catheter is present:</td>
</tr>
<tr>
<td></td>
<td>a) recovery of fever within 48 hours after start of antibiotic treatment</td>
</tr>
<tr>
<td></td>
<td><strong>Probable</strong> 1. Clinical signs of infection with at least one of the following criteria:</td>
</tr>
<tr>
<td></td>
<td>a) fever (&gt;38°C)</td>
</tr>
<tr>
<td></td>
<td>b) chills</td>
</tr>
<tr>
<td></td>
<td>c) hypotension (systolic pressure &lt;100 mmHg) or need for vasopression</td>
</tr>
<tr>
<td></td>
<td>and positive or unknown blood culture: culture of peripheral blood (venapuncture) or blood obtained from other catheter line is positive, or no culture</td>
</tr>
<tr>
<td></td>
<td>and suspected catheter line with at least one of the following criteria:</td>
</tr>
<tr>
<td></td>
<td>• In case catheter is removed:</td>
</tr>
<tr>
<td></td>
<td>a) catheter tip is positive for same pathogen that was recovered from blood culture (or positive for common skin contaminant if no paired blood culture);</td>
</tr>
<tr>
<td></td>
<td>b) culture of catheter not performed because fever disappears within 48 hours after catheter line removal</td>
</tr>
<tr>
<td></td>
<td>• In case catheter is present:</td>
</tr>
<tr>
<td></td>
<td>a) recovery of fever within 48 hours after start of antibiotic treatment</td>
</tr>
<tr>
<td></td>
<td><strong>Definite</strong> 1. Clinical signs of infection (see at probable)</td>
</tr>
<tr>
<td></td>
<td>and positive blood culture: culture of peripheral blood (venapuncture) or blood obtained from other catheter line is positive</td>
</tr>
<tr>
<td></td>
<td>and positive catheter tip culture with same pathogen (i.e., catheter colonization), whereby the same microorganism (species and antibiogram) is isolated from the catheter segment</td>
</tr>
<tr>
<td></td>
<td>and peripheral blood and confirmed absence of other sources with same pathogen</td>
</tr>
</tbody>
</table>
## Endocarditis

<table>
<thead>
<tr>
<th>Clinical setting</th>
<th>Patients presenting with SIRS/sepsis without an evident clinical focus, or with persistent SIRS/sepsis despite adequate therapy for any suspected alternative source</th>
</tr>
</thead>
</table>

### Modified Duke criteria

#### Major criteria include:

1. **Positive blood culture with typical IE microorganism, defined as one of the following:**
   - **Typical microorganism consistent with IE from 2 separate blood cultures, as noted below:** Viridans-group streptococci, or Streptococcus bovis including nutritional variant strains, or HACEK group, or Staphylococcus aureus, or Community-acquired Enterococci, in the absence of a primary focus
   - **Microorganisms consistent with IE from persistently positive blood cultures defined as:**
     - a. Two positive cultures of blood samples drawn >12 hours apart, or
     - b. All of 3 or a majority of 4 separate cultures of blood (with first and last sample drawn 1 hour apart)
     - c. Coxiella burnetii detected by at least one positive blood culture or antiphase I IgG antibody titer >1:800

2. **Evidence of endocardial involvement with positive echocardiogram defined as:**
   - Oscillating intracardiac mass on valve or supporting structures, in the path of regurgitant jets, or on implanted material in the absence of an alternative anatomic explanation
   - Abscess
   - New partial dehiscence of prosthetic valve or new valvular regurgitation (worsening or changing of preexisting murmur not sufficient)

#### Minor criteria include:

1. **Predisposing factor:** known cardiac lesion, recreational drug injection
2. **Fever >38°C**
3. **Evidence of embolism:** arterial emboli, pulmonary infarcts, Janeway lesions, conjunctival hemorrhage
4. **Immunological problems:** glomerulonephritis, Osler’s nodes
5. **Positive blood culture (that doesn’t meet a major criterion) or serologic evidence of infection with organism consistent with IE but not satisfying major criterion**

---

### Rejected (none)

1. **One of the following criteria**
   - Evidence for alternative diagnosis
   - Symptoms disappearing within 4 days after start of antibiotic treatment
   - No pathological evidence for endocarditis form surgery or autopsy, with less than four days of antibiotic treatment
   - Does not meet the criteria for possible endocarditis, see below

### Possible

1. **Two of the minor criteria without any other apparent cause**

### Probable

1. **Three minor criteria without any other apparent cause**
2. **One major and one minor criterion**

### Definite

1. **Clinical criteria with at least 2 major criteria, 1 major and three minor criteria, or 5 minor criteria**
2. **Histopathologic evidence: pathogen confirmed in vegetation or abscess**
Platelets are associated with nosocomial bacterial infections in the ICU

Myocarditis and pericarditis

Possible
1. At least two of the following signs or symptoms with no other recognized cause:
   a) fever (>38°C)
   b) chest pain
   c) paradoxical pulse
   d) increased heart size on X-chest or ultrasound

Probable
1. See criteria possible and at least one of the following criteria:
   a) abnormal EKG consistent with myocarditis or pericarditis;
   b) fourfold rise in type-specific antibody with or without isolation of virus from pharynx or feces;
   c) radiographic indications for infection (echocardiogram, CT scan, magnetic resonance imaging (MRI))

Definite
1. Positive culture or PCR of pericard tissue or fluid, obtained by puncture or surgical operation
2. Evidence of myocarditis or pericarditis on histopathologic examination of heart tissue

Mediastinitis

Clinical setting
Patients after cardiothoracic surgery or other causes of an open chest presenting with symptoms suggestive of an infection of the mediastinum

Possible
1. At least two of the following signs or symptoms in patients after cardiothoracic surgery or other causes of an open chest:
   a) fever (>38°C)
   b) chest pain
   c) sternal instability

Probable
1. See criteria ‘possible’ and at least one of the following:
   a) organisms cultured from blood
   b) mediastinal widening on x-ray

Definite
1. See criteria ‘possible’ and at least one of the following:
   a) purulent discharge from mediastinal area;
   b) organisms cultured from discharge from mediastinal area
2. Evidence of mediastinitis seen during a surgical operation or histopathologic examination
3. Organisms cultured from mediastinal tissue or fluid obtained during a surgical operation or needle aspiration
**Central nervous system**

### Intracranial infections: abscess

<table>
<thead>
<tr>
<th>Possible</th>
<th>1. At least two of the following signs or symptoms with no other recognized cause:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a) headache</td>
</tr>
<tr>
<td></td>
<td>b) fever (&gt;38°C)</td>
</tr>
<tr>
<td></td>
<td>c) localizing neurologic signs</td>
</tr>
<tr>
<td></td>
<td>d) changing level of consciousness</td>
</tr>
<tr>
<td></td>
<td>e) confusion</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Probable</th>
<th>1. See criteria 'possible' and at least one of the following:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a) Organisms seen on microscopic examination of brain or abscess tissue obtained by needle aspiration or by biopsy during a surgical operation or autopsy</td>
</tr>
<tr>
<td></td>
<td>b) Radiographic evidence of infection, e.g. abnormal findings on ultrasound, CT scan, magnetic resonance imaging (MRI), or arteriogram</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Definite</th>
<th>1. Organisms cultured from brain tissue or dura</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2. Abscess or evidence of intracranial infection seen during a surgical operation or histopathologic examination</td>
</tr>
</tbody>
</table>

### Primary meningitis and/or ventriculitis, encephalitis

<table>
<thead>
<tr>
<th>Possible</th>
<th>1. At least two of the following signs or symptoms with no other recognized cause:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a) fever (&gt;38°C)</td>
</tr>
<tr>
<td></td>
<td>b) headache</td>
</tr>
<tr>
<td></td>
<td>c) stiff neck</td>
</tr>
<tr>
<td></td>
<td>d) meningeal signs</td>
</tr>
<tr>
<td></td>
<td>e) cranial nerve signs</td>
</tr>
<tr>
<td></td>
<td>f) changing level of consciousness</td>
</tr>
<tr>
<td></td>
<td>g) petechia seen during meningococcemia</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Probable</th>
<th>1. See criteria at possible and at least one of the following:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a) positive antigen test of cerebrospinal fluid (CSF), blood or urine</td>
</tr>
<tr>
<td></td>
<td>b) organisms cultured from blood</td>
</tr>
<tr>
<td></td>
<td>c) uncertain abnormal CSF findings</td>
</tr>
<tr>
<td></td>
<td>d) In case no CSF is obtained due reasons other than clinical motives</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Definite</th>
<th>1. Organisms cultured from CSF (if low numbers of skin contaminants, then take chemical and clinical signs into account)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2. See criteria at possible and at least one of the following:</td>
</tr>
<tr>
<td></td>
<td>a) Increased white cells, elevated protein, and/or decreased glucose in CSF (if bleedy LP then positive is when leukocytes:erythrocytes ratio is &gt;1:100; if not bleedy then positive when leukocytes &gt; 100x10^6/L)</td>
</tr>
<tr>
<td></td>
<td>b) organisms seen on Gram stain of CSF</td>
</tr>
<tr>
<td></td>
<td>c) positive PCR on viral etiologic agents</td>
</tr>
</tbody>
</table>
### Secundaire meningitis

**Clinical setting**  
Patients presenting with symptoms of meningitis up to 1 year after neurotrauma, neurosurgery, ENT-surgery, external ventricular drain, external lumbar drain, or ventriculo-peritoneal drain if there is a suspected infection in the peritoneal part

**Possible**  
1. At least two of the following signs or symptoms with no other recognized cause:  
   a) fever (>38°C)  
   b) headache  
   c) stiff neck  
   d) meningeal signs  
   e) cranial nerve signs  
   f) changing level of consciousness

**Probable**  
1. See criteria at possible and at least one of the following:  
   a) positive urine antigen test (pneumococcal)  
   b) organisms cultured from blood

**Definite**  
1. Organisms cultured from CSF (if low numbers of skin contaminants, then take chemical and clinical signs into account)  
2. See criteria at possible and increased elevated protein, and/or decreased glucose in CSF (if bleedy LP then positive is when leukocytes:erythrocytes ratio is >1:100; if not bleedy then positive when leukocytes >100x10⁶/L) and organisms seen on Gram stain of CSF

### Spinal abscess without meningitis

**Possible**  
1. At least one of the following signs or symptoms with no other recognized cause:  
   a) fever (>38°C)  
   b) back pain  
   c) focal tenderness  
   d) radiculitis  
   e) paraparesis  
   f) paraplegia

**Probable**  
1. See criteria at possible and at least one of the following:  
   a) organisms cultured from blood  
   b) radiographic evidence of a spinal abscess

**Definite**  
1. Organisms cultured from abscess in the spinal epidural or subdural space  
2. Abscess in the spinal epidural or subdural space seen during a surgical operation or at autopsy of evidence of an abscess seen during a histopathologic examination
### Urinary tract

#### Urosepsis in noncatheterized patients

<table>
<thead>
<tr>
<th>Possible</th>
<th>1. At least two of the following signs or symptoms with no other recognized cause</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a) fever (&gt;38 °C)</td>
</tr>
<tr>
<td></td>
<td>b) urgency</td>
</tr>
<tr>
<td></td>
<td>c) frequency</td>
</tr>
<tr>
<td></td>
<td>d) dysuria</td>
</tr>
<tr>
<td></td>
<td>e) pyuria</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Probable</th>
<th>1. See criteria at ‘possible’ and at least one of the following</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a) positive dipstick for leukocyte esterase and/or nitrate</td>
</tr>
<tr>
<td></td>
<td>b) pyuria (&gt;10 white blood cells/mm³ or &gt;3 white blood cells/high-power field of unspun urine)</td>
</tr>
<tr>
<td></td>
<td>c) organisms seen in Gram stain of unspun urine</td>
</tr>
<tr>
<td></td>
<td>d) frank pus expressed around the urinary catheter</td>
</tr>
<tr>
<td></td>
<td>e) at least two urine cultures with repeated isolation of the same uropathogen with ≥10² colonies/ml in nonvoided specimens</td>
</tr>
<tr>
<td></td>
<td>f) urine culture with ≤ 10⁵ colonies/mL of a single uropathogen in a patient being treated with appropriate antimicrobial therapy</td>
</tr>
<tr>
<td></td>
<td>g) radiographic evidence of infection (e.g., ultrasound, computed tomography, magnetic resonance imaging, radiolabeled scan)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Definite</th>
<th>1. See criteria at possible and urine culture with &gt;10⁵ colonies/mL with no more than two species of microorganisms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2. Abscess or other evidence of infection seen on direct examination, during surgery, or by histopathologic examination</td>
</tr>
</tbody>
</table>
### Urosepsis in catheterized patients

**Clinical setting**  Patients who have an urinary catheter or that has been removed within the past 6 days presenting with symptoms of urinary tract infection

**Possible**  1. At least two of the following signs or symptoms with no other recognized cause
   a) Fever (>38 °C)
   b) positive dipstick for leukocyte esterase and/or nitrate
   c) pyuria (>10 white blood cells/mm³ or >3 white blood cells/high-power field of unspun urine)
   d) organisms seen in Gram stain of unspun urine
   e) frank pus expressed around the urinary catheter
   f) two urine cultures with repeated isolation of the same uropathogen with \( \geq 10^2 \) colonies/ml in nonvoided urine (Gram negative pathogen or S. Saprophyticus)
   g) urine culture with \( \leq 10^5 \) colonies/mL of single uropathogens in a patient being treated with appropriate antimicrobial therapy
   h) hematuria

**Probable**  1. See criteria at possible
   and urine culture with >105 colonies/mL and no more than 2 species of pathogens

**Definite**  1. See criteria at probable
   and purulent drainage from the affected site (nefrostomy)
   and organisms cultured from blood that are compatible with urine culture
   2. Abscess or other evidence of infection seen on direct examination, during surgery, or by histopathologic examination
   and bacteremia

**Comment**  • Urine culture must be obtained using appropriate technique, such as clean catch collection or catheterization
### Upper urinary tract infection (kidney, ureter, bladder, urethra, or tissues surrounding the retroperitoneal or perinephric spaces)

**Possible**
1. At least two of the following signs or symptoms with no other recognized cause:
   a) fever (>38 °C)
   b) urgency
   c) localized pain or tenderness at involved site

**Probable**
1. See criteria at possible
   and at least one of the following:
   a) purulent drainage,
   b) pyuria,
   c) hematuria,
   d) positive culture,
   e) positive Gram stain
   f) radiographic evidence of infection (e.g., ultrasound, computed tomography, magnetic resonance imaging, radiolabeled scan)

**Definite**
1. Organism isolated from culture (other than urine) or tissue from the affected site
2. Abscess or other evidence of infection seen on direct examination, during surgery, or by histopathologic examination

### Skin infection, erysipelas, phlebitis

**Possible**
1. At least two of the following signs or symptoms with no other recognized cause:
   a) pain or tenderness
   b) localized swelling
   c) redness
   d) heat

**Probable**
1. See criteria at possible
   and organisms cultured from blood

**Definite**
1. Purulent drainage, pustules, vesicles, or boils
2. See criteria at possible
   and organisms cultured from aspirate or drainage of affected site; if organisms are normal skin flora (e.g. coagulase-negative staphylococci, micrococci, diphtheroids) they must be a pure culture
### Skin and soft tissue

#### Surgical site infections: Superficial wounds

<table>
<thead>
<tr>
<th>Clinical setting</th>
<th>Patients presenting with symptoms or signs of wound infection within 30 days following surgery or trauma</th>
</tr>
</thead>
</table>
| Possible         | 1. Infection that arises within 30 days of an operative procedure and at the site of surgical intervention  
    and infection involves the skin or subcutaneous tissue alone  
    and at least two of the following:  
    a) pain or tenderness  
    b) localized swelling  
    c) redness  
    d) heat |
| Probable         | N.A.                                                                                                  |
| Definite         | 1. See criteria at possible  
    and at least one of the following:  
    a) purulent discharge from incision or drain  
    b) abscess seen during observation, (re)surgery, histopathologic or radiographic examination  
    c) organisms cultured from tissue or drainage of affected site |

**Comment**  
- Infections around surgical sutures and infected burn wounds are not classified as superficial POWI's

#### Surgical site infections: Deep wounds

<table>
<thead>
<tr>
<th>Clinical setting</th>
<th>Patients presenting with symptoms or signs of wound infection within 30 days following surgery or trauma</th>
</tr>
</thead>
</table>
| Possible         | 1. Infection that arises within 30 days of an operative procedure and at the site of surgical intervention or within 1 year after implant placement  
    and Infection involves the fascia or muscle layers  
    and at least two of the following:  
    a) pain or tenderness  
    b) localized swelling  
    c) redness  
    d) heat |
| Probable         | N.A.                                                                                                  |
| Definite         | 1. See criteria at possible  
    and at least one of the following:  
    a) purulent discharge from incision or drain  
    b) abscess seen during observation, (re)surgery, histopathologic or radiographic examination  
    c) organisms cultured from tissue or drainage of affected site |
## Soft tissue infections

**Clinical setting**  
Patients presenting with symptoms of infections of soft tissue such as necrotizing fasciitis, infectious gangrene and necrotizing cellulitis and myositis, lymphadenitis and lymphangitis

| Possible | 1. At least two of the following signs or symptoms with no other recognized cause:  
|          | a) pain or tenderness  
|          | b) localized swelling  
|          | c) redness  
|          | d) heat  |

| Probable | 1. See criteria at possible  
|          | and at least two of the following:  
|          | a) organisms cultured from blood;  
|          | b) signs suggestive for infection on computed tomography or magnetic resonance imaging  |

| Definite | 1. Organisms cultured from tissue or drainage of affected site  
|          | 2. Purulent drainage from affected site  
|          | 3. Abscess or other evidence of infection seen during observation, surgery, histopathologic or radiographic examination  |

## Decubitus infections

| Possible | 1. Clinical signs of infection (stage 3 or more) with the following signs or symptoms with no other recognized cause:  
|          | a) redness  
|          | b) tenderness  
|          | c) swelling of decubitus wound edges  |

| Probable | 1. See criteria at possible  
|          | and organisms cultured from blood and absence of other evidence of infections  |

| Definite | 1. See criteria at possible  
|          | and organisms cultured from a decubitus ulcer by needle aspiration of fluid or biopsy of tissue from the ulcer margin  
|          | and evident pus from wound  |

**Comments**  
- Purulent drainage alone is not sufficient evidence of an infection  
- Organisms cultured from the surface of a decubitus ulcer are not sufficient evidence that the ulcer is infected. A properly collected specimen from a decubitus ulcer involves needle aspiration of fluid or biopsy of tissue from the ulcer margin
Platelets are associated with nosocomial bacterial infections in the ICU

**Mastitis**

Possible 1. The following signs or symptoms with no other recognized cause:
   a) fever (>38°C)
   b) local inflammation of the breast

Probable 1. See criteria at possible
   and radiographic evidence for breast abscess or mastitis on computed tomography

Definite 1. Organisms cultured from affected breast tissue site or fluid obtained by incision and drainage or needle aspiration
   2. Breast abscess or other evidence of infection seen during observation, surgery, histopathologic or radiographic examination

**Infections of the GI tract**

**Intra-abdominal infection/abscess (IAI)**

Clinical setting Patients presenting with a localized pus collection in peritoneal cavity or nearby abdominal structures in the absence of a documented gastrointestinal perforation

Possible 1. At least two of the following signs or symptoms with no other recognized cause:
   a) pain
   b) diarrhea
   c) fever
   d) vomiting

Probable 1. See criteria at possible
   and radiographic and/or surgical evidence of abscess
   and purulent drainage and/or positive Gram stain of drainage

Definite 1. See criteria at probable
   and organisms cultured from aspiration and/or blood

**Pancreatic infection, infected necrotizing pancreatitis**

Possible 1. Radiographic or direct surgical inspection with evidence suggestive of pancreatic abscess or other type of infection

Probable 1. The presence of surgical or radiographic evidence of an abnormal collection of an inflammatory focus within the substance of the pancreas or surrounding structures
   and a positive Gram stain from the pancreatic collection in the absence of culture documentation

Definite 1. This requires direct confirmation of positive microbial cultures from the pancreas or surrounding structures by percutaneous aspiration or direct visualization and culture at the time of surgery or from the bloodstream
### Biliary tract infection

**Possible** 1. At least two of the following clinical symptoms of biliary tract infection:
   - a) pain right upper quadrant
   - b) fever
   - c) jaundice
   - d) colic pain

**Probable** 1. See criteria at possible
   - and positive Gram stain from the biliary system
   - and radiographic evidence on ultrasound/CT

**Definite** 1. See criteria at probable
   - and at least one of the following:
     - a) the isolation of pathogenic microorganisms obtained via percutaneous or direct surgical collection of samples in the lumen of the gall bladder or the biliary tract or the blood
     - b) ERCP with quick recovery
     - c) cholecystectomy with recovery

**Comments**
- A positive blood culture in a patient with ascending cholangitis is sufficient to diagnose a biliary tract infection
- A positive culture from the biliary tract in a patient without symptoms is insufficient to diagnose a biliary tract infection
- A positive culture from a T-tube drain from the biliary tract is insufficient to diagnose a biliary tract infection when the tube is more than 24 hours in situ

### Primary peritonitis (spontaneous bacterial peritonitis)

**Clinical setting** Patients presenting with an infection of the peritoneal fluid in the absence of a gastrointestinal perforation, abscess, or other localized infection within the gastrointestinal tract

**Possible** 1. At least two of the following signs or symptoms with no other recognized cause:
   - a) fever
   - b) abdominal pain in more than 1 quadrant (not localized)
   - c) ileus
   - d) feeding intolerance
   - and inflammatory peritoneal fluid (>500 leucocytes/mL) in the absence of a positive culture (in peritoneal fluid or blood) or Gram stain

**Probable** 1. See criteria at possible
   - and evidence of an inflammatory ascitic fluid (>500 leukocytes/mL with a neutrophil predominance) in the presence of a positive Gram stain but negative peritoneal fluid cultures or in the presence of a positive blood culture for a pathologic organism with inflammatory cells in ascitic fluid

**Definite** 1. Clinical signs of peritonitis
   - and isolation of microbial pathogens (in peritoneal fluid or blood)
### Secondary peritonitis

**Clinical setting**  
Patients presenting with an infection of the peritoneal space following perforation, abscess formation, ischemic necrosis, or penetrating injury of the intra-abdominal contents

**Possible**  
1. Upper gastrointestinal perforation or penetrating abdominal trauma that is surgically repaired without further evidence of microbiologic confirmation or clinical signs or symptoms supportive of a diagnosis of bacterial or fungal peritonitis
2. A finding of an inflammatory peritoneal fluid in the presence of a documented but localized intra-abdominal abscess in the absence of culture confirmation
3. At least two of the following signs or symptoms with no other recognized cause:
   a) fever
   b) abdominal pain in more than 1 quadrant (not localized)
   c) ileus
   d) feeding intolerance

**Probable**  
1. See clinical signs at possible and at least one of the following:
   a) Documented evidence of perforation (free air in the abdomen on radiographic studies or surgical confirmation of peritoneal inflammation following luminal perforation in the absence of microbiologically confirmed peritonitis)
   b) Gram stain in the absence of a positive culture from the peritoneum

**Definite**  
1. See clinical signs at possible and isolation of one or more microbial pathogens found in the peritoneum or the blood 24 hrs after a gastrointestinal perforation of the stomach, esophagus or duodenum, or any perforation of the small bowel distal to the ligament of Treitz

**Comments**  
- Spillage of luminal contents during an operative procedure is not sufficient evidence of perforation that allows for definitive diagnosis of peritonitis
- Furthermore, a penetrating abdominal wound or documented perforation that is surgically repaired within 12 hrs of its occurrence is not sufficient evidence to support diagnosis of secondary bacterial peritonitis

### Tertiary peritonitis

**Clinical setting**  
Patients presenting with a persistent intra-abdominal inflammation and clinical signs of peritoneal irritation following secondary peritonitis from nosocomial pathogens

**Possible**  
1. Clinical signs of peritonitis (see secondary peritonitis) and persistent signs of systemic inflammation but without clear documented evidence of persistent inflammation within the peritoneal space following secondary bacterial peritonitis

**Probable**  
1. Clinical signs of peritonitis with documented secondary peritonitis and persistent peritoneal inflammation (500 leukocytes/mL peritoneal fluid) in the absence of microbiologically confirmed microbial persistence in the peritoneal space

**Definite**  
1. Isolation of one or more nosocomial pathogens from peritoneal fluid or blood in an appropriate clinical situation (48 hrs after treatment for primary or secondary peritonitis)
### Peritoneal dialysis-related peritonitis

**Clinical setting**
Patients with peritoneal dialysis presenting with an infection of the peritoneal fluid

**Possible**
1. Clinical signs of peritonitis in patients receiving peritoneal dialysis (see secondary peritonitis) and abnormal accumulation of inflammatory cells in the peritoneum (100 leukocytes/mL) but without absence of Gram stain and culture evidence of infection

**Probable**
1. Clinical signs of peritonitis and an inflammatory process (100 leukocytes/mL) of the peritoneum during the course of peritoneal dialysis and positive Gram stain but without culture documentation from blood or the peritoneal space

**Definite**
1. Clinical signs of peritonitis and an inflammatory process (100 leukocytes/mL) of the peritoneum and presence of culture documentation in peritoneal fluid or blood of a pathogenic microorganism

### Gastroenteritis

**Possible**
1. Acute onset of diarrhea (liquid stools for more than 12 hours) of unknown cause (infectious or non-infectious) and at least 5 times a day of minimal 500 mL liquid stool (patients with laxatives are excluded in case no evidence of infection)

**Probable**
1. Acute onset of diarrhea (liquid stools for more than 12 hours) and no likely noninfectious cause

**Definite**
1. At least two of the following symptoms:
   a) nausea
   b) vomiting
   c) abdominal pain
   d) headache
   and enteric pathogen is cultured from stool or rectal swab
### Viral hepatitis

<table>
<thead>
<tr>
<th>Level</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Possible</td>
<td>N/A</td>
</tr>
</tbody>
</table>
| Probable | 1. At least two of the following signs or symptoms with no other recognized cause:  
   a) fever  
   b) anorexia  
   c) nausea  
   d) vomiting  
   e) abdominal pain  
   f) jaundice |
| Definite | 1. See criteria at probable and at least one of the following criteria  
   a) positive antibody (IgM) test for HAV, HBV, HCV, HDV, HEV  
   b) abnormal liver function tests (e.g. elevated ALT, AST, bilirubin)  
   c) cytomegalovirus (CMV) detected in urine or oropharyngeal secretions  
   d) positive antigen for HBV (HbsAg)  
   e) positive PCR for EBV, CMV, HBV, HCV |

### Necrotizing enterocolitis (typhlitis)

<table>
<thead>
<tr>
<th>Level</th>
<th>Criteria</th>
</tr>
</thead>
</table>
| Possible | 1. At least two of the following signs or symptoms with no other recognized cause in a neutropenic patient (neutrophils < 500/μL):  
   a) fever  
   b) abdominal pain (right upper quadrant)  
   c) abdominal distension  
   d) diarrhea |
| Probable | 1. See possible and evidence for enterocolitis on ultrasound |
| Definite | 1. Enterocolitis seen during surgery or histology  
  2. See possible and evidence for enterocolitis on CT (thickened wall of the cecum; air, hemorrhage, presence of bowel wall edema) |
## Infections of the reproductive system

### Endometritis, epididymitis, prostatitis

<table>
<thead>
<tr>
<th>Possible</th>
<th>1. Purulent drainage from the uterus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2. Male: at least one of the following</td>
</tr>
<tr>
<td></td>
<td>a) fever</td>
</tr>
<tr>
<td></td>
<td>b) enlarged testicle / epididymis</td>
</tr>
<tr>
<td>Probable</td>
<td>1. Purulent drainage from the uterus</td>
</tr>
<tr>
<td></td>
<td>and at least two of the following:</td>
</tr>
<tr>
<td></td>
<td>a) fever (&gt;38°C),</td>
</tr>
<tr>
<td></td>
<td>b) abdominal pain,</td>
</tr>
<tr>
<td></td>
<td>c) uterine tenderness</td>
</tr>
<tr>
<td></td>
<td>2. Male: see criteria at possible</td>
</tr>
<tr>
<td></td>
<td>and purulent drainage from the urethra</td>
</tr>
<tr>
<td>Definite</td>
<td>1. Organisms cultured from fluid or tissue from endometrium obtained during surgical operation, by needle aspiration, or by brush biopsy</td>
</tr>
<tr>
<td></td>
<td>2. Male: see criteria at probable</td>
</tr>
<tr>
<td></td>
<td>and evidence for infection on ultrasound</td>
</tr>
</tbody>
</table>

### Bones and joints

### Osteomyelitis

<table>
<thead>
<tr>
<th>Possible</th>
<th>1. At least two of the following signs or symptoms with no other recognized cause:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a) fever (&gt;38°C)</td>
</tr>
<tr>
<td></td>
<td>b) localized swelling</td>
</tr>
<tr>
<td></td>
<td>c) tenderness</td>
</tr>
<tr>
<td></td>
<td>d) heat</td>
</tr>
<tr>
<td></td>
<td>e) drainage at suspected site of bone infection</td>
</tr>
<tr>
<td>Probable</td>
<td>1. See criteria at possible</td>
</tr>
<tr>
<td></td>
<td>and radiographic evidence of infection (CT, MRI)</td>
</tr>
<tr>
<td>Definite</td>
<td>1. Organisms cultured from bone</td>
</tr>
<tr>
<td></td>
<td>2. Evidence of osteomyelitis on direct examination of the bone during a surgical operation or histopathologic examination</td>
</tr>
<tr>
<td></td>
<td>3. See criteria at probable</td>
</tr>
<tr>
<td></td>
<td>and organisms cultured from blood</td>
</tr>
</tbody>
</table>
Discitis

Possible 1. Both of the clinical signs or symptoms with no other recognized cause:
   a) fever (>38°C) with no other recognized cause
   b) pain at the involved vertebral disc space

Probable 1. See criteria at possible
   and radiographic evidence of infection

Definite 1. Organisms cultured from vertebral disc space tissue obtained during a surgical operation or needle aspiration
   2. Evidence of vertebral disc space infection seen during a surgical operation or histopathologic examination
   3. See criteria at possible
   and radiographic evidence of infection (CT, MRI)
   and organisms cultured from blood

Bursitis

Possible 1. At least two of the following signs or symptoms with no other recognized cause:
   a) joint pain
   b) swelling
   c) tenderness
   d) heat
   e) evidence of effusion
   f) limitation of motion

Probable 1. See criteria at possible
   and at least one of the following:
   a) organisms and white blood cells seen on Gram stain of joint fluid
   b) cellular profile and chemistries of joint fluid compatible with infection and not explained by an underlying rheumatologic disorder
   c) radiographic evidence of infection

Definite 1. Organisms cultured from joint fluid or synovial biopsy
   2. Evidence of joint or bursa infection seen during a surgical operation or histopathologic examination
### Eye

#### Conjunctivitis

<table>
<thead>
<tr>
<th>Status</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Possible</td>
<td>1. Pain or redness of conjunctiva or around eye</td>
</tr>
<tr>
<td>Probable</td>
<td>1. See criteria at possible and at least one of the following:</td>
</tr>
<tr>
<td></td>
<td>a) WBCs and organisms seen on Gram stain</td>
</tr>
<tr>
<td></td>
<td>b) purulent exudate</td>
</tr>
<tr>
<td></td>
<td>c) multinucleated giant cells seen on microscopic examination of conjunctival exudate or scrapings</td>
</tr>
<tr>
<td></td>
<td>d) positive viral culture</td>
</tr>
<tr>
<td>Definite</td>
<td>1. Pathogens cultured from purulent exudate obtained from the conjunctiva or contiguous tissues, such as eyelid, cornea, meibomian glands, or lacrimal glands</td>
</tr>
</tbody>
</table>

#### Endophtalmitis

<table>
<thead>
<tr>
<th>Status</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Possible</td>
<td>1. At least two of the following signs or symptoms with no other recognized cause:</td>
</tr>
<tr>
<td></td>
<td>a) eye pain</td>
</tr>
<tr>
<td></td>
<td>b) visual disturbance</td>
</tr>
<tr>
<td></td>
<td>c) hypopyon</td>
</tr>
<tr>
<td>Probable</td>
<td>1. See criteria at possible and organisms cultured from blood</td>
</tr>
<tr>
<td>Definite</td>
<td>1. See criteria at possible and organisms cultured from anterior or posterior chamber of vitreous fluid</td>
</tr>
</tbody>
</table>
## Ear

<table>
<thead>
<tr>
<th>Possible</th>
<th>1. Externa: at least one of the following</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a) fever (&gt;38°C)</td>
</tr>
<tr>
<td></td>
<td>b) pain</td>
</tr>
<tr>
<td></td>
<td>c) redness</td>
</tr>
<tr>
<td></td>
<td>1. Media: at least one of the following:</td>
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<tr>
<td></td>
<td>a) pain in the eardrum</td>
</tr>
<tr>
<td></td>
<td>b) inflammation, retraction or decreased mobility of eardrum</td>
</tr>
<tr>
<td></td>
<td>c) fluid behind eardrum</td>
</tr>
<tr>
<td></td>
<td>1. Mastoid: at least two of the following and with no other recognized cause:</td>
</tr>
<tr>
<td></td>
<td>a) fever (&gt;38°C)</td>
</tr>
<tr>
<td></td>
<td>b) pain, tenderness</td>
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<tr>
<td></td>
<td>c) erythema</td>
</tr>
<tr>
<td></td>
<td>d) headache</td>
</tr>
<tr>
<td></td>
<td>e) facial paralysis</td>
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<table>
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<tr>
<th>Probable</th>
<th>1. Externa:</th>
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<tr>
<td></td>
<td>See criteria at possible and purulent drainage from ear canal</td>
</tr>
<tr>
<td></td>
<td>1. Media:</td>
</tr>
<tr>
<td></td>
<td>See criteria at possible and purulent drainage</td>
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<tr>
<td></td>
<td>1. Mastoid:</td>
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<tr>
<td></td>
<td>See criteria at possible</td>
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<tr>
<td></td>
<td>And organisms seen on Gram stain of purulent material from mastoid</td>
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</table>

| Definite | 1. Pathogens cultured from purulent drainage from ear canal, middle ear, inner ear or mastoid |
Infections of mouth, tongue or gums

**Possible**
1. At least one of the following signs or symptoms:
   a) abscess
   b) ulceration
   c) raised white patches on inflamed mucosa
   d) plaques on oral mucosa

**Probable**
1. See criteria at possible
   and at least one of the following:
   a) organisms seen on Gram stain;
   b) positive KOH (potassium hydroxide);
   c) multinucleated giant cells seen on microscopic examination of mucosal scrapings;
   d) diagnostic single antibody titer (IgM) or fourfold increase in paired sera (IgG) for pathogen

**Definite**
1. Abscess or other evidence of oral cavity infection seen on direct examination, during a surgical operation, or during a histopathologic examination
2. See criteria at probable
   and organisms cultured from purulent material from tissues or oral cavity

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**Other definitions**

<table>
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<tr>
<th>CPIS</th>
<th>None or scant</th>
<th>0</th>
<th>Non-purulent</th>
<th>1</th>
<th>Purulent</th>
<th>2</th>
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<tbody>
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<td><strong>Tracheal secretions</strong></td>
<td>None or scant</td>
<td>0</td>
<td>Non-purulent</td>
<td>1</td>
<td>Purulent</td>
<td>2</td>
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<tr>
<td><strong>Infiltrates on chest radiograph</strong></td>
<td>Absent</td>
<td>0</td>
<td>Diffuse</td>
<td>1</td>
<td>Localized</td>
<td>2</td>
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<td><strong>Fever (°C):</strong></td>
<td>&gt;36.5 en &lt; 38.4</td>
<td>0</td>
<td>&gt; 38.4 en &lt; 38.9</td>
<td>1</td>
<td>&gt; 38.9 of &lt; 36</td>
<td>2</td>
</tr>
<tr>
<td><strong>Leukocytosis (/mm3):</strong></td>
<td>&gt; 4000 and &lt; 11,000</td>
<td>0</td>
<td>&lt; 4000 or &gt; 11,000</td>
<td>1</td>
<td>&lt; 4000 or &gt; 11,000 en &gt; 500 band forms</td>
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<td><strong>PaO2/FiO2:</strong></td>
<td>&gt; 240 or ARDS</td>
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<td>&lt; 240 and no ARDS</td>
<td>2</td>
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<td><strong>Microbiology</strong></td>
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<td>Positive</td>
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Chapter 10

Summary and general discussion
SUMMARY AND DISCUSSION

The aims of this thesis

This thesis discusses effects of transfusion of red blood cells, platelets as well as FFP in critically ill patients. Although frequently administered, a blood transfusion with any of these components is not without risk. This thesis aims to increase the understanding of effects of a blood transfusion to allow the clinician to better outweigh the advantages against the disadvantages and thereby minimize the risk of the patients for an adverse outcome.

The three aims of this thesis are:
Red blood cells: To investigate the role of storage red blood cells as well as the presence of an inflammatory condition on the association between RBC transfusion and host response (part I)
Fresh frozen plasma: To assess the effect of a FFP transfusion on host response and haemostatic balance in critically ill patients (part II)
Platelets: To assess the effect of transfusion on the development of infections in critically ill patients (part III)

Part I – red blood cells

Summary

Part I contains studies investigating red blood cell transfusions. Chapter 2 gives an overview of the knowledge of mechanisms of the clearance of red blood cells when the red blood cells normally age (senesence) and the clearance of red blood cells in patients with an inflammatory condition. We discuss that anemia of inflammation is multifactorial. There is reduced production of erythropoietin, impaired bone marrow response to erythropoietin, reduced iron availability and increased red blood cell (RBC) clearance. Given that this thesis shows that inflammation contributes to increased clearance of RBCs in organs and we hypothesize that clearance is an important mechanism of anemia of inflammation, this review elaborates on the pathogenesis of the increased red blood cell clearance. We state that PS expression in inflammation is mainly enhanced due to an increase in ceramide, caused by an increase in sphingomyelinase activity due to either Platelet Activating Factor, tumor necrosis factor-α or direct production by bacteria. Phagocytosis of RBCs during inflammation is mediated via RBC membrane protein Band 3. Reduced deformability of RBCs seems an important feature in inflammation, also mediated by Band 3, as well as by nitric oxide, reactive oxygen species and sialic acid residues. Also, adherence of RBCs to the endothelium is increased during inflammation, most likely due to increased expression of endothelial adhesion molecules as well as phosphatidylserine on the RBC membrane, in combination with decreased capillary
blood flow. Thereby, clearance of RBCs during inflammation shows similarities to clearance of senescent RBCs, but also has distinct entities, including increased adhesion to the endothelium. This is further investigated in Chapter 6.

Chapter 3 focuses on models of transfusion. In the literature, the effect of human RBCs have been evaluated in animal models. As it is unsure how this affects clearance, the routing of the post transfusion recovery following a RBC transfusion was studied in a rat model using fresh and stored human RBCs. No human RBCs could be detected 24 hours after transfusion in the circulation of any of the recipients. Instead, donor RBCs were detected in the organs of the recipients. Given that the standard of appropriate transfusion requires more than 75 % posttransfusion survival, we concluded that the use of human RBCs in rodent recipients is not an appropriate model to study the efficacy of RBC transfusion and that transfusion models should preferably be syngeneic.

Chapter 4 shows the results of a study performed in endotoxemic rats receiving a transfusion with either fresh or stored labeled rat RBCs. We hypothesized that endotoxemia contributes to increased clearance of donor RBCs from the circulation, with concomitant trapping of RBCs in organs, thereby accounting for transfusion-related organ failure. Indeed, endotoxemia induced trapping of transfused RBCs in different organs, predominantly the lung and kidney, which are the two sites most often associated with transfusion-related organ failure. This trapping of donor RBCs in organs was associated with lung injury, but not with kidney injury. Furthermore, endotoxemia did not induce trapping of donor RBCs in the spleen and liver. In accordance, there is no clinical association between transfusion and the occurrence of spleen or liver injury. We also hypothesized that stored RBCs would further enhance clearance of donor RBCs compared to fresh RBCs. Indeed, stored RBCs yielded a significantly lower post-transfusion recovery compared to fresh RBCs, both in healthy and endotoxemic rats. As an increased storage time did not result in enhanced trapping of donor RBCs in the organs, further research to the underlying mechanism of a decreased 24-hour PTR of transfused stored RBCs is warranted. In conclusion, endotoxemia results in an increased percentage of donor RBCs trapped in the lung and kidney, associated with lung injury following transfusion. Trapping of donor RBCs in the microvasculature of the lungs during an inflammatory state may explain the association between lung injury and RBC transfusion in the critically ill.

Chapter 5 constitutes the results of an in vitro experiment in which whole blood was incubated with supernatant from RBC units stored for different time periods, either containing (different numbers of) EVs or depleted from EVs. We hypothesized that EVs accumulating in RBC products during storage contribute to a pro-inflammatory host response in recipients, which is related both to their amount as well as to their storage duration. Indeed, incubation with both fresh and stored supernatant containing EVs induced a strong host response with production of TNF, IL-6 and IL-8. In supernatant
depleted from EVs, this host response was completely abrogated. This EV-induced pro-inflammatory host response was both dependent on the number of EVs as well as on storage time of the blood product from which the EVs were isolated. In conclusion, EVs from RBC units illicit a strong dose-dependent inflammatory host response in recipients and may therefore contribute to the adverse outcome observed in patients receiving RBC transfusion.

**Chapter 6** further elaborates on the effects of EVs. Human umbilical vein endothelial cells (HUVECs) were incubated with supernatant from fresh and stored RBC units either containing EVs or depleted from EVs to which immune cells were added. We hypothesized that RBC-derived EVs can activate endothelial cells through activation of host immune cells and that this effect is a function of storage time. Incubation of endothelial cells with monocytes alone did not induce upregulation of adhesion markers, but the addition of both monocytes and supernatant from RBCs containing EVs resulted in upregulation of endothelial expression of ICAM-1 and E-Selectin when compared to baseline. Upregulation was absent when HUVECs were incubated with RBC supernatant depleted from EVs. RBC-derived EVs also directly induce endothelial secretion of vWF. Both the upregulation of endothelial adhesion markers and the endothelial secretion of vWF were independent of storage time. We also studied phagocytosis of EVs by monocytes by using confocal microscopy and flow cytometry and showed that EVs are phagocytosed by monocytes, which was partly abrogated after co-incubation with two different complement receptor 3 (CR3)-blocking antibodies. Herefore, we conclude that EVs from RBC transfusion bags induce a pro-inflammatory and pro-coagulant endothelial cell response, which is irrespective of storage time, and is mediated by activation of monocytes through complement receptor 3. Thereby, EVs again were found to mediate activation of the host inflammatory response. Of note, this effect can not entirely be explained as part of the ‘storage lesion’, as effects are equal when MVs from fresh and stored products were added.

**Discussion**

The aim of part I of this thesis was to investigate the effect of inflammation on clearance and adverse effects of red blood cells and to investigate whether age of red blood cells augments this interaction, in particular the role of extracellular vesicles that accumulate in a RBC product during storage.

Since anemia is such a frequent finding in the ICU and a hallmark of sepsis, we began this thesis with the description of the pathogenesis of the anemia of inflammation (chapter 2). The mechanisms of red blood cell clearance in physiologic senescence, namely phosphatidylserine exposure, erythrocyte phagocytosis and reduced deformability, are augmented in an inflammatory state. In addition, during inflammation,
another mechanism of clearance plays a role, namely adherence of red blood cells to endothelium. Incubation of both endothelial cells and red blood cells with endotoxin increased adherence of RBCs to endothelial monolayers. This effect was also observed after stimulation with TNF-α. This is a mechanism of anemia of inflammation which has hithertho not received much attention.

If adherence of donor RBCs to the endothelium plays a role, may this be a mechanism of clearance? To investigate this, we used an animal model in which rats were transfused with human RBCs, in order to retrace these cells after transfusion (chapter 3). However, although we detected human RBCs in the circulation of all recipients, we could not detect any human RBCs 24 hours after transfusion in the circulation of any of the recipients. Unfortunately, we had to conclude that the use of human RBCs in rodent recipients are not appropriate to study the efficacy of RBC transfusion and that transfusion models should preferably be syngeneic. This is an important finding, as models of cross species have been frequently used. This chapter argues against the use of those models. For further experiments, a syngeneic model was used (chapter 4). We found that endotoxemia induced trapping of transfused RBCs in different organs, predominantly the lung and kidney, the two sites most often associated with transfusion-related organ failure. Trapping of donor RBCs in the microvasculature of the lungs during an inflammatory state may explain the association between lung injury and RBC transfusion in the critically ill. Of note, although storage reduced post transfusion recovery, donor RBCs were not found in the spleen. As hemolysis was increased during storage, this rather suggests that stored donor RBCs are not phagocytosed in the spleen but undergo hemolysis.

We did not provide direct evidence that the RBCs were stuck in the organs following adherence to the endothelium. Thereby, how RBCs interact with the endothelium in the development of organ failure is not known. We previously found that washing of RBCs attenuates organ injury in a rat transfusion model, suggesting a soluble factor in the blood product. We also found that EVs from both fresh and stored RBC units illicited a strong dose-dependant inflammatory host response in recipients in an in vitro whole blood stimulation experiment (chapter 5).

Thereby, the question remained whether EVs could also induce upregulation of adhesion markers on the endothelium. We found that EVs and monocytes alone could not induce upregulation of adhesion markers, but that the addition of both monocytes and EVs were required for upregulation of adhesion molecules on the endothelial cells. Finally, we found that RBC-derived EVs also directly induce endothelial secretion of vWF.

In conclusion, EVs seem to play a pivotal role in the complex system leading to the development of RBC transfusion-related organ failure. RBC-derived EVs are phagocytozed by monocytes and monocytes can subsequently activate the endothelium, which leads
to the adherence of RBCs to the endothelium. We hypothesize that this process results in impeding the microcirculation. This may be further aggravated by the EV-induced endothelial secretion of vWF, which can lead to a pro-coagulant state and the formation of microthrombi and thereby leading to further damage. Taken these results together, depletion of the RBC transfusion product of EVs directly before transfusion, e.g. by washing or filtration, can abrogate this pathophysiological cascade, thereby leading to less transfusion-related organ failure.

**Part II – fresh frozen plasma**

*Summary*

**Part II** contains studies investigating the effect of fresh frozen plasma transfusions on coagulation and host response. **Chapter 7** shows the results of a trial in coagulopathic non-bleeding critically ill patients receiving a prophylactic transfusion of FFP (12 ml/kg). We investigated whether INR prolongation parallels changes in the results of other tests investigating hemostasis and evaluated the coagulant effects of a fixed dose of FFP. We found that at baseline, levels of factor II, FV, FVII, protein C, protein S and antithrombin were reduced, and thrombin generation was impaired. Thromboelastometry variables were within reference ranges, except for a prolonged INTEM clot formation time. FFP transfusion increased the levels of coagulation factors FII, FV and FVII and the levels of anticoagulant proteins. Thrombin generation was unaffected by FFP transfusion, whereas ROTEM EXTEM clotting time and maximum clot firmness slightly improved in response to FFP. In conclusion, critically ill patients with coagulopathy as reflected by an increased INR show delayed thrombin generation, whereas viscoelastic test results were mostly preserved. Prophylactic FFP transfusion at a dose of 12 mL kg\(^{-1}\) increased the levels of individual coagulation factors, with concomitant increases in the levels of anticoagulants. However, the effects of FFP transfusion on thrombin generation and thromboelastometry were very limited, and failed to induce a more procoagulant state.

**Chapter 8** describes the effect of transfusion of FFP on endothelial and cytokine host response in ICU patients. We found that at baseline, systemic cytokine levels were mildly elevated. FFP transfusion resulted in a decrease of levels of TNF-α and other cytokines were not affected. FFP also resulted in a decrease in systemic syndecan-1 levels and a decrease in factor VIII levels, suggestive of an improved endothelial condition. This was associated with an increase in ADAMTS13 levels and a concomitant decrease in vWF levels. Therefore, we concluded that a fixed dose of FFP transfusion in critically ill patients decreases syndecan-1 and factor VIII levels, suggesting a stabilized endothelial condition, possibly by increasing ADAMTS13 which is capable of cleaving vWF.
Discussion

The aim of part II of this thesis was to assess the effect of an FFP transfusion on host response and haemostatic balance in critically ill patients. Although substantial units of Fresh Frozen Plasma (FFP) are utilized in the ICU, only a few small clinical trials have studied the efficacy of FFP in critically ill patients in terms of correction of coagulopathy.

We showed that critically ill patients with an increased INR show delayed thrombin generation, suggesting that INR prolongation does reflect a coagulopathy. Prophylactic FFP transfusion increased the levels of individual coagulation factors, with concomitant increases in the levels of anticoagulants. However, the effects of FFP transfusion on thrombin generation and thromboelastometry were very limited, and failed to induce a more procoagulant state. One could argue that this dose was too low to induce procoagulant effects. We chose for this dose as this dose reflects clinical practice. Also, as there was not even a trend towards improved thrombin generation, we do not think that higher doses of FFP would have resulted in a more procoagulant profile.

We conclude that an FFP transfusion should not be used to correct for a coagulopathy in the critically ill patient population. Therefore, one may state that over the past decades FFP has been transfused too liberally. It is needless to say that a more restrictive transfusion strategy will lead to less transfusion-related adverse events.

Of note, above mentioned conclusion may not be applicable to trauma patients requiring a massive transfusion. In this group, resuscitation with a higher ratio of FFP to red blood cell units is associated with decreased exsanguination. Of interest however, this association is independent of the effect of FFP on correction of coagulopathy, as found before and underlined by our findings in chapter 7, in which we showed that FFP fails to alter haemostatic balance. Thereby, there may be another cause for the beneficial effects of FFP as observed in studies on traumatic bleeding. This may be related to the restoration of injured endothelium as further investigated in chapter 8. Syndecan-1 is a proteoglycan on the luminal surface of endothelial cells which inhibits leukocyte adhesion. During endothelial damage, syndecan-1 is shed, resulting in increased levels of syndecan-1 in the systemic compartment. Patients in hemorrhagic shock have a disrupted endothelial integrity and glyocalix layer, with decreased syndecan-1 expression. Vascular integrity is also disrupted in various populations of critically ill patients, as demonstrated by increased systemic levels of syndecan-1. Of interest, in a hemorrhagic shock model, FFP was found to improve endothelial integrity, associated with increased expression of syndecan-1 on endothelial cells.

To further study this mechanism, we investigated the inflammatory and endothelial host response to a fixed dose of FFP transfusion in a study investigating the risk-benefit ratio of FFP transfusion in non-bleeding critically ill patients with a coagulopathy (chapter 8).
We found FFP transfusion decreased levels of TNF-α. FFP also resulted in a decrease in systemic syndecan-1 levels and a decrease in factor VIII levels, suggestive of an improved endothelial condition. This was associated with an increase in ADAMTS13 levels and a concomitant decrease in von Willebrand (vWF) levels. Therefore, we conclude that a fixed dose of FFP transfusion in critically ill patients decreases syndecan-1 and factor VIII levels, suggesting a stabilized endothelial condition, possibly by increasing ADAMTS13 which is capable of cleaving vWF.

We hypothesize that in contrast to patients with a coagulopathy, an FFP transfusion may be beneficial for patients with endothelial dysfuction, such as patients in shock. Thereby, patients receiving multiple units of red blood cells may benefit from FFP, by counteracting the increased shedding of vWF induced by RBC-derived vesicles. In conclusion, we call for more research into this interesting phenomena.

Part III – platelets

Summary

Part III contains a study investigating effects of platelet transfusions. Chapter 9 describes the effects of a transfusion on the development of nosocomial infections in critically ill patients. Whereas the transfusion of both RBCs and FFP was not a risk factor for the development of a nosocomial bacterial infection, platelet transfusion was. We found that the hazard ratio of infection was higher in patients receiving platelets, an effect that was independent of the other transfusion products, independent of disease severity and also seemed independent of the reason of receiving platelets, as patients receiving platelets did not differ from those who did not receive platelets.

Discussion

We found a strong association between the transfusion of platelets and the development of a nosocomial bacterial infection. This finding is different from previous studies, which have associated RBCs with the development of infections. An important explanation for these seemingly conflicting results may be that we corrected for exposure time. This study highlights the importance of the design of a statistical model. At the same time, this study also shows that observational studies are limited. A clear answer to whether the observed association is truly present would require an RCT with different platelet triggers, adequately powered to yield a possible difference in the occurrence of infections. This would require a very large number of patients. Most likely, such a trial will not be conducted. Thereby, we rely on observational studies which may suggest associations, which then need to be confirmed in appropriate models. In the mean time, as long as the pathophysiological mechanism behind the development of TRIM has not been elucidated, it is unlikely that preventive measures can be taken. Therefore, the only
preventive measure at our disposal nowadays is to reduce the amount of transfused platelets to a minimum.

Since this study did not look investigate the effect on overall outcome, we cannot state that a platelet transfusion should be omitted. This study highlights the need for research on appropriate platelet transfusion triggers in the critically ill patient populations.

In conclusion

There are two main findings of this thesis. The first is that there are multiple unexpected effects of transfusion, due to influences of the product on the host coagulation and inflammatory response. Thereby, the balance between adverse and beneficial effects of a blood transfusion in the critically ill patient is very delicate. A decision to transfuse should be carefully made, considering the intended aim, transfusion product and clinical condition of the recipient. This thesis may contribute to making this decision.

Even in the era of restrictive transfusion triggers, transfusion of blood products remains a cornerstone of treatment in the Intensive Care Unit, as anemia and thrombocytopenia are highly prevalent. Also, major bleeding often occurs. Thereby, it remains important to investigate interventions that may decrease pro-coagulant and pro-inflammatory adverse effects of transfusion products. This thesis may contribute to this aim by making the suggestion that the effect of depletion of RBC products from vesicles may warrant further study.
LITERATURE


Chapter 11

Nederlandse samenvatting

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**INTRODUCTIE**

**De geschiedenis van de bloedtransfusie**

In 1628 ontdekte de Engelse arts William Harvey de bloedsomloop en kort daarna vond de eerste poging tot een bloedtransfusie plaats. In 1665 vond echter pas de eerste succesvolle bloedtransfusie plaats, de arts Richard Lower hield honden in leven door ze een transfusie te geven met het bloed van andere honden. Het duurt nog tot 1818 voordat de eerste succesvolle humane bloedtransfusie het leven van een vrouw redt die leed aan een massale postpartum bloeding. De behandelende gynaecoloog, James Blundell, verrichtte in totaal 10 bloedtransfusies, waarvan er 5 ook daadwerkelijk voordeel opleverden voor zijn patiënten.


*Rode bloedcellen (erytrocyten)*

In de late jaren ’70 werden zowel het nieuwe anticoagulans CPDA-1 en de eerste bewaarvloeistof voor rode bloed cellen, saline-adenineglucose (SAG) ontwikkeld. In 1981 ontdekte dezelfde groep wetenschappers dat het toevoegen van mannitol aan SAG het membraan van de rode bloedcel beschermt en daarmee hemolyse reduceert. Deze nieuwe bewaarvloeistof heet SAGM en tegenwoordig is dit wereldwijd de meest gebruikte bewaarvloeistof. Echter, omdat de Food and Drugs Administration (FDA) geen licentie heeft afgegeven voor SAGM wordt dit niet in de Verenigde Staten gebruikt. Nadat werd vastgesteld dat een lagere concentratie leukocyten in het transfusieproduct leidde tot minder bijwerkingen is leukoreductie door middel van filtratie de standaard geworden in Nederland. Het volume van een zak rode bloedcellen is ongeveer 280 ml en het hematocriet varieert tussen de 50% en 65%. De transfusie van één eenheid rode bloedcellen leidt tot een stijging van de hemoglobine concentratie met ongeveer 0.5 mmol/L. In Nederland mag een zak rode bloedcellen maximaal 35 dagen bewaard worden bij een temperatuur tussen de 2 en 6 °C.
Vers bevroren plasma

De eerste plasmatransfusie in dieren vond plaats rond 1870, toen Bowditch en Luciani op zoek waren naar een bloedvervanger en schapenserum injecteerden in kikkers.1,3 De eerste plasmatransfusie in mensen werd gebruikt als behandeling van de Spaanse griep in 19181,4. Eind jaren '20 en begin jaren '30 werd plasma frequent gebruikt in ziekenhuizen1,3,5. In eerste instantie werd vers plasma gebruikt, maar wetenschappers toonden aan dat zowel gedroogd plasma als vers bevroren plasma even effectief waren en ook nog eens makkelijker te bewaren1,3,5,6. Tijdens de Tweede Wereldoorlog gebruikte het leger van de Verenigde Staten gedroogd plasma als een bloedvervanger omdat dat makkelijk te bewaren was1,7. Echter, het werd al snel duidelijk dat trauma slachtoffers ook zuurstofvervoerende capaciteiten nodig hadden en als gevolg daarvan werd het gedroogde plasma vervangen door volbloed1,7. Na de Tweede Wereldoorlog werden de indicatie voor vers bevroren plasma (Fresh Frozen Plasma, FFP) geleidelijk uitgebreid naar sepsis, brandwonden, voedingsdeficiënties, nefrotisch syndroom, sikkelcel anemie en pediatrische acute lymfoblastaire leukemie1,8-12. In 1964 werd de eerste gerandomiseerde gecontroleerde studie naar FFP gepubliceerd en ongeveer 70 andere zouden volgen1,13. Tegenwoordig is volgens de geldende richtlijnen een FFP transfusie echter alleen geïndiceerd in het geval van: enkele stollingsfactor deficiënties waarvoor geen virus-veilig product beschikbaar is, meerdere stollingsfactor deficiënties (b.v. diffuse intravasale stolling, DIS) in de aanwezigheid van een ernstige bloeding, thrombotische thrombocytopenische purpura (TTP), het antagoneren van warfarine in de aanwezigheid van een ernstige bloeding, een chirurgische bloeding en massale transfusie14.

Tegenwoordig wordt in Nederland Omniplasma gebruikt in plaats van FFP. Omniplasma wordt gemaakt door het ontdooien en poolen van meerdere units enkele-donor-FFP. Het belangrijkste voordeel van Omniplasma is dat het gepooled is uit 600-1200 donaties, waardoor adequate hoeveelheden van de verschillende stollingsfactoren gewaarborgd is en dilutie van eventueel aanwezige antistoffen bewerkstelligd wordt, wat resulteert in een reductie van het risico op transfusion-related-acute-lung-injury (TRALI). Eén eenheid Omniplasma bevat ongeveer 310 ml en kan gedurende 4 jaar bewaard worden, indien in het donker opgeslagen bij -18 °C.

Bloedplaatjes (trombocyten)

De eerste trombocytentransfusie werd beschreven in de jaren ’50 en werd gebruikt om mortaliteit door bloedingen in patiënten met acute leukemie te reduceren15-17. Sinds die tijd is het gebruik langzaamaan toegenomen. De overstap van glazen flessen naar de plastic zakken die we tegenwoordig nog steeds gebruiken was een belangrijke ontwikkeling, aangezien dit de verzameling en bereidende van trombocyten in een gesloten systeem mogelijk maakte. Hierdoor werd het risico op bacteriële contaminatie gereduceerd en maakte dit de implementatie van het eenvoudige twee-stappen centrifugatieprotocol
mogelijk. In de jaren ’70 begonnen wetenschappers met het verwijderen van de leukocytenrijke en trombocytenrijke buffy coats van de rode bloedcelconcentraten teneinde de witte cellen te gebruiken voor interferon-productie en de leukocyten-gerelateerde transfusiereacties te verminderen. Het regelmatige gebruik van deze procedure leidde tot een nieuwe procedure voor de bereiding van plaatjesconcentraten, namelijk de buffy coat methode, die we heden ten dage nog steeds gebruiken in Nederland. Eerst wordt volbloed afgedraaid in een centrifuge waardoor alle cellen zullen neerslaan. De dunne laag witte bloedcellen en bloedplaatjes bovenop de rode bloedcellen heet de buffy coat. Deze laag wordt verwijderd en vervolgens worden vijf buffy coats van dezelfde bloedgroep (ABO/Rh) verzameld, gepooled en verdund in autoloog plasma of een cristalloïde oplossing. De gepoolde buffy coats worden dan zacht gecentrifugeerd en het plaatjes-rijke supernatant wordt verzameld. Één zak bloedplaatjes bevat ongeveer 330 ml, de hoeveelheid plaatjes is minimaal 250 x 10^9 /ml en de bewaarvloeistof is Platelet Additive Solution type III (PAS-III). Een zak bloedplaatjes is maximaal zeven dagen houdbaar, mits op een zwenkapparaat bewaard bij 20-24 °C.

Transfusie in ernstig zieke patiënten – twee zijdes van de medaille

Transfusie van rode bloedcellen

Anemie is een veel voorkomend probleem bij ernstig zieke patiënten. Als gevolg hiervan wordt deze patiëntengroep veel getransfundeerd, waarbij tot 44% van de patiënten een bloedtransfusie krijgt. Als deze patiënten sepsis hebben krijgt zelfs 73% van hen een bloedtransfusie. De voor de hand liggende oorzaken van anemie in ernstig zieke patiënten zijn een verminderde productie van rode bloedcellen (bijvoorbeeld een slechte voedingsstatus, het bestaan van comorbiditeiten, een verminderde productie van erytropoëtine en een verminderde beschikbaarheid van ijzer) en een toegenomen verlies van rode bloedcellen (bijvoorbeeld toegenomen bloedverlies door chirurgische procedures of herhaalde bloedafnames of een bloeding). Daarbij komt nog dat er redenen zijn om aan te nemen dat in een inflammatoire staat een toegenomen klaring van rode bloedcellen een grote rol speelt. Hoewel algemeen wordt aangenomen dat de klaring van rode bloedcellen tijdens inflammatie wordt gemedieerd in de milt is dit nooit bewezen. Mogelijk spelen andere mechanismen van klaring een rol. Deze zijn onder andere een toegenomen PS-expressie op de rode bloedcel, ten gevolge van toegenomen plasmaconcentraties van sfingomyelinase geïnduceerd door tumor necrosis factor-α of door directe productie van bacteriën, wat vervolgens kan dienen als een “eat-me” signaal voor circulerende macrofagen. Ook kunnen rode bloedcellen direct gefagocyteerd worden door toegenomen expressie van Band op de rode bloedcel, een toename die wel wordt gezien in septische muizen, maar niet in gezonde muizen. Inflammatie kan ook leiden tot een verminderde deformabiliteit van de rode bloedcel in inflammatoire condities geïnduceerd door LPS of sepsis, dat uiteindelijk weer kan
leiden tot het vastlopen van rode bloedcellen in de microcirculatie. Ook kan het vastlopen van rode bloedcellen in de microcirculatie tijdens inflammatie in vitro gemedieerd worden door adhesie van de rode bloedcellen aan het endothelium^{38,39}.

Omdat de rode bloedcellen de zuurstof vanuit de longen naar de weefsels transporteren is het sinds jaar en dag gebruikelijk om rode bloedcellen genereus te suppleren teneinde een adequate zuurstofvoorziening van de weefsels te bewerkstelligen. Echter, de Transfusion Requirements in Critical Care (TRICC) studie toonde aan dat ernstig zieke patiënten lagere Hb waardes toch goed tolereren^{36}. Ook bleek dat jongere patiënten en net wat minder zieke patiënten die getransfundeerd werden volgens een restrictievere transfusiestrategie een betere 30-dagen mortaliteit hadden dan vergelijkbare patiënten die getransfundeerd werden volgens een liberale transfusiestrategie^{36}. Hierdoor werd een associatie gelegd tussen de transfusie van rode bloedcellen en een ongunstige uitkomst. Hiermee in overeenstemming zijn enkele observationele studies die ook een associatie legden tussen een transfusie met rode bloedcellen en een ongunstige uitkomst^{19,37}.

Vooral een associatie tussen een rode bloedceltransfusie en de ontwikkeling van acute lung injury^{38-45} en acute kidney injury^{41,46} wordt herhaaldelijk gevonden. TRALI (Transfusion-Related Acute Lung Injury) is een klinische diagnose en gedefinieerd als het ontstaan van acute lung injury (ALI) binnen 6 uur na een bloedtransfusie zonder de aanwezigheid van een andere risicofactor voor acute lung injury^{47-49}. Ook zijn possible TRALI (dat is TRALI in de aanwezigheid van een andere risicofactor) en delayed TRALI (dat is TRALI ontstaan binnen 6-72 uur na een bloedtransfusie) beschreven als afzonderlijke klinische ziektebeelden^{47-49}. De incidentie van TRALI varieert tussen de 0.08 en 15.1% per getransfundeerde patiënt en de 0.01 en 1.12% per getransfundeerd product^{50,51}. TRALI komt vaker voor in de ernstig zieke patiënten populatie^{45,50,51}.

Hoewel het aantonen van een associatie tussen transfusie en orgaanschade in veel Intensive Cares (ICs) heeft geleid tot het implementeren van een lagere transfusiegrens^{19,52}, blijft er een grote variatie in transfusiepraktijk bestaan^{53}. Aangezien een anemie ook geassocieerd is met een ongunstige uitkomst en de huidige lage transfusiegrens voor bepaalde specifieke patiënten populaties al te laag kan zijn, vormt dit een grote uitdaging voor zowel de behandelend arts als de wetenschappelijke gemeenschap die oproept tot interventies om rode bloedceltransfusies te verbeteren en het risico op orgaansfalen te reduceren. Overigens is het belangrijk om te vermelden dat de mechanismen achter de associatie tussen een rode bloedceltransfusie en een ongunstige uitkomst nog steeds onbekend zijn.

In dierstudies is langdurige opslag van de getransfundeerde rode bloedcellen duidelijk gerelateerd aan de ontwikkeling van longschade^{54-61}. Klinische data is echter tegenstrij-
dig. Zo zijn er observationele studies die een associatie tonen tussen een transfusie met langer opgeslagen rode bloedcellen en een ongunstige uitkomst\textsuperscript{41,62}, maar zijn er ook studies die geen associatie vinden tussen de leeftijd van bloed en de uitkomst\textsuperscript{45,63-65}. Deze verschillen kunnen verklaard worden door de heterogene studiepopulaties en het feit dat de meeste patiënten zowel verse als opgeslagen rode bloedcellen ontvingen. Recent zijn er twee grote gerandomiseerde gecontroleerde studies verschenen die geen verschil vonden in klinisch relevante uikomstparameters tussen ernstig zieke patiënten die verse en ernstig zieke patiënten die opgeslagen rode bloedcellen ontvingen\textsuperscript{71,72}. Deze bevindingen sluiten het bestaan van “storage lesion” (vrij vertaald: opslagschade) echter niet uit. Aangezien er een associatie bestaat tussen transfusie en orgaanfalen, blijft de noodzaak tot het verbeteren van de productie en bewaarcondities bestaan.

Er wordt gedacht dat orgaenschade wordt veroorzaakt door bio-actieve substanties die accumuleren tijdens de opslag van cellulaire bloedproducten. Bio-actieve lipiden\textsuperscript{50,54,59,60,66-69} en soluble CD40 ligand (sCD40L)\textsuperscript{50,58,70,71} zijn geïmpliceerd als oplosbare mediatoren in TRALI, maar er zijn ook studies die dit verband niet leggen\textsuperscript{72}. Recent zijn ook “extracellular vesicles” (EVs, vrij vertaald: extracellulaire vesikels) aangedragen als verantwoordelijke mediatoren. EVs zijn kleine vesikels van fosfolipiden die door de meeste celtypes uitgescheiden kunnen worden. EVs faciliteren de intercellulaire uitwisseling van receptoren, liganden, signaalmoleculen, genetische informatie etcetera zonder direct cel-tot-cel contact. Hoge concentraties EVs van rode bloedcellen zijn aanwezig in het supernatant van rode bloedcel transfusiezakken\textsuperscript{73}. Deze EVs initiëren en propageren trombine vorming en ze verkorten de stollingstijd \textit{in vitro}\textsuperscript{73-75}. Deze effecten van EVs zijn waarschijnlijk afhankelijk van de opslagduur van de zakken rode bloedcellen, omdat de concentratie en trombine-vormende eigenschappen van EVs toenemen met een toenemende opslagduur\textsuperscript{74,75}.

Naast een mogelijk verantwoordelijke oplosbare factor ondergaat ook de rode bloedcel zelf veranderingen tijdens opslag in de bloedbank. Het verliest bijvoorbeeld zijn Duffy antigeen expressie en daardoor zijn mogelijkheid tot het wegvangen van chemokines. Het Duffy antigeen is een minor bloedgroep antigeen dat aan een aantal verschillende inflammatoire chemokines bindt, waardoor deze aan de rode bloedcel gebonden chemokines onbereikbaar worden gemaakt voor circulerende neutrofielen\textsuperscript{76}. Ook verliezen rode bloedcellen hun deformabiliteit naarmate ze ouder worden, waardoor hun passage door de microcirculatie van de organen wordt bemoeilijkt\textsuperscript{77}. Dit vastlopen kan in inflammatoire condities nog eens extra versterkt worden door activatie van het endothelium\textsuperscript{78,79}, waardoor adhesiemoleculen op de endothecel worden opgereguleerd.

Concluderend blijft er ondanks de overduidelijke voordelen van een transfusie van rode bloedcellen en optimalisatie van het opslagproces nog steeds een associatie bestaan tussen een transfusie en het optreden van een ongunstige uitkomst. Dit vraagt om ver-
der onderzoek naar de mechanismen achter deze associatie in een poging de optimale bewaarcondities vast te stellen.

*Transfusie van vers bevroren plasma*

Aanzienlijke hoeveelheden vers bevroren plasma (FFP) worden getransfundeerd in de Intensive Care. In de praktijk wordt FFP gebruikt om afwijkende stollingstesten te corrigeren teneinde een bloeding te voorkomen. Studies tonen aan dat de prevalentie van stollingsstoornissen onder ernstige zieke patiënten hoog is: 30-66% heeft een International Normalized Ratio (INR) van >1.5 of een protrombine (PT) ratio van >1.5 en 8-45% heeft een trombocytopenie op enig tijdstip gedurende hun IC-opname. De meest voorkomende oorzaken van een gestoorde stolling zijn sepsis, multitrauma, hersenschade, massaal bloedverlies, leveraandoeningen, diffuse intravasale stolling, gebruik van vitamine K antagonisten voorafgaand aan de IC-opname, nierfalen, hartchirurgie en trombotische micro-angiopathieën.

Vers bevroren plasma corrigeert multipele stollingsfactor deficiënties zeer effectief en de richtlijnen adviseren het gebruik bij ernstig bloedverlies, maar ook patiënten die een coagulopathie hebben zonder ernstig bloedverlies ontvangen substantiële hoeveelheden FFP. Zelfs terwijl bewijs ontbreekt dat profylactische toediening bloedingscomplicaties voorkomt blijft ongepast gebruik wijdverspreid. In Nederland worden jaarlijks 80.000 eenheden FFP uitgegeven en de meeste hiervan worden getransfundeerd op de Intensive Care. Drie misvattingen worden verantwoordelijk gehouden voor dit ongepaste gebruik van vers bevroren plasma: allereerst nemen artsen aan dat een verhoogde PT/INR een toegenomen bloedingsrisico voorspelt voor patiënten die een invasieve ingreep ondergaan, ten tweede dat het voorafgaand aan de procedure toedienen van FFP de PT/INR zal corrigeren en tot slot dat de profylactische transfusie resulteert in minder bloedingscomplicaties.

Maar voorspelt een verhoogde INR/PT wel een toegenomen bloedingsrisico? Het stollingsstelsel bestaat uit drie belangrijke componenten. De pro-coagulante component omvat het endothelium, de trombocyten, de individuele stollingsfactoren en het fibrinogeen. De anti-coagulante component omvat proteïnes C en S en antitrombine. De derde component is het fibrinolytische systeem. De meeste standaard stollingstesten (aPTT, PT, trombocytopenie, fibrinogen en D-dimeer) reflecteren slechts een deel van dit complexe systeem en geven dus niet het netto resultaat van de actuele balans tussen de drie componenten weer. Derhalve kunnen de standaard stollingstesten het bloedingsrisico dus niet betrouwbaar voorspellen. In contrast met deze standaard testen, beoordeelt trombo-elastografie zowel de stolselvorming als de stolselafbraak. Het resulterende trombo-elastogram geeft de initiatiel van stolselvorming, fibrineformatie en stolselafbraak weer. In ernstig zieke patiënten die verdacht worden van een
coagulopathie kan trombo-elastografie de patiënten identificeren die ook daadwerkelijk een verhoogd bloedingsrisico hebben en bij wie een FFP transfusie geïndiceerd is. Echter, exacte waardes die een coagulopathie weergeven zijn nog niet gevalideerd voor patiënten op een Intensive Care.

Bovendien, verbetert een FFP transfusie voorafgaand aan een invasieve ingreep de PT/INR en meer in het algemeen, herstelt een FFP transfusie de stollingscapaciteit? Slechts een klein aantal klinische studies hebben het effect van een FFP transfusie in ernstig zieke patiënten met een stollingsstoornis onderzocht. Deze studies gebruikten verschillende doseringen, keken niet naar het effect van de FFP transfusie op het uiteindelijke bloedingsrisico en includeerden zowel bloedende als niet-bloedende patiënten\textsuperscript{86,95}. Bij het beoordelen van de effectiviteit van een FFP transfusie is de dosis van belang. Een adequate dosis zal de INR/PT corrigeren aangezien het alle stollingsfactoren suppleert\textsuperscript{95,96}. Echter, sinds de PT/INR slechts een deel van het stollingssysteem weergeeft hoeft dit niet te betekenen dat een FFP transfusie ook daadwerkelijk de coagulopathie corrigeert. Alles samengenomen is het bewijs dat de effectiviteit van FFP in het corrigeren van een coagulopathie ondersteunt flinterdun. Verder bestaat er ook geen bewijs dat een FFP transfusie bloedingscomplicaties vermindert in Intensive Care patiënten met een stollingsstoornis.

Opvallend is is dat epidemiologische studies suggereren dat in traumapatiënten die een massale transfusie nodig hebben, resuscitatie met een hogere ratio van FFP versus rode bloedcellen geassocieerd is met een lagere mortaliteit\textsuperscript{97,98}. Belangrijk om toe te lichten is dat deze associatie onafhankelijk was van het effect van de FFP transfusie op de correctie van de coagulopathie\textsuperscript{98,99}. Aangezien FFP normaal gesproken gunstig is in bloedende patiënten door het corrigeren van de stollingsfactordeficiëntie suggereert deze observatie een ander werkingsschematische van FFP.

Vergelijkbaar met een rode bloedceltransfusie is er een associatie tussen een FFP transfusie en een ongunstige uitkomst in de ernstige zieke patiënten populatie, zoals multiorgaanfalen,\textsuperscript{100,101} transfusion-related circulatory overload\textsuperscript{102,103} en een verhoogd risico op infecties\textsuperscript{104}. Maar vooral is een FFP transfusie geassocieerd met TRALI\textsuperscript{39,63,100,105,106}. Meer specifiek met antilichaam-gemedieerde TRALI, wat wordt veroorzaakt door de passieve infusie met human leucocyte antigen (HLA) en human neutrophil antigen (HNA) antilichamen afkomstig uit het donorbloed\textsuperscript{107,108}. Deze antilichamen worden voornamelijk gevonden in het bloed van multipara vrouwen, aangezien zij tijdens hun zwangerschappen zijn gesensitiseerd doordat zij blootgesteld zijn geweest aan de antilichamen van hun foetus\textsuperscript{109-113}.

Het risico van plasmadonatie door vrouwen was bevestigd in twee studies in ernstig zieke patiënten\textsuperscript{114,115}. Het Verenigd Koninkrijk implementeerde als eerste een FFP
transfusiebeleid waarbij alleen mannelijke donoren plasma mochten doneren en vele landen volgden. De incidentie van TRALI is sinds die tijd significant afgenomen en twee recente meta-analyses rapporteerden dat het uitsluiten van vrouwelijke donoren de incidentie van plasma-gerelateerde TRALI verminderden met 73% en twee recente meta-analyses rapporteerden dat het uitsluiten van vrouwelijke donoren de incidentie van plasma-gerelateerde TRALI verminderden met 73% en twee recente meta-analyses rapporteerden dat het uitsluiten van vrouwelijke donoren de incidentie van plasma-gerelateerde TRALI verminderden met 73%. Echter, het beleid om alleen gebruik te maken van mannelijke plasmadonoren heeft dan wel de incidentie van TRALI verminderd, maar het risico is nog niet volledig geweken.

Concluderend ontbreekt, ondanks het wijdverbreide gebruik in de Intensive Care, nog steeds kennis van alle effecten van een FFP transfusie. Kennis van deze effecten is noodzakelijk om de voordelen en nadelen van een plasmatransfusie in ernstig zieke patiënten af te wegen.

**Transfusie van bloedplaatjes (trombocyten)**

Een trombocytopenie is een veel voorkomende bevinding in ernstig zieke patiënten. Ongeveer 40% van de patiënten opgenomen op een Intensive Care heeft een trombocytopenie (trombocytengetal lager dan 150 x 10^9 per L). Er zijn veel oorzaken voor een trombocytopenie, maar sepsis, diffuse intravasale stolling, massaal bloedverlies, trombotische micro-angiopathieën, heparine-geïnduceerde trombocytopenie, immuun trombocytopenie en medicatie-geïnduceerde trombocytopenie zijn de meest voorkomende.

Een transfusiegrens voor bloedplaatjes specifiek voor ernstig zieke patiënten is niet vastgesteld. De gebruikte transfusiegrenzen zijn vastgesteld op basis van gerandomiseerde studies en de resulterende richtlijnen, hoewel niet specifiek voor de ernstig zieke patiënt ontworpen, adviseren verschillende transfusiegrenzen, variërend van 10 x 10^9 tot 50 x 10^9 per L, voor profylactische transfusies voor verschillende indicaties.

Hoewel een trombocytopenie een risicofactor is voor een ongunstige uitkomst, zoals een massale bloeding, toegenomen length-of-ICU-stay en sterfte, zijn de studies niet éénsgezind of een FFP transfusie nu de overleving verbetert of verslechtert. Het kan natuurlijk zijn dat verslechtering van de overleving een confounder is voor de ernst van de ziekte, maar er kunnen ook ongunstige effecten van een plaatjestransfusie zijn die zwaarder wegen dan de gunstige effecten. Deze zijn onder andere transmissie van infectie, allergische reacties, TRALI, transfusion-related immunomodulation (TRIM) en veneuze trombo-embolie. TRIM is geassocieerd met vertraagde transplantaatafstoting, toegenomen terugkeer van kanker en een hogere vatbaarheid voor nosocomiale bacteriële infecties. Inderdaad, studies tonen aan dat een trombocytentransfusie geassocieerd is met het ontstaan van nosocomiale infecties in een aantal ernstig zieke patiëntenpopulaties. Derhalve is het belangrijk de voordelen van een trombocytentransfusie af te wegen tegen de nadelen en is het belangrijk een transfusiegrens zorgvuldig vast te stellen.
Waar naïeve bloedplaatjes een natuurlijke levensduur van 8-12\textsuperscript{137} dagen hebben, kunnen bloedplaatjes die zijn geprepareerd voor transfusie slechts 2-7 dagen bewaard worden bij 20-24\textsuperscript{0}C onder continue zwenking. Dit vermindert allereerst het risico op bacteriële groei, maar minimaliseert ook de “storage lesion”, wat weer gecorreleerd is met een toegenomen opbrengst en overleving \textit{in vivo} alsmede een betere hemostatische activiteit na transfusie\textsuperscript{150,151}. Hoewel de transfusie met oudere bloedplaatjes in dierstudies duidelijk gecorreleerd is met de ontwikkeling van TRALI\textsuperscript{50,56,66,68,138}, zijn de klinische studies hier niet éénduidig over\textsuperscript{45,50,63-65,139}.

Concluderend zijn de voor- en nadelen van een trombocytentransfusie in de Intensive Care nog steeds verbazingwekkend onduidelijk.

**DOELEN VAN DIT PROEFSCHRIFT**

Dit proefschrift bevat zowel klinische als pre-klinische studies naar de effecten van een bloedtransfusie met verschillende componenten in ernstig zieke patiënten. De studies richten zich vooral op de mechanismen achter de associatie tussen een bloedtransfusie en een ongunstige uitkomst.

**Rode bloedcellen:** Het onderzoeken van de rol van opslagduur en inflammatie op de “host response” na een bloedtransfusie (deel I)

**Vers bevroren plasma:** Het onderzoeken van het effect van een FFP transfusie op de hemostatische balans en “host response” in Intensive Care patiënten (deel II)

**Bloedplaatjes:** Het onderzoeken van het effect van een trombocytentransfusie op de ontwikkeling van infecties in Intensive Care patiënten

**HOOFDLIJNEN VAN DIT PROEFSCHRIFT**

**Hoofdstuk 2** bevat een samenvatting van de kennis van de mechanismen van de klaring van rode bloedcellen in patiënten met een inflammatoire aandoening

**Hoofdstuk 3** onderzoekt de routing van donor rode bloedcellen na een transfusie in een rattenmodel, waarbij gebruik wordt gemaakt van verse en opgeslagen humane rode bloedcellen

**Hoofdstuk 4** toont de resultaten van een studie in endotoxemische ratten die een transfusie met verse of opgeslagen gelabelde rode bloedcellen van ratten krijgen. De hypothese was dat endotoxemie leidt tot een toegenomen klaring van donor rode
bloedcellen uit de circulatie, met gelijktijdig vastlopen van rode bloedcellen in de
organen, wat uiteindelijk bijdraagt aan transfusie-gerelateerde orgaanschade. Verder
hypothetiseerden we dat opgeslagen donor rode bloedcellen sneller geklaard worden
uit de circulatie dan verse donor rode bloedcellen.

**Hoofdstuk 5** bevat de resultaten van een *in vitro* experiment dat de hypothese onder-
zoekt dat EVs die zich tijdens de opslag ophopen in rode bloedcelproducten bijdragen
aan een pro-inflammatoire “host response” en dat dit gerelateerd is aan zowel de hoe-
veelheid EVs als de opslagduur van de rode bloedcelproducten.

**Hoofdstuk 6** bevat de resultaten van een *in vitro* experiment dat de hypothese onder-
zoekt dat EVs afkomstig uit rode bloedcelproducten endotheelcellen kunnen activeren
door activatie van immuuncellen van de ontvanger en dat dit effect afhankelijk is van de
opslagduur van de rode bloedcelproducten.

**Hoofdstuk 7** toont de resultaten van een prospectieve vooraf gedefinieerde substudie
van een gerandomiseerde studie in coagulopathische niet-bloedende ernstig zieke
patiënten die een profylactische FFP transfusie (12 ml/kg) krijgen voorafgaand aan een
invasieve ingreep. We onderzochten of INR verlenging correleert met visco-elastiche
metingen en evalueerden het effect van een vaste dosis FFP op de hemostatische balans
van deze patiënten.

**Hoofdstuk 8** bevat de resultaten van een prospectieve vooraf gedefinieerde substudie
van een gerandomiseerde studie in coagulopathische niet-bloedende ernstig zieke
patiënten die een profylactische FFP transfusie (12 ml/kg) krijgen voorafgaand aan een
invasieve ingreep. We hypothetiseerden dat een FFP transfusie een pro-inflammatoire
“host-response” opwekt.

**Hoofdstuk 9** verkent de effecten van verschillende transfusieproducten op het voorko-
men van nosocomiale infecties in een prospectieve multicenter observationele studie in
ernstig zieke patiënten. We hypothetiseerden dat de hoeveelheid van elk bloedproduct
is geassocieerd met een hoger risico op nosocomiale infecties.

Dit proefschrift eindigt met een samenvatting van bovenstaande studies en een alge-
mene discussie in **Hoofdstuk 10**, waarvan hieronder de Nederlandse samenvatting
wordt gegeven.
SAMENVATTING EN DISCUSSIE

Deel I – Rode bloedcellen

Samenvatting

Deel I bevat studies naar rode bloedcel transfusies. Hoofdstuk 2 geeft een overzicht van de kennis van de mechanismen van de klaring van rode bloedcellen als deze verouderen en de klaring van rode bloedcellen in patiënten met een inflammatoire aandoening. We bespreken dat anemie der inflammatie multifactorieel is. Er is een verminderde erytroëtine productie, verminderde beenmerg respons op erytropoëtine, verminderde beschikbaarheid van ijzer en een toegenomen klaring van rode bloedcellen. Aangezien dit proefschrift laat zien dat inflammatie bijdraagt aan een toegenomen klaring van rode bloedcellen in de organen en we hypothetiseren dat klaring een belangrijk onderdeel is van anemie der inflammatie, borduurt deze review met name voort op de pathogene nese van de toegenomen rode bloedcelklaring. We stellen dat de PS expressie op het membraan van de rode bloedcel in inflammatie voornamelijk is toegenomen door een toename van ceramide, danwel veroorzaakt door een toename in sphingomyelinase activiteit danwel door een toename in Platelet Activating Factor, tumor necrosis factor-α of directe productie door bacteriën. Fagocytose van rode bloedcellen tijdens inflamm atie wordt gemedieerd door het rode bloedcel membraaneiwit Band 3. Verminderde deformabiliteit van rode bloedcellen lijkt ook een belangrijk kenmerk in inflammatie en kan ook gemedieerd worden door Band 3, maar ook door stikstofoxide, reactieve zuurstofradicalen en saalzuurresiduen. Verder is ook de binding van rode bloedcellen aan het endothelium toegenomen onder inflammatoire omstandigheden, waarschijnlijk door toegenomen expressie van endotheliale adhesi moleculen, door de toegenomen PS expressie op het membraan van de rode bloedcellen en door de verminderde capilla laire bloeddoorstroming. Daarmee vertoont de klaring van rode bloedcellen tijdens inflammatie gelijkenissen met de klaring van rode bloedcellen vanwege het natuurlijke verouderingsproces, maar speelt er ook een ander mechanisme een rol: namelijk de toegenomen adhesie aan het endotheel. Dit wordt verder onderzocht in Hoofdstuk 6.

Hoofdstuk 3 richt zich op tranfusiemodellen. In de literatuur wordt het effect van humane rode bloedcellen vaak geëvalueerd in diermodellen. Aangezien het onduidelijk is hoe dit de klaring beïnvloedt, hebben we de route van klaring na een rode bloedceltransfusie bestudeerd in een rattenmodel waarbij we gebruik maakten van verse en opgeslagen humane rode bloedcellen. Kort na de transfusie vonden we de humane rode bloedcellen nog terug in de circulatie van de ontvangers, maar 24 uur na de transfusie konden we geen humane rode bloedcel meer detecteren in de circulatie van de ratten. In plaats daarvan vonden we de bloedcellen terug in de organen van de dieren. Aangezien een belangrijk kwaliteitskenmerk van een tranfusieproduct is dat
de opbrengst 24 uur na transfusie minimaal 75% moet zijn, concludeerden we dat het gebruik van humane rode bloedcellen in een knaagdiermodel geen geschikt model is om de effectiviteit van een rode bloedceltransfusie te onderzoeken en dat er bij voorkeur een syngeen model gebruikt moet worden.

**Hoofdstuk 4** toont de resultaten van een studie verricht in endotoxemische ratten die een bloedtranfusie ontvingen met verse of met opgeslagen gelabelde rode bloedcellen van ratten. We hypothetiseerden dat endotoxemie bijdraagt aan een toegenomen klaring van donor rode bloedcellen uit de circulatie, met gelijktijdig vastlopen van deze rode bloedcellen in de organen en daarbij bijdragend aan transfusie-gerelateerde orgaanschade. Inderdaad leidde endotoxemie tot toegenomen vastlopen van de ge-transfundeerde rode bloedcellen in verschillende organen, met name in de longen en de nieren. Dit vastlopen was geassocieerd met longschade, maar niet met nierschade. Verder leidde endotoxemie niet tot het vastlopen van donor rode bloedcellen in de milt en lever. Overeenkomstig hiermee is er geen klinische associatie bekend tussen een bloedtranfusie en het optreden van schade van de lever of milt. Verder hypothetiseerden we dat opgeslagen donor rode bloedcellen sterker uit de circulatie zouden worden geklaard dan verse donor rode bloedcellen. Inderdaad leverden opgeslagen donor rode bloedcellen een significant lagere post-transfusie opbrengst op in vergelijking tot verse donor rode bloedcellen, zowel in gezonde als endotoxemische ratten. Maar aangezien een langere opslagduur niet leidde tot toegenomen vastlopen van de donor rode bloedcellen in de organen is verder onderzoek naar het onderliggende mechanisme van een verminderde 24-uurs opbrengst van opgeslagen rode bloedcellen nodig. Conclusief leidt endotoxemie tot een toegenomen percentage donor rode bloedcellen dat vastloopt in de longen en nieren en dit is geassocieerd met longschade. Het vastlopen van donor rode bloedcellen in de microcirculatie tijdens een inflammatoire aandoening kan de associatie tussen longschade en een rode bloedceltransfusie in ernstig zieke patiënten verklaren.

**Hoofdstuk 5** bevat de resultaten van een in vitro experiment waarin volbloed werd geïncubeerd met supernatant van rode bloedcelproducten die gedurende een verschillende periode in de bloedbank zijn bewaard en welke danwel (verschillende hoeveelheden) EVs bevatten, danwel geen EVs bevatten. We hypothetiseerden dat EVs die tijdens opslag ophopen in rode bloedcelproducten bijdragen aan een pro-inflammatoire “host-response” in ontvangers van het transfusieproduct en dat dit gerelateerd is aan zowel de hoeveelheid EVs als aan de opslagduur van het betreffende transfusieproduct. Inderdaad induceerde incubatie met supernatant dat EVs bevatte van zowel verse als opgeslagen rode bloedcel transfusieproducten tot een sterke “host-response” waarbij TNF, IL-6 en IL-8 geproduceerd werden. Als dit supernatant geen EVs bevatte, dan was deze “host-response” volledig afwezig en deze “host-response” was afhankelijk van zo-
wel het aantal EVs in het supernatant alsmede de opslagduur van het transfusieproduct waarvan het supernatant afkomstig was. Concluderend lokken EVs uit rode bloedcel-transfusieproducten een sterke, dosis-afhankelijke “host-response” uit in de ontvanger en kunnen ze daarom bijdragen aan de ongunstige uitkomst die wordt gezien in ernstig zieke patiënten die een rode bloedceltransfusie ontvangen.

**Hoofdstuk 6** werkt het effect van EVs uit rode bloedcel transfusieproducten verder uit. Human umbilical vein endothelial cells (HUVECs) werden geïncubeerd met supernatant van rode bloedcelproducten die gedurende lange of korte tijd in de bloedbank zijn bewaard en welke danwel EVs bevatten, danwel geen EVs bevatten, zowel in de aanwezigheid als in de afwezigheid van immuuncellen. We hypothetiseerden dat EVs uit de rode bloedceltransfusieproducten endotheelcellen kunnen activeren door activatie van immuuncellen van de ontvanger en dat deze eigenschap afhankelijk is van de opslagduur van het transfusieproduct in de bloedbank. Incubatie van HUVECs met alleen monocyten leidde niet tot de opregulatie van adhesiemoleculen, maar de incubatie met zowel monocyten als supernatant mét EVs leidde tot een opregulatie van ICAM-1 en E-selectine op de endotheelcellen. Deze opregulatie was afwezig wanneer de HUVECs werden geïncubeerd met zowel monocyten als supernatant zonder EVs. De EVs induceerden verder zonder tussenkomst van monocyten endotheliale secretie van von Willebrandfactor (vWF). Zowel de opregulatie van endotheliale adhesiemarkers als de endotheliale secretie van vWF waren onafhankelijk van de opslagduur van de transfusieproducten waaruit de EVs werden geïsoleerd. Verder onderzochten we de fagocytose van EVs door monocyten en toonden we aan dat EVs worden gefagocyteerd door monocyten, wat deels werd opgeheven door co-incubatie met twee verschillende complement receptor 3 (CR3) blokkerende antilichamen. Hieruit concluderen we dat EVs uit rode bloedcel transfusieproducten een pro-inflammatoire en pro-coagulante endotheelresponse induceren, welke onafhankelijk is van opslagduur en gemedieerd wordt door activatie van monocyten via complement receptor 3. Hiermee tonen we weer aan dat EVs uit rode bloedcel transfusieproducten een pro-inflammatoire response in de ontvanger induceren. Belangrijk is dat dit effect niet volledig verklaard kan worden door de “storage lesion”, aangezien de effecten gelijk zijn als EVs uit verse of opgeslagen transfusieproducten gebruikt werden.

**Discussie**

Het doel van **deel I** van dit proefschrift was om het effect van inflammatie op de klaring en ongunstige effecten van rode bloedcellen te onderzoeken en om te onderzoeken of de leeftijd van de getransfundeerde rode bloedcelproducten ook een invloed heeft op dit effect. Wij richtten ons dan met name op de rol van extracellulaire vesikels (EVs) die zich ophopen in een rode bloedcel transfusieproduct tijdens opslag in de bloedbank.
Aangezien anemie een veelvuldige bevinding is in de Intensive Care en karakteristiek is voor sepsis, begonnen we dit proefschrift met de beschrijving van de pathogenesis van anemie der inflammatie (hoofdstuk 2). De mechanismen van rode bloedcelklering in het natuurlijke verouderingsproces, namelijk phosphatidylserine expositie, fagocytose van erytrocyten en vermindere deformabiliteit zijn allen toegenomen in een inflammatoire conditie. Tijdens inflammatie speelt echter ook een ander mechanisme van klaring een rol, namelijk de binding van rode bloedcellen aan het endotheel. Incubatie van zowel endotheelcellen als rode bloedcellen met endotoxine leidde tot een toename van de binding van rode bloedcellen aan endotheelcellen\(^79\). Dit effect werd ook geobserveerd na stimulatie met TNF-\(\alpha\)\(^78\). Dit is een mechanisme van anemie der inflammatie dat tot dusver weinig aandacht heeft gekregen.

Als binding van donor rode bloedcellen aan het endothelium een rol speelt, kan dit dan tevens een mechanisme zijn van klaring? Om dit te onderzoeken gebruikten we een diermodel waarin ratten werden getransfundeerd met humane rode bloedcellen teneinde deze cellen te kunnen volgen na transfusie (hoofdstuk 3). Echter, hoewel we direct na transfusie humane rode bloedcellen vonden in de circulaties van alle ontvangers, konden we na 24 uur in geen van de circulaties van de ontvangers nog humane rode bloedcellen terugvinden. Helaas moesten we concluderen dat het gebruik van humane rode bloedcellen in knaagdierontvangers geen geschikt model is om de effectiviteit van een rode bloedceltransfusie te onderzoeken en dat er bij voorkeur syngene modellen gebruikt dienen te worden. Dit hoofdstuk pleit dus tegen het gebruik van dat soort modellen. Voor verdere experimenten hebben we dan ook een syngene model gebruikt (hoofdstuk 4). We vonden dat endotoxemie leidt tot het vastlopen van getransfundeerde rode bloedcellen in verschillende organen, met name de longen en nieren, de twee organen het meest frequent geassocieerd met transfusie-gerelateerde orgaanschade. Het vastlopen van donor rode bloedcellen in de microcirculatie van de longen tijdens inflammatie kan de associatie tussen longschade en rode bloedceltransfusies in de ernstig zieke patiëntenpopulatie verklaren. Belangrijk is dat hoewel een langere opslagduur leidde tot een verminderde posttransfusie opbrengst, donor rode bloedcellen niet werden gevonden in de milt. Aangezien hemolyse was toegenomen in dieren die langer opgeslagen rode bloedcellen ontvingen, sugge- reert dit eerder dat opgeslagen donor rode bloedcellen niet gefagocyteerd worden in de milt, maar hemolyse ondergaan.

We leverden geen direct bewijs dat de rode bloedcellen vastliepen in de organen na daadwerkelijke binding aan het endotheel. Hierdoor blijft het onduidelijk hoe rode bloedcellen interacteren met het endotheel. We vonden eerder dat het wassen van rode bloedcellen voorafgaand aan de transfusie leidde tot vermindering van orgaanschade
in een ratten transfusiemodel, wat suggereert dat er een oplosbare factor in het bloedproduct verantwoordelijk is voor de orgaanschade. Verder vonden we dat EVs van zowel verse als opgeslagen rode bloedceltransfusieproducten een sterke dosis-afhankelijke inflammatoire “host-response” uitlokten in ontvanger in een in vitro volbloedstimulatie experiment (hoofdstuk 5).

De vraag bleef echter of EVs ook opregulatie van adhesiemoleculen op het endotheel kunnmen geven. In hoofdstuk 6 vonden we dat EVs en monocyten los van elkaar geen opregulatie induceerden, maar de combinatie van beiden wel. Tevens vonden we dat EVs ook direct endotheliale secretie van vWF kunnen induceren.

Concluderend lijken EVs een doorslaggevende rol te spelen in het complexe systeem dat leidt tot transfusie-gereeleteerde orgaanschade. EVs uit rode bloedceltransfusieproducten worden gefagocyteerd door monocyten en monocyten kunnen vervolgens het endotheel activeren dat leidt tot de binding van rode bloedcellen aan dit endotheel. We hypothetiseren dat dit proces resulteert in het obstrueren van de microcirculatie. Dit kan verder verergerd worden door de EV-geënduicte endotheliale secretie van vWF, wat kan leiden tot een pro-coagulantie staat en de formatie van micro-trombi en daarmee weer tot verdere orgaanschade. Alles samengenomen kan de depletie van het rode bloedcel transfusieproduct van EVs direct voorafgaand aan de transfusie, bijvoorbeeld door wassen of filtratie, deze pathofysiologische cascade onderbreken en daarmee leiden tot minder transfusie-gereeleteerde orgaanschade.

Deel II – vers bevroren plasma

Samenvatting

Deel II bevat studies die het effect van een FFP transfusie op de stolling en de “host response” onderzoeken. Hoofdstuk 7 toont de resultaten van een studie in coagulopathische niet-bloedende ernstig zieke patiënten die een profylactische FFP transfusie (12 ml/kg) krijgen. We onderzochten of INR verlenging correleert met visco-elastische metingen en evalueerden het effect van een vaste dosis FFP op de hemostatische balans van deze patiënten. We vonden dat de uitgangswaarden van factoren II, V en VII, alsmede proteïne C, proteïne S en antitrombine waren verlaagd en dat de aanmaak van trombine was verminderd. Trombo-elastografische waarden waren binnen de normale range, met uitzondering van een verlengde “INTEM clot formation time”. Een FFP transfusie verhoogde de waardes van de stollingsfactoren FII, FV en FVII en de waardes van de antostollings eiwitten. Trombine aanmaak werd echter niet beïnvloed door een FFP transfusie, terwijl de trombo-elastografische “EXTEM clotting time” en “maximum clot firmness” licht verbeterden. Concluderend hebben wij aangetoond dat ernstig zieke patiënten met een verlengde INR een vertraagde trombine aanmaak hebben, terwijl visco-elastische meetresultaten vrijwel volledig behouden bleven. Een
Hoofdsteuk 8 beschrijft de effecten van een FFP transfusie op de “host response” van het endotheel en de cytokines van ernstig zieke patiënten. We vonden dat de uitgangswaardes van alle systemische cytokines licht verhoogd waren. Een FFP transfusie resulteerde in een daling van TNF-α, maar de overige cytokines werden niet beïnvloed. Een FFP transfusie leidde ook tot een daling van het systemische syndecan-1 en een daling van factor VII, wat een verbetering van de endotheliale conditie suggereert. Dit was geassocieerd met een toename van ADAMTS13 en een gelijktijdige afname van vWF. Hieruit concluderen wij dat een FFP transfusie in ernstig zieke patiënten syndecan-1 en factor VIII doet dalen, wat een stabilisatie van het endotheel suggereert, mogelijk door een toename van ADAMTS13 dat vWF knipt.

Discussie
Het doel van deel II van dit proefschrift was om het effect van een FFP transfusie op de “host response” en hemostatische balans van ernstig zieke patiënten te onderzoeken. Hoewel substantiële hoeveelheden FFP worden getransfundeerd in de Intensive Care, hebben slechts enkele kleine klinische studies het effect hiervan op de coagulopathie bestudeerd.

Wij toonden aan dat ernstig zieke patiënten met een verhoogde INR een vertraagde trombine aanmaak vertoonden, wat suggerereert dat een verlengde INR inderdaad wijst op een coagulopathie. Een FFP transfusie verhoogde de hoeveelheid individuele stollingsfactoren en dit ging gepaard met een verhoging van de natuurlijke anticoagulantia. De effecten van een FFP transfusie op trombine aanmaak en trombo-elastografische metingen was echter minimaal en de transfusie induceerde dan ook geen procoagulantere staat in de ontvangers. Men zou kunnen zeggen dat de gebruikte dosis te laag was voor het induceren van procoagulant effecten, maar wij hebben voor deze dosis gekozen omdat dit de praktijk in de kliniek weergeeft. Ook was er zelfs geen trend naar een verbeterde trombine aanmaak, wat ons doet vermoeden dat een hogere dosis ook niet had geleid tot een meer procoagulant profiel.

Wij concluderen dan ook dat een FFP transfusie niet gebruikt zou moeten worden voor het corrigeren van een coagulopathie in de ernstig zieke patiëntenpopulatie. Men zou zelfs kunnen zeggen dat FFP gedurende de afgelopen tientallen jaren te liberaal is getransfundeerd. Het lijkt ons overbodig te vermelden dat een restrictievere transfusie-strategie zal leiden tot minder transfusie-gerelateerde bijwerkingen.
Het is belangrijk om te vermelden dat bovenstaande conclusies wellicht niet van toepassing zijn op traumapatiënten die een massale transfusie nodig hebben. In deze groep patiënten is een transfusie met meer FFPs ten opzichte van rode bloedcellen geassocieerd met vermindere exsanguiatie\textsuperscript{140}. Opvallend is echter dat deze associatie onafhankelijk is van het effect van FFP op de correctie van een coagulopathie, zoals eerder is gevonden\textsuperscript{98,99} en wat onderschreven wordt door onze bevindingen uit hoofdstuk 7, waarin we aantonen dat een FFP transfusie de hemostatische balans niet verandert. Daarom is er wellicht een andere oorzaak voor de gunstige effecten van FFP die worden gevonden in studies naar traumatisch bloedverlies. Deze effecten zouden te maken kunnen hebben met het herstel van beschadigd endotheel, zoals wij hebben onderzocht in hoofdstuk 8. Syndecan-1 is een proteoglycaan op het luminale oppervlak van de endotheelcellen dat de adhesie van leukocyten verhindert. Indien er endotheelschade optreedt wordt syndecan-1 afgescheiden, waardoor de hoeveelheid syndecan-1 in het systemische compartiment toeneemt\textsuperscript{141}. De integriteit van het endotheel en de glyco-calix van patiënten met een hemorragische shock is verstoord, waardoor er een vermindere expressie van syndecan-1 op het endotheel is\textsuperscript{142}. De vasculaire integriteit is tevens verstoord in diverse populaties van ernstig zieke patiënten zoals wordt aangetoond door een toename van de systemische hoeveelheden syndecan-1\textsuperscript{143,144}. Belangrijk is dat een FFP transfusie in een model van hemorrhagische shock leidde tot een verbetering van de integriteit van het endotheel en dat dit geassocieerd was met een toegenomen expressie van syndecan-1 op de endotheelcellen\textsuperscript{145}.

Om dit mechanisme verder te bestuderen onderzochten we de inflammatoire en endotheeliale “host response” op een FFP transfusie in een studie waarin we eerder keken naar de risico-batenverhouding van een FFP transfusie in niet-bloedende ernstig zieke patiënten met een coagulopathie (hoofdstuk 8). We vonden dat een FFP transfusie de hoeveelheid TNF-\(\alpha\) liet afnemen. Verder resulteerde de FFP transfusie in een daling van de systemische hoeveelheid syndecan-1 en een toename van factor VIII en was dit geassocieerd met een toename van ADAMTS13 en een gelijktijdige afname van vWF. Hieruit concluderen we dat een FFP transfusie in ernstig zieke patiënten leidt tot een stabilisatie van het endotheel, wat mogelijk veroorzaakt wordt door een daling van de hoeveelheid vWF doordat dit geknipt wordt door de toegenomen hoeveelheid ADAMTS13.

We hypothetiseren dan ook dat een FFP transfusie in patiënten met een endotheeliale dysfunctie, zoals bijvoorbeeld wordt gezien tijdens shock, in tegenstelling tot patiënten met een coagulopathie gunstig kan zijn. Hierdoor zou het ook mogelijk zijn dat patiënten die meerdere eenheden rode bloedcellen ontvangen baat kunnen hebben van een gelijktijdige FFP transfusie, teneinde het effect van de toegenomen secretie van vWF door EVs tegen te gaan. Concluderend roepen wij dan ook op tot meer onderzoek naar dit interessante fenomeen.
Deel III - bloedplaatjes

Samenvatting

Deel III bevat een studie die het effect van een transfusie met bloedplaatjes onderzoekt. Hoofdstuk 9 beschrijft de effecten van een transfusie op de ontwikkeling van nosocomiale infecties in ernstig zieke patiënten. Terwijl een transfusie met zowel rode bloedcellen als FFP geen verhoogd risico gaf op het ontwikkelen van een nosocomiale infectie, was een transfusie met bloedplaatjes hiermee wel geassocieerd. Wij vonden dat de “hazard ratio” van infectie hoger was in patiënten die bloedplaatjes getransfundeerd kregen en dat dit effect onafhankelijk was van de andere transfusieproducten, de ernst van de ziekte en de reden van de transfusie, aangezien patiënten die bloedplaatjes ontvingen niet verschilden van de patiënten die geen bloedplaatjes ontvingen.

Discussie

Wij vonden alleen een sterke associatie tussen de transfusie van bloedplaatjes en het ontwikkelen van een nosocomiale infectie. Deze bevinding is anders dan die van eerdere studies, die ook een transfusie met rode bloedcellen associeerden met de ontwikkeling van nosocomiale infecties. Een belangrijke verklaring voor deze onwaarschijnlijk conflicterende resultaten kan zijn dat wij hebben gecorrigeerd voor de tijd van de blootstelling. Deze studie benadrukt het belang van het ontwerp van een statistisch model. Tegelijkertijd laat deze studie ook zien dat observationele studies hun beperkingen hebben. Een duidelijk antwoord op de vraag of de beschreven associatie daadwerkelijk bestaat vereist een gerandomiseerde gecontroleerde studie met verschillende transfusiegrenzen die voldoende “gepowerd” is om een mogelijk verschil te kunnen detecteren. Hiervoor is een groot aantal patiënten vereist en het is onwaarschijnlijk dat een dergelijke studie ooit plaats zal vinden. Daarom blijven we op observationele studies terugvallen die associaties suggereren, die vervolgens in geschikte modellen bevestigd dienen te worden. Ondertussen is het onmogelijk om preventieve maatregelen te nemen zolang het pathofysiologische mechanisme achter TRIM nog onduidelijk blijft. Daarom is de enige preventieve maatregel die we op dit moment kunnen nemen het reduceren van de hoeveelheid getransfundeerde bloedplaatjes.

Aangezien deze studie niet heeft gekeken naar het effect op de uiteindelijke uitkomst, zoals mortaliteit, kunnen we niet zeggen dat een transfusie met bloedplaatjes volledig vermeden dient te worden. Deze studie onderstreept echter wel de noodzaak tot verder onderzoek naar de optimale transfusiegrens in de ernstig zieke patiëntengroep.

Conclusie

Dit proefschrift heeft twee belangrijke bevindingen. De eerste is dat er vele onverwachte effecten van een transfusie zijn door de invloed van het transfusieproduct op de stolling en de inflammatoire respons van de ontvanger. Hierdoor is de balans tussen de gunstige
en ongunstige effecten van een bloedtransfusie in ernstig zieke patiënten zeer delicaat. De beslissing over te gaan tot transfusie moet dan ook goed afgewogen worden, waarbij het beoogde doel, het transfusieproduct en de klinische conditie van de ontvangen in ogenschouw genomen dienen te worden. Dit proefschrift kan bijdragen aan het maken van die beslissing.

Verder blijft een transfusie met de verschillende bloedproducten zelfs in het tijdperk van restrictieve transfusiegrenzen een hoeksteen van de behandeling in de Intensieve Care, aangezien anemie en trombocytopenie frequent voorkomen. Ook komt massaal bloedverlies veel voor. Daarom blijft het belangrijk om interventies te onderzoeken die de procoagulante en pro-inflammatoire bijwerkingen van een transfusie te kunnen verminderen. Dit proefschrift kan hieraan bijdragen door te suggereren dat het verwijderen van EVs uit rode bloedcelproducten verdere studie behoeft.
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Appendices

- Contributing authors and affiliations
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PUBLICATIONS


PHD PORTFOLIO

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<td>Clinical Data Management</td>
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Presentations

*Mechanical ventilation and LPS increase susceptibility to lung injury in a murine TRALI model,* oral presentation at the annual congress of the European Society of Intensive Care Medicine, Berlijn, Duitsland

*Inflammatory properties of microparticles in stored red blood cell products,* poster presentation at the International Symposium on Intensive Care and Emergency Medicine, Brussel, België

Effect of Fresh Frozen Plasma on systemic inflammation and endothelial damage in non-bleeding critically ill patients; *poster presentation at the International Symposium on Intensive Care and Emergency Medicine, Brussel, België*

(International) national conferences

Attendance annual congress of the European Society of Intensive Care Medicine, Berlijn, Duitsland

Attendance International Symposium on Intensive Care and Emergency Medicine, Brussel, België

Other activities

Intensive Care Journal Club (monthly) 2010-2014 3

Intensive Care Research Meeting (weekly) 2010-2014 13.5

Laboratory of Experimental Intensive Care and Anesthesiology (LEICA) research meeting (weekly) 2010-2014 13.5

Teaching

Master student, medicine, University of Amsterdam

Lecturing

*Diagnose van stollingsstoornissen en transfusie protocollen in trauma patiënten,* Regiobijeenkomst Klinisch Chemici regio Noord-Holland, AMC, Amsterdam, Nederland

Grants

CSL Behring – Prof. Heimburger Award 2014 0.5
CURRICULUM VITAE

A
cKnoWledgements

En dan nu het laatste, maar derhalve niet per se makkelijkste hoofdstuk: het dankwoord. Want is een enkele regel wel voldoende om eenieder te bedanken zonder wie dit boekje niet tot stand was gekomen? Ik ga het toch proberen, maar genoemden, weet alsjeblieft dat mijn dank verder gaat dan één regel!

Mijn co-promotor, inmiddels prof. dr. N.P. Juffermans. Beste Nicole, dank voor jouw begeleiding van de afgelopen jaren. Hoe had ik kunnen weten dat het oriënterende gesprek over wetenschappelijk onderzoek zou kunnen uitmonden in een proefschrift? En hoewel je een risico hebt genomen door mij erg vrij te laten tijdens het gehele traject, ligt er uiteindelijk een boekje klaar én heb ik veel geleerd over time-management. Nicole, de titel professor heb je méér dan verdiend!

Mijn co-promotor, dr. R. van Bruggen. Beste Robin, hartelijk dank voor al je adviezen en alle antwoorden op mijn vele vragen. Als arts ondergedompeld worden in de wonderlijke wereld van Sanquin en de rode bloedcellen was niet altijd even gemakkelijk, maar gelukkig wees jij mij altijd de weg. Wat was het een geruststelling te merken dat ook gerenommeerde wetenschappers van Hazes houden!

Mijn promotor, prof. dr. M.B. Vroom. Beste Margreeth, ook al was Nicole mijn wetenschappelijke begeleider, toch wil ik jou hartelijk danken voor alle mogelijkheden die je mij geboden hebt.


Alvorens verder te gaan wil ik hier graag even stil staan bij alle patiënten die hebben deelgenomen aan klinisch wetenschappelijk onderzoek op de Intensive Care. Ik kan mij niet voorstellen hoe moeilijk jullie situatie en die van jullie naasten moet zijn geweest op het moment dat mijn collega's en ik u om deelname vroegen. Ik heb niets dan diep respect voor de open houding die jullie hadden en de gesprekken die jullie hierover wilden aangaan.

Daarnaast wil ik graag alle IC-verpleegkundigen van het AMC bedanken voor alle moeite die zij hebben gedaan voor alle studies die wij nú wéér hadden bedacht! Immers, zonder jullie hulp was geen enkele studie geslaagd. In het bijzonder wil ik graag Vivian, Vivian, Debby, Joyce en Jay-Jay bedanken voor alle gezellige uitjes, die een welkome afleiding waren van het harde onderzoekswerk!
Uiteraard wil ik ook alle arts-assistenten, fellows en stafleden van de IC van het AMC bedanken. Zonder jullie aanmeldingen en behulpzaamheid was er geen wetenschappelijk onderzoek mogelijk op de IC.

Dan over naar de collega’s van het onderzoek. Laat ik maar beginnen met de MARSers omdat we zoveel lief en leed hebben gedeeld. Roosmarijn, je bent begonnen als stagiaire maar hebt je supersnel opgewerkt tot een meer dan volwaardige collega die mij vaak heeft geholpen. Niet alleen wetenschappelijk-inhoudelijk, maar vooral ook met veel gezelligheid, vrijdagmiddagborrels, relativerende opmerkingen en geweldig sarcastische humor. Je bent een echte vriendin geworden! Laura, je kwam later bij de groep maar hebt harder gewerkt dan wij allemaal. Was je niet zo gezellig, had je niet net zulke harde, sarcastische humor als Roos en ik en ging je niet elke dag terug om de -80s te checken, dan had ik kunnen denken dat je een machine was. Ook jij bent een vriendin voor het leven geworden! Lieuwe, ik heb enorm veel respect voor je en dat weet je: je bent niet alleen een wetenschappelijk wonder, je bent ook nog eens een hartstikke toffe vent, verander alsjeblieft nooit! Luuk, niet alleen was jij de initiator van de VrijMiBo's avant-la-lettre in Stevens, ook was jij het diplomatieke geweten van onze onderzoeksgroep. Dank voor al jouw adviezen, zowel professioneel als privé. Friso, dank voor alle gezelligheid waarmee je mij altijd door de moeilijkere momenten sleepte! Lonneke, dushi, dank voor al jouw optimisme. Na kort even met jou gesproken te hebben leek alles altijd weer mogelijk. Maryse, in het begin moest je even wennen op de IC, maar uiteindelijk heb je je draai helemaal gevonden. Daar ben ik heel blij om, want ik had je niet willen missen! Gerie, jij bent een voorbeeld voor mij: hoe je privé en werk hebt gecombineerd, ondanks alle tegenslagen als een soort stoomschip bent doorgestoomd en dit ook nog eens deed zonder ook maar een beetje ongezellig te zijn op de werkvloer! Esther, als ik aan jou denk, denk ik aan die proeven die dagenlang leken te duren. Je draaide er je hand niet voor om!

Dan de andere collega’s van de IC-onderzoeksgroep. Tineke, heel erg bedankt voor al jouw mode-praat, ondersteuning bij het onderzoek, gezelligheid en het vele lachen samen. Annelou, dankjewel voor jouw niet-aflatende waakzaamheid de afdeling te verbeteren en het onderzoek te ondersteunen. Sabrine, dankjewel voor al je gezelligheid, Karin en de vele borrels. Frank, hoewel je af en toe wel wat weg had van een spammer, kan ik me geen betere collega indenken: altijd in voor advies, gezelligheid, motivatie en brainstorm-sessies. Hemmik, ook jij begon als stagiair en bent uitgegroeid tot een volwaardige collega. Jij bent het levende bewijs dat een computernerd niet altijd een nerd hoeft te zijn. Pieter-Roel, Aline, Marcella, Sophia, Fabiënne, Maike, en Marjolein, er zit zo’n grote tijdspanne tussen jullie dat jullie elkaar waarschijnlijk niet kennen, maar jullie zijn allemaal geweldige collega’s en ik kijk met plezier terug op onze samenwerking. Mary-Ann en Erica, hartelijk dank voor al jullie ondersteuning en de oneindige energie
waarmee jullie altijd mijn vragen en verzoeken hebben beantwoord. Jullie zijn topspers!
Fredérique, Jan, Mark, Marjon, Janneke en Wim, heel erg bedankt voor jullie zijdelingse
bemoeienissen met mijn werkzaamheden!

Over naar “beneden”, naar de collega’s van het LEICA. Rianne, we hebben altijd lief en
leed gedeeld en dat heeft me altijd enorm gesteund. Anna-Linda, jouw motivatie is een
voorbeeld voor ons allen. Soms is het verbazingwekkend hoe je jouw enorme producti-
viteit kan combineren met alle hartelijkheid en gezelligheid die je uitstraalt. Charlotte,
dank voor alle vragen die ik jou mocht stellen, het inwerken met de ratten, maar vooral
veel dank voor alle gezelligheid! Hamid, dank voor je hulp bij het ambieteuz plan om
ratten te beadem in de MRI, alle vragen die ik mocht stellen en de gezelligheid tijdens
ons zeilweekje! Hendrik, ook al zat je de meerderheid van de tijd in Leiden, het leek wel
alsof je er altijd was. Jouw werklust, spontaniteit en collegialiteit zijn overweldigend!
Djai, Daniel, Gezina, Kirsten en Ilse, ook wel de oude garde van het LEICA. Dank voor
alle gezelligheid! Matt en Margit, jullie belichamen het nieuwe LEICA en het doet me
deugd dat er dus kennelijk niets van de werklust, humor en gezelligheid verloren zal
gaan! Anita, Gaertsje en Jacolie, mijn oneindige dank voor alle hulp die jullie mij hebben
geboden. Zonder jullie was dit boekje waarschijnlijk nog steeds niet af… Coert, Nina,
Koen en Jessica, dank voor alle energie waarmee jullie het lab draaiende houden.

Collega’s van het LEKC, hoe vaak heb ik niet geroepen dat jullie wel een hele pagina van
mijn dankwoord in beslag zouden moeten nemen. Nu, daar komt het niet van, maar
ik wil jullie heel hartelijk danken voor alles dat jullie voor mij hebben gedaan. Anita B.,
zoener jou hadden we onze mooie roze FACS nog steeds niet aan de praat en waren
al mijn HUVECs-proeven genadeloos mislukt. Ondanks de verschillende levensfasen
waarin we ons bevonden bleken we toch verrassend hetzelfde te zijn. Dank voor alles!
Chi, zonder jou had ik nooit leren FACSen en was ik nu nog steeds aan het klooi en met
die honderden buisjes. Dankjewel voor de niet aflaten energie waarmee je al mijn
vragen beantwoordde! Anita G., dank voor al je hulp, maar in het bijzonder je hulp met
dat walgelijk gecompliceerde kolom-experiment. Najjat, zelfs in de korte tijd dat je bij
het LEKC werkt moet ik je al bedanken: dank voor al je hulp! Tot slot Rienk, dank voor
al jouw enthoische meedenken en de kritische opmerkingen waarmee je mijn onder-
zoekspozetten hebt bijgeschaafd.

Een proefschrift over transfusie is natuurlijk niet volledig zonder samenwerking met
Sanquin. Boukje en Thomas, heel erg bedankt voor alle hulp met de TETRIS en de dier-
studies. Robin, ik heb je al eerder genoemd, maar nogmaals dank!

Naast alle collega’s zijn er natuurlijk nog meer mensen die indirect veel hebben bijge-
dragen aan dit proefschrift. Of moet ik zeggen, hebben geleden onder zit proefschrift?
Lieve Niesje, Dorris, Jessica, Wanda, Suzan en Tessa, ik kan niet uitleggen hoe fijn het was
om jullie begrip te hebben voor de vele uren die in dit project zijn gaan zitten. Zeker toen ik ook weer in de kliniek ging werken bleef er nog maar weinig tijd voor jullie over. Ik kan niet wachten tot ik jullie weer wat vaker kan zien!

Lieve Frans, wat geweldig om een oom te hebben die grafisch ontwerper én bereid is je proefschrift op te maken. Ik weet dat het alleen om de inhoud zou moeten gaan, maar ik ben toch wel verdomd trots op de presentatie, dankjewel! Lieve Herjanne, ik weet dat jij altijd achter Frans staat en dit soort projecten zonder jouw hulp niet zo geslaagd zouden zijn, dus dankjewel!!!

Lieve paranimfen, Jessica en Laura, beiden al genoemd maar ik wil toch de gelegenheid nemen jullie nog eens expliciet te bedanken voor jullie steun op een voor mij heel bijzondere dag. Het is heerlijk om te weten dat er twee van zulke vrouwen naast je staan.

Lieve Aafke, zusje, heel erg bedankt voor het begrip dat je kon opbrengen als ik weer eens te druk was voor het één of het ander. Ik zal onze “roadtrip” naar Rotterdam nooit meer vergeten!

Lieve Thea en Ruud, mam en pap, niet alleen dank voor al jullie steun en begrip in de afgelopen jaren, maar ook tijdens mijn kinderjaren en puberteit. Zonder jullie stimulatie in die tijd was ik natuurlijk nooit tot een proefschrift gekomen!

Lieve Joas, hoewel ik altijd wel mijn woordje klaar heb, schieten woorden mij nu toch echt tekort. Want hoe beschrijf ik alles dat je voor mij gedaan hebt? Niet alleen bood jij altijd praktische oplossingen, maar ook stapte jij grootmoedig over de grillen die ik vertoonde als ik het weer eens drukker had dan ik eigenlijk aan kon. Ik kan nog veel meer voorbeelden noemen, maar daar is dit dankwoord niet voor bedoeld. Ik denk dat je de volledige strekking wel begrijpt als ik zeg: behind every successful man there is a woman and behind every successful woman there is a man. Ik zou willen dat ik je kon beloven dat je mij nooit meer zou hoeven delen, maar je weet dat ik dat niet kan. Gelukkig doen we dit project samen!