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PLATELET TRANSFUSIONS IN HEMATOLOGICAL DISORDERS

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Platelet transfusions in hematological disorders

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To Dexter and Lexie,

without the two of you I would be less crazy but always missing a part.

ABSTRACT

Platelets are important for clot formation and vascular integrity. Thrombocytopenic patients with hematological disorders are at risk of bleeding and platelet transfusions are important in supportive care and treatment of life-threatening bleedings. Transfusion response in terms of platelet increment is highly variable and hard to predict in the individual patient. Platelet transfusion refractoriness is often due to non-immune factors such as fever, bleeding or infection. Of the immune-causes, antibodies against Human Leukocyte Antigen (HLA) class I is the most common, and these patients can receive HLA-matched platelet transfusions. In a more experimental approach HLA deficient platelets are created by acid treatment.

The overall aim of this thesis was to gain a better knowledge of factors affecting platelet transfusion outcome and platelet function, and to improve transfusion practice to patients with platelet refractoriness due to anti-HLA antibodies. In the included research papers, we focused on platelet function and clot formation in patients before and after platelet transfusion (**paper I**), response to HLA matched platelet transfusions (**paper II**) and function and survival of HLA deficient platelets (**paper III and IV**).

In **paper I**, we evaluated the effect of platelet transfusions in 40 hematology patients, measuring platelet count increment, clot formation and response to agonist activation. Platelet count increment correlated with improved clot formation, but the response was highly variable and 34 % of the patients did not respond to the transfusion. In responder patients, efficient clot formation was predicted by good platelet responsiveness to agonist stimulation, but transfusion did not, however, restore platelet function to that of healthy controls. In **paper II** we evaluated HLA matched platelet transfusions to platelet refractory hematology patients. We found that selection of platelets with either a complete HLA match, or an acceptable HLA mismatch, was highly predictable for a successful transfusion response. For HLA-mismatched transfusions, the degree of matching correlated with the fraction of successful transfusions. Many transfusions were successful despite the presence of donor-specific antibodies (DSAs). In **paper III** we evaluated the function of HLA deficient platelets after acid treatment and found that they were viable with a normal upregulation of activation markers. The HLA deficient platelets aggregated to a similar extent as untreated platelets in response to stimulation with agonists. Acid treatment removed 70 to 90% of all HLA Class I complexes, which was followed by protection from anti-HLA antibody-mediated complement lysis and reduced phagocytosis by monocytes *in vitro*. In **paper IV** we further evaluated the function of HLA deficient platelets in whole transfusion units and investigated the survival of HLA deficient platelets in a mouse model. Recovery, HLA reduction, and viability in acid-treated platelet bags were comparable to the small-scale treatment protocol, and platelets contributed normally to clot formation. The HLA deficient platelets also contributed to endothelial integrity. Acid-treated platelets showed a lower *in vivo* recovery compared to untreated platelets in the mouse model. They were protected from rejection by single HLA allele-specific antibodies, but still cleared by a pan-HLA antibody.

In summary, the results and conclusions presented in this thesis provide a basis for adjusted transfusion practices and several hypotheses for future research. Our results also take HLA deficient platelets closer to a clinical trial.

LIST OF SCIENTIFIC PAPERS

- I. Platelet transfusion improves clot formation and platelet function in severely thrombocytopenic hematology patients.
Karlström C, Gryfelt G, Schmied L, Meinke S, Höglund P.
Manuscript 2020.
- II. HLA-selected platelets for platelet refractory patients with HLA antibodies: a single-center experience.
Karlström C, Linjama T, Edgren G, Lauronen J, Wikman A, Höglund P.
Transfusion. 2019 Mar; 59(3):945-52.
- III. Platelets made HLA deficient by acid treatment aggregate normally and escape destruction by complement and phagocytes in the presence of HLA antibodies.
Meinke S, Sandgren P, Mörtberg A, **Karlström C**, Kadri N, Wikman A, Höglund P.
Transfusion. 2016 Feb;56(2):370-82.
- IV. HLA removal by acid treatment - *In vitro* and *in vivo* studies of platelet function and survival.
Meinke S, **Karlström C**, Heshmati Y, Gryfelt G, Hultenby K, Sandgren P, Miyazawa B, Pati S, Walfridsson J, Höglund P.
Manuscript 2020.

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LIST OF ABBREVIATIONS

ADP	Adenosine Diphosphate
AML	Acute Myeloid Leukemia
ASCT	Allogeneic Stem Cell Transplantation
β 2m	β 2 microglobulin
BC	Buffy Coat
CCI	Corrected Count Increment
CD62P	P-selectin
CML	Chronic Myeloid Leukemia
CREG	Cross-reactive Epitope Group
CRP	C-reactive Protein
CT	Clotting Time
CFT	Clot Formation Time
DSA	Donor Specific Antibody
ECM	Extracellular Matrix
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HPA	Human Platelet Antigen
HSC	Hematopoietic Stem Cell
IgG	Immunoglobulin G
IL	Interleukin
iPSC	Induced Pluripotent Stem Cell
ITP	Immune Thrombocytopenia
MCF	Maximum Clot Firmness
MDS	Myelodysplastic Syndrome
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex

NET	Neutrophil Extracellular Trap
PAC-1	Platelet Activation, clone number 1
PAR	Protease Activated Receptor
PD	Platelet Dose
PI	Platelet Increment
PLC β	Phospholipase C β
PPR	Percentage Platelet Recovery
PRP	Platelet Rich Plasma
ROTEM	Rotational Thromboelastometry
SCF	Stem Cell Factor
TLR	Toll-like receptor
TPO	Thrombopoetin
TRALI	Transfusion Related Acute Lung Injury
TRAP	Thrombin Receptor Activating Peptide
TxA ₂	Thromboxane A ₂
WHO	World Health Organization
VWF	Von Willebrand Factor

1 INTRODUCTION

Platelets are the cellular mediators of clot formation and therefore an important part of the defence against bleeding. Many patients with hematological disorders experience low platelet counts in peripheral blood, a condition also known as thrombocytopenia. The thrombocytopenia may result from the hematological disorder itself or be due to bone marrow suppression after intensive chemotherapy[1]. Low platelet counts increase the risk of bleeding and patients with thrombocytopenia may need platelet transfusions[2]. The platelet transfusions can be given either prophylactically to prevent bleeding or therapeutically at signs of bleeding. The presented thesis has two main objectives: 1) platelet function in patients with hematological malignancies and what effect platelet transfusions have on platelet function in such patients and 2) patients that do not obtain the full effect of platelet transfusions due to alloimmunization with Human Leukocyte Antigen (HLA) antibodies, including exploration of an alternative transfusion strategy with HLA deficient platelets.

1.1 HEMATOPOIESIS

Hematopoiesis refers to the formation of all blood cells from hematopoietic stem cells (HSCs), a process that in adult humans primarily takes place in the bone marrow. HSCs are multipotent and have the capacity to self-renew[3]. HSCs are defined by the expression of CD49f and other markers[4]. Their multipotency give rise to two specific paths, the myeloid pathway via common myeloid progenitors (CMPs) and the lymphocytic pathways via multi-lymphoid progenitors (MLPs)(Figure 1). CMPs differentiate into red blood cells (erythrocytes) and platelets (thrombocytes) via myeloid erythroid progenitors (MEPs), and cells involved in cellular immunity such as granulocytes via granulocyte-macrophage progenitors (GMPs). MLPs differentiate into T cells (T stands for thymus, the organ in which the T cells mature), B cells (B comes from the name of the place they were discovered, the Bursa of Fabricius which is an organ in birds) and NK (natural killer) cells.

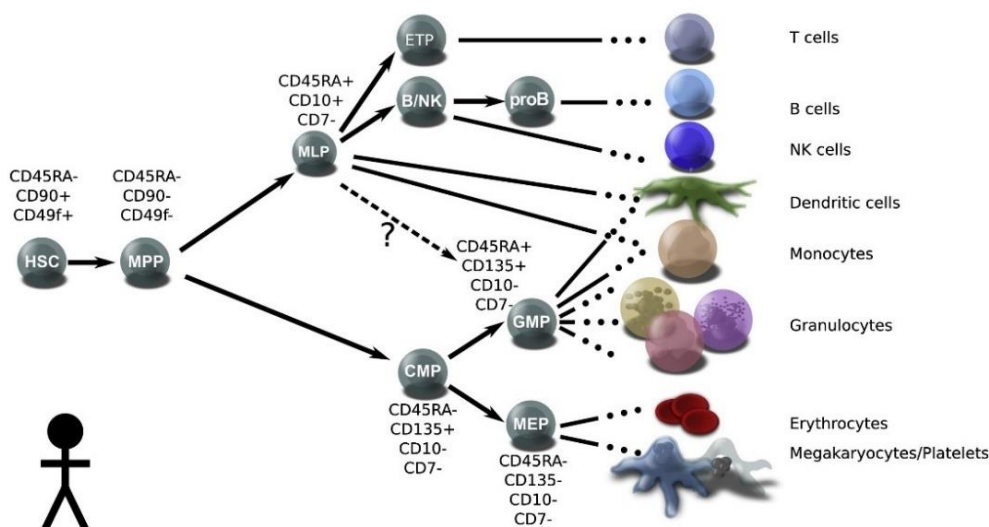


Figure 1. Schematic overview of HSCs developmental journey. HSCs develop into the terminally differentiated cell populations on the far right in this figure. Multipotent progenitors (MPPs) can be distinguished from HSCs by the loss of CD49f expression. Adapted from [4].

1.1.1 Megakaryocytopoiesis and platelet formation

A long time has passed since William Osler first described what is now believed to be platelets in his papers in 1873 and 1874[5]. Identification of the platelet's role in clot formation is accredited to Giulio Bizzozero who in 1881–1882 performed elegant intravascular microscopy and *in vitro* flow chamber studies[6]. Bizzozero was likely the first to describe megakaryocytes in the bone marrow but it was not until the early 20th century that James Homer Wright suggested that they were the progenitors of platelets, at this time called “dust of the blood”[7]. The description is easily understood knowing that the platelet is in average 2-4 μm in diameter, and the smallest of the blood cells. However, the platelet is enucleated, basically being just little shreds of membrane-enclosed cytoplasm from the megakaryocytes and therefore does not count as the smallest cell of the body, despite being smaller than the title-holding sperm.

Megakaryocytes are rare in the bone marrow, constituting around 0,05% of the nucleated cells[8]. The development of megakaryocytes in the bone marrow is a unique process, induced by the growth factor thrombopoietin (TPO). By injecting plasma from patients with thrombocythemia (abnormally high platelet counts) into mice and observing the following transient increase in platelet counts, TPO was proven to exist in 1958[9]. The maturation of megakaryocytes involve duplication of the nucleus without cell division, resulting in giant cells averaging 50–100 μm in diameter[10]. Upon leaving the bone marrow some megakaryocytes reach the blood stream, some end up trapped in the lungs. The cytoplasm from the megakaryocyte fragments at shear stress and pieces of cytoplasm can be released in the bone marrow as well as in the lungs and in the blood stream. However, it has been suggested that it is only in the circulating blood that the fragments, or proplatelets, form into mature platelets. The maturation process of proplatelets involves elongation, curving, and finally change shape to form the platelets[11].

Approximately 100 billion platelets are produced every day in the human body, enough to maintain levels of 150 000 to 400 000 platelets per microliter of blood ($150\text{-}400 \times 10^9/\text{L}$)[12]. Platelets have a short life span, surviving only around 10 days in the circulation[1].

1.2 THE ROLE OF PLATELETS

The process to prevent and stop bleeding is called hemostasis. The platelets are the cellular mediators of clot formation (thrombosis). They patrol vessels enabling quick cover and closure of tissue and endothelium wounds, an important part of hemostasis. In the last decade, important immune and inflammatory roles of platelets have also been continuously acknowledged.

Platelet function, a recurring concept in this thesis, is defined as the ability of platelets to respond to different stimuli in the environment.

1.2.1 Platelet activation and clot formation

Clot formation is usually described in a three-step model containing adhesion, activation and aggregation[13]. These steps develop successively but are closely integrated and partially overlap. Platelet adhesion, as the first step of clot formation, usually start when extracellular matrix (ECM) is exposed due to a vessel or tissue injury. Platelets get in contact with, and can adhere to, matrix components such as collagen, laminin and Von Willebrand Factor (VWF). For example, platelets are very quickly tethered to exposed subendothelial VWF by GPIb-IX-V, a receptor complex located in the platelet membrane. Adhesion to other matrix components are mediated through integrin receptors in the platelet membrane, such as $\alpha 2\beta 1$ for collagen and $\alpha 6\beta 1$ for laminin[14].

Platelet agonists, for example thrombin, adenosine diphosphate (ADP) and thromboxane A2 (TxA2), play an important role in the following steps of clot formation: activation and extension of the platelet plug (aggregation). Thrombin is perhaps the most effective platelet activator, even at a very low concentration. Platelet activation with thrombin is largely mediated by two protease activated receptors (PARs), in humans PAR-1 and PAR-4[15]. Thrombin also seems to be the agonist most efficiently coupled to phospholipase C (PLC β) activation, generating second messengers and leading to the release of intracellular calcium. Other thrombin-induced platelet responses are shape change, secretion, TxA2 generation, protein phosphorylation and aggregation. Schematic overview of clot formation is shown in figure 2.

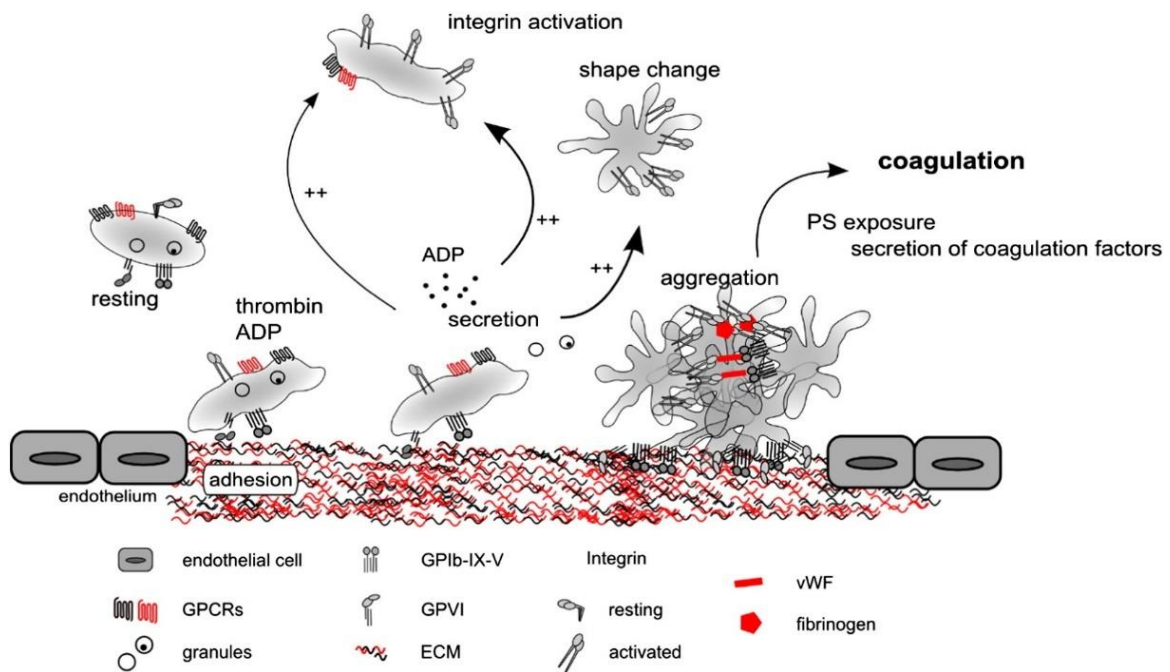


Figure 2. Schematic of platelet activation and clot formation. Clot formation starts with platelets adhering to exposed extracellular matrix via integrin or GPVI receptors. Release of bioactive molecules from dense and α -granules in the platelets act to activate additional surrounding platelets. Formation of the clot is further promoted by activated integrin $\alpha IIb\beta 3$ and fibrin. Adapted from [16].

In recent years it has been acknowledged that platelets are not equally activated throughout thrombus formation, and the core- and shell model has emerged. One study could show two distinct platelet populations in the clot, a core of highly activated platelets and a looser outer shell with less activated platelets[17].

1.2.1.1 Some other mediators and receptors important for platelet function

ADP is stored in dense granules in the platelets and platelet activation leads to secretion. Red blood cells at sites of vascular injury also release ADP. Events following platelet activation by ADP includes for example intraplatelet Ca^{2+} elevation, synthesis of TxA_2 , shape change, granule secretion, activation of the receptor $\alpha\text{IIb}\beta_3$, and aggregation[15].

P-selectin (CD62P) belongs to a family of receptors (selectins) with a pivotal role in adhesion, but also plays a role in attachment of neutrophils and monocytes to the platelets and endothelial cells[14]. It is present in α -granules of resting platelets and is translocated to the plasma membrane after activation. The expression of CD62P on circulating platelets indicates *in vivo* activation.

The integrin receptor $\alpha\text{IIb}\beta_3$ (CD41/CD61), also known as GPIIb-IIIa, is expressed on resting platelets in its low affinity state[14]. Upon activation this receptor goes through a conformational change to reach the high affinity state, promoting platelet aggregation by facilitating binding of for example fibrinogen, fibrin and VWF.

$\text{Fc}\gamma\text{RIIA}$ (CD32) is a member of the immunoglobulin (Ig) super family with low affinity for the constant fragment (Fc) of immunoglobulin G (IgG). Cross-linking of this receptor initiates platelet activation by for example tyrosine phosphorylation, phospholipase C activation, calcium signaling and cytoskeletal rearrangement[14].

1.2.2 Inflammation and immune responses

The role of platelets as immune cells has gained increased understanding the last decade. Several studies have demonstrated that platelets impact many inflammatory processes ranging from atherosclerosis to infectious diseases, making platelets the most numerous circulating cell type with an immune function[18]. Platelets have for example been suggested to induce the acute phase response, the earliest response to infection or vascular injury. Furthermore, platelets have been shown to attract and capture circulating leukocytes by secretion of pro- and anti-inflammatory molecules such as CXCL4 and CXCL7, thus guiding them to inflamed tissues[19].

Platelets express Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), that recognize various molecular structures found on bacteria, viruses, and fungi[20, 21]. For example, TLR4 on platelets has been shown to induce platelet binding to adherent neutrophils in severe disease settings such as sepsis, thus promoting formation of so-called NETs (neutrophil extracellular traps)[22]. NETs are structures with proteolytic activity and the

ability to trap and kill microbes in tissues. NETs are suggested to be the key activators of coagulopathy in the infection setting, and activated platelets seem to be able to enhance formation of these neutrophil traps[23].

CLRs on platelets have been shown to interact with several viruses, for example Human Immunodeficiency Virus 1 (HIV-1), facilitating capture by platelets[24]. Platelets are even able to directly kill microorganisms and evidence has been presented that activated platelets are able to internalize the bacteria *Staphylococcus aureus* and HIV-1, the latter probably mediated by VAMP (vesicle-associated membrane protein)-3[25, 26].

In the current COVID-19 pandemic, patients with severe disease sometimes present with significant thrombocytopenia correlated to a worse prognosis[27]. The thrombocytopenia has been suggested to be due to bone marrow inhibition, increased platelet destruction or increased platelet consumption[28]. It is unclear whether ACE-2, the suggested SARS-CoV-2 receptor, is expressed on platelets[19]. Other potential receptors for platelet interaction with SARS-CoV-2 are TLR7 and 9, and FcγRIIA via immune complexes formed in patients with cross-reacting antibodies to SARS-CoV-2[29]. Further research is needed to clarify the role of platelets in the pathophysiology of COVID-19.

1.3 REGULATION OF PLATELET LIFE SPAN

Due to the numerous functions of platelets, it is extremely important to maintain the balance between production and clearance. Platelets are removed from circulation by several different mechanisms, including signals induced by aging and apoptosis as well as immune mediated responses. The following three main mechanisms responsible for platelet clearance have been described[12]. The first mechanism is glycan-lectin mediated clearance in which the platelets lose sialic acid in the aging process. This leads to clearance via the hepatic Ashwell-Morell Receptor, which in turn regulates TPO in hepatocytes as a feed-back loop. The second mechanism is antibody mediated. Platelets coated with antibodies directed against the integrin αIIbβ3 and the glycoprotein GPIbα, as part of the von Willebrand receptor complex (GPIbIX), are cleared via Fc receptors on macrophages and CD8+ cytotoxic T lymphocytes. Apoptosis mediated clearance is the third mechanism described. Platelet survival is described to also depend on the balance between pro-survival and pro-apoptotic members of the Bcl-2 family, critical regulators of the intrinsic apoptotic pathway[30].

1.4 HEMATOLOGICAL DISORDERS

Hematology refers to the knowledge of blood, blood-forming organs, and blood disorders, both benign and malignant. Examples of benign hematological disorders are inherited diseases such as the group of hemoglobinopathies or acquired diseases such as immune thrombocytopenia (ITP). The malignant hematological diagnoses will be described in more detail below, as being related to the work in this thesis.

1.4.1 Myeloid malignancies

Myeloid malignancies are clonal diseases originating from HSCs or later progenitor cells[31]. They consist of both chronic and acute diseases. In the group of chronic diseases, we find chronic myeloid leukemia (CML), myelodysplastic syndromes (MDS) and the myeloproliferative neoplasms: essential thrombocytosis (ET), polycythemia vera (PV) and primary myelofibrosis (PMF). Acute myeloid leukemia (AML) is the primary acute myeloid disease. In around 80% of the cases of AML it is a de novo diagnosis, but in some cases a secondary AML occur after radiation or chemotherapy for another cancer, such as breast cancer, or following a chronic myeloid malignancy such as MDS. They all result from genetic mutations that disturb important processes in the cells, such as self-renewal, proliferation, and differentiation. Treatment usually include chemotherapy of different kinds and in different combinations[32]. For some diagnoses, tremendous breakthroughs have been made in the last two decades. The most successful example is the treatment with Tyrosine Kinase Inhibitors (TKIs) for CML which has dramatically changed the prognosis and outcome of CML. The life expectancy of CML patients is now approaching that of the general population[33].

1.4.2 Lymphoid malignancies

Lymphoid malignancies include a large group of lymphomas, both indolent and more aggressive, as well as myeloma and lymphoid leukemia. These diseases originate from the malignant transformation of normal lymphoid cells at various stages of differentiation[34]. As a group, lymphoid malignancies are the sixth most common type of malignancies in the world. Lymphoid malignancies can present as leukemia with primary involvement of bone marrow and blood, or as lymphomas with solid tumors. A few of the lymphoid malignancies can present as either lymphoma or leukemia, for example Burkitt lymphoma/leukemia[35].

1.5 PLATELET TRANSFUSIONS

Transfusions of whole blood were first reported to stop bleedings in hemorrhagic patients by William Duke in 1910. He had invented a bleeding time test and could conclude that the bleeding time was inversely correlated with the platelet count[36]. However, it was not until 35 years later that major progress was made in the area of platelet transfusions, when it was recognized that thrombocytopenia was a major cause of death after radiation exposure from atomic weapons[37]. In the years that followed, new chemotherapeutic agents emerged that resulted in thrombocytopenia in the patients. Therefore, platelet transfusions have had an important role in management of side effects, improving survival and thus enabling the development of modern chemotherapy. The first prospective randomized comparative study of supportive therapy with platelets was published in 1959. Platelets were then given as whole blood transfusions, and the study compared fresh and stored whole blood transfusions to children with bleeding because of leukemia treatment. Nine patients were included, of which 6 had a reduction or cessation of bleeding. Five out of these 6 received fresh blood. They concluded that the clinical effect on bleeding was significantly better in fresh products[38]. In 1974 a double-blinded study was performed on thrombocytopenic leukemia

patients where they prophylactically received either platelets or platelet-poor plasma[2]. Patients that had received platelets had a significantly lower frequency of bleedings and it was the first study to show the benefit of prophylactic platelet transfusions.

The numbers of platelet transfusions given have been steadily increasing and approximately 2 million platelet units are transfused yearly in the US[39]. In 2019, as many as 49,784 platelets units were transfused in Sweden[40]. Owing to initiatives in patient blood management, a short-term reduction can be expected. However, in the coming 5-10 years the demand will increase due to demographic shifts with the aging of the population, not only in developed countries but also low- and middle-income countries, facing an enormous challenge[41, 42].

1.5.1 Platelet transfusions in patients with hematological disorders

Today, the most common indication for platelet transfusions is bone marrow failure. The inability of the bone marrow to produce enough platelets can be due to hematological malignancies such as MDS, or because of intensive chemotherapy for more acute diagnoses such as AML. Allogeneic stem cell transplantation (ASCT) is a treatment with high doses of cytostatic drugs, often in combination, with the aim to destroy residual malignant cells and to clear the bone marrow for the new donor stem cells. Understandably, this always results in thrombocytopenia.

In the US as an example, between one-third and half of the total platelet concentrates transfused yearly are transfused to hematology/oncology patients, and approximately as many to surgical patients (general, orthopedic, and cardiac surgery combined)[39]. From the UK it is reported that up to 67% are issued to hematological patients[43]. The majority of transfusions to hematology/oncology patients are given prophylactically[44].

1.5.2 Platelet concentrates

Platelet concentrates for transfusion were not routinely available until the 1970s[45]. Around this time, techniques had developed that allowed the collection of sufficient numbers of platelets in a platelet concentrate, and clinical effectiveness of platelets could be demonstrated. Recovery and survival studies demonstrated that cold stored (2° to 6°C) platelets had a shorter survival time in the circulation than room temperature–stored platelets, and room temperature storage was therefore implemented as routine for prophylactic transfusions in hematological patients with thrombocytopenia[46].

There are three methods to prepare platelets for transfusion: single-unit preparation from whole blood using a platelet rich plasma (PRP), single-unit preparation from whole blood using pooled buffy coats (BC) and collection by apheresis[47]. These different methods have their separate advantages and disadvantages. Platelets from whole blood requires pooling to reach an adult platelet dose. At the Karolinska University hospital blood bank, a total of 8 donors are pooled to make two platelet units. Pooling is associated with increased donor exposure and higher risk of viral transmission compared to apheresis platelets from a single

donor, but it also reduces the impact of individual variability in platelet function, and thus secures a more predictable function of the unit.

Apheresis platelets, on the other hand, are produced at a relatively higher cost and can also be limited by the number of available platelet donors. A major advantage of apheresis platelets is that the platelet donor can be chosen for specific properties, such as HLA- and human platelet antigen (HPA) type and cytomegalovirus status. This may be of importance for ASCT patients, where it is important to reduce the risk of alloimmunization as much as possible and where the risk for transmission of cytomegalovirus must be kept to a minimum[48]. The Platelet Dose (“PLADO”) study found significantly lower increase in platelet count with platelets derived from whole blood compared to apheresis platelets but could not show the equivalence in clinical effectiveness as there was no difference in the incidence of bleeding[49]. The quality of apheresis-derived and buffy coat-derived platelets are generally considered equal nowadays, and they are handed out equally to patients in need of a platelet transfusion.

Platelet concentrates can be stored for 5-7 days but it has been shown that storage time has a negative effect on platelet quality, a process called platelet storage lesion[50]. The loss of function is accompanied by morphological changes such as decreased mean platelet volume and increased release of platelet α -granules and cytosolic proteins. A recent study implicates that there might be differences in platelet storage lesions between buffy coat and apheresis platelets, with apheresis platelets expressing higher pH and higher lactate, especially at the end of the storage period[51]. Storage time also seems to influence platelet increment in the transfused patient, as elaborated on in the next part.

1.5.3 Platelet transfusion efficacy

The most important aim of a platelet transfusion is to stop or prevent bleeding. Patients with severe thrombocytopenia are at high risk of bleeding, and when bleeding occurs it can often be life threatening. One of the challenges in transfusion medicine research is to measure the clinical efficacy of transfused platelets. Ceasing of actual bleeding, rated according to the World Health Organization’s (WHO) bleeding scale or indirect methods like bleeding time, has been used historically. The WHO bleeding scale is graded from 0-4 where grade 0 equals no clinical signs of bleeding and grade 2 or higher usually is associated with a clinically relevant bleeding. For example, grade 2 can be epistaxis or hematemesis, and a grade 4 bleeding is associated with severe hemodynamic instability or fatal bleeding[52].

Out of simplicity, in the clinical setting, the post-transfusion platelet increment (PI) is most often used as a surrogate marker of transfusion efficacy where post-transfusion platelet count is usually performed at 1 and/or 24 hours after the transfusion. PI is the basis for other efficacy measurements shown below, and is performed as follows:

PI = Post-transfusion platelet count - Pre-transfusion platelet count.

The percentage platelet recovery (PPR) is calculated from the platelet increment $\times 10^9/L$ (PI), the blood volume in liters (BV) and the platelet dose transfused $\times 10^{11}$ (PD):

$$R (\%) = PI \times BV \times PD^{-1} \times 100.$$

The corrected count increment $\times 10^9/L$ (CCI) is calculated from the PI, the body surface area of the patient in m^2 (BSA) and PD:

$$CCI = PI \times BSA \times PD^{-1}.$$

More direct and precise methods for studying recovery and survival of the transfused platelets require that they can be discriminated from the patient's own circulating platelets. Some studies have used radioisotope-labeled autologous platelets to track transfused platelets in healthy volunteers[53]. Newer methods include transfusing apheresis platelets of different HLA Class I types than the patient, and the HLA Class I discrepancy between donor and recipient can then be used to identify the transfused platelets using for example multicolor flow cytometry[54].

The response from a random platelet concentrate unit varies to a large extent and the increase is hard to predict. Some studies have attempted to address this question. Results from the Trial to Reduce Alloimmunization to Platelets (TRAP) study showed a mean CCI of 13.1-16.5, depending on the preparation technique, one hour after transfusion. Recovery measured as PPR varied between 40.4 and 50.7 % [55]. They further reported that significant increases in platelet increments were associated with ABO-compatible platelets and platelets stored for 48 hours or less[56]. Recently a publication used flow cytometry to identify apheresis platelets of non-self HLA Class I types transfused in eight patients undergoing allogeneic stem cell transplantation. One hour after transfusion fresh platelets (5-72 hours old) showed a recovery of 43% with a median CCI of 18.2. For stored platelets (73-148 hours old) recovery was 30% and median CCI was 11.4. At 15-28 hours after transfusion, recovery was 31% for fresh platelets and 17% for stored platelets, with a median CCI of 13.2 for fresh platelets and 6.4 for stored platelets[54].

Measuring the number of viable platelets after transfusion does not give the whole picture. A burning question is how to assess platelet function after transfusion and some attempts have been made to address this issue, even though most studies assess viability more than function. Some recent studies have used flow cytometry to investigate platelet function after transfusion. One study assessed platelet reactivity toward the agonists adenosine diphosphate (ADP), thrombin receptor-activating peptide (TRAP) and convulxin. They used a flow cytometry-based assay and found a higher mean fluorescence intensity of platelet activation markers after transfusion. They concluded that platelet transfusion improves platelet function in thrombocytopenic patients[57]. A similar approach was used in another recent study, but agonist-induced platelet reactivity was instead correlated to clinically significant bleeding. A moderate correlation was observed between platelet count and expression of the platelet activation marker P-selectin, using four different agonists. The platelet count was not

significantly correlated to clinically relevant bleeding (WHO grade 2 or higher) but activation capacity of the platelets was. As an example, the risk of developing a significant bleeding was reduced by 72% for every unit increase of mean fluorescence intensity (MFI) on the platelets after stimulation with ADP[58].

1.5.4 Platelet transfusion approaches

Prophylactic platelet transfusions have been standard of care to prevent bleeding in hematological patients since the late 1970's. This strategy was based on a number of small, randomized controlled trials (RCTs) that were published around this time[2]. In a more recent study, standard- and low-dose platelet transfusions were compared in patients with chemotherapy induced thrombocytopenia. This study was stopped early because of higher grade 4 bleeding in patients receiving the low dose platelet transfusions, making further randomization unethical[52].

Prophylactic versus therapeutic transfusions have been compared in several studies[59, 60]. As an example, one study randomized 600 patients to receive, or not to receive, prophylactic platelet transfusions when morning platelet counts were less than $10 \times 10^9/L$. There were significantly more bleeding episodes of WHO grade 2, 3, or 4 in the no-prophylaxis group among patients treated with chemotherapy or undergoing ASCT[59]. However, in patients undergoing autologous stem-cell transplantation the rates of bleeding events of WHO grade 2, 3, or 4 were similar in the two study groups. This differs slightly from a previous study showing a higher incidence of grade 2, but not grade 3 or 4, bleedings in the no-prophylaxis group in patients undergoing autologous stem-cell transplantation[60]. Overall, the results of these studies support the continuation of use of prophylactic platelet transfusions to hematologic patients with chemotherapy induced thrombocytopenia and patients undergoing ASCT.

In the last decade, cold storage of platelets has gained new research interest, as has cryo-preservation of platelets. In a recent study, cold-stored platelets increased platelet aggregation *in vitro* after transfusion compared to room temperature-stored platelets[61]. There was also no significant difference in platelet count or postoperative blood loss between transfusion with cold- or room temperature-stored platelets. In a different study, cold-stored platelets were transfused to bleeding patients during different surgical procedures[62]. All patients had adequate surgical hemostasis, based on reports from the clinicians. Cold-stored platelets clearly come with a moderately reduced recovery and markedly reduced survival but seem to have a short term hemostatic effect in actively bleeding patients, with the advantage of increasing storage time significantly[63].

1.5.5 Platelet transfusion complications

Common side effects of platelet transfusions are non-hemolytic transfusion reactions with symptoms such as fever or even rigor and chills. These reactions are most often not reported and the true incidence is hard to estimate[64]. The effect of universal leukodepletion on the incidence of febrile non-hemolytic transfusion reactions has been studied with findings that

the incidence decreased from 0.45% to 0.11% after leukodepletion. The reduction was significant[65].

Despite great efforts there is still a small risk of transfusing a platelet unit with bacterial contamination[66]. This is due to the fact that platelets, unlike other transfusion products, are preferably stored at room-temperature[67]. However, the number of cases where bacterial contamination leads to clinically relevant infection is likely small. Newer techniques for pathogen inactivation also have a potential to further reduce this risk[68].

Transfusion across the ABO barrier may be necessary if no compatible product is at hand. Guidelines say that this is acceptable but several studies have shown that transfusion of ABO incompatible platelets give a significantly lower platelet increment compared to ABO-matched transfusions[69].

A rare, but serious, complication is called TRALI (transfusion related acute lung injury). It is defined as an acute lung injury that develops during or within 6 hours after transfusion of one or more units of blood or blood components. The most common symptoms are dyspnea, hypoxia, hypotension, and fever. Research led to the understanding that plasma-containing components from female donors with leukocyte antibodies were responsible for the majority of TRALI fatalities[70]. A hypothesis has been stipulated that antibodies in the blood component unit bind to correspondent antigens in the recipient and causes an immunological reaction involving granulocytes in the recipient. Granulocytes reside to a large extent in the lungs and the reaction leads to injury of the lung endothelium, after which pulmonary oedema develops[71]. Reduction strategies, such as leukodepletion of blood products and excluding women as plasma donors, have lowered the incidence of TRALI from 2.57 cases per 10 000 units transfused in the US in 2006[72] to only 11 suspected TRALI reports overall and no TRALI deaths reported in 2012.

1.6 PLATELET REFRACTORINESS AND THE COMPLEXITY OF HLA

Platelet refractoriness is described as the repeated failure to obtain expected increases in platelet count following platelet transfusions[73]. Guidelines and previous studies[69, 74, 75] have often used a CCI of more than $7.5 \times 10^9/L$ as a cut-off for a successful transfusion, while a CCI of less than $7.5 \times 10^9/L$ is usually considered a poor response. Platelet refractoriness is commonly defined as a one-hour increment of less than $5 \times 10^9/L$ in at least two consecutive transfusions. Refractoriness is often related to non-immune causes such as fever, infection, or bleeding. Of the immune causes, alloimmunization against HLA with the formation of anti-HLA antibodies is the most important. A small part of refractoriness is also caused by immunization against HPA[76].

The genes for HLA in humans are located on the short arm of chromosome 6, in other species this is referred to as Major Histocompatibility complex (MHC) system[77]. MHC or HLA are important structures in the recognition of foreign antigens, allogeneic (non-self) cells and autologous (self) abnormal cells[78]. HLA class I, II and III have different structure and functions. Class I, present on all nucleated cells and on platelets, is divided into HLA A-, B-

and -C. Platelets predominantly express HLA-A and -B. The main function of HLA Class I is to present antigenic peptides to responding T cells, and the HLA molecule consist of two non-covalently linked polypeptide chains: an α chain or heavy chain (with three globular domains ($\alpha 1$, $\alpha 2$, and $\alpha 3$) and a subunit called $\beta 2$ microglobulin ($\beta 2m$).

The HLA system is the most polymorphic gene cluster in the human genome, and this diversity affect which amino acids are in the peptide-binding site of the HLA molecule and thus their binding specificity. The polymorphism of the HLA system is important in an evolutionary aspect, increasing the likelihood that virtually any microbe can be recognized by at least some individuals[77]. More than 9.000 HLA alleles have been recognized, of which more than 7.000 belong to HLA Class I. Each HLA allele name has a unique number consisting of up to four sets of digits separated by colons[79]. The HLA denotation system is explained in figure 3. In the context of platelet refractoriness, organ transplantation, and ASCT the polymorphism of the HLA system is problematic and compatible donors may be hard to identify.

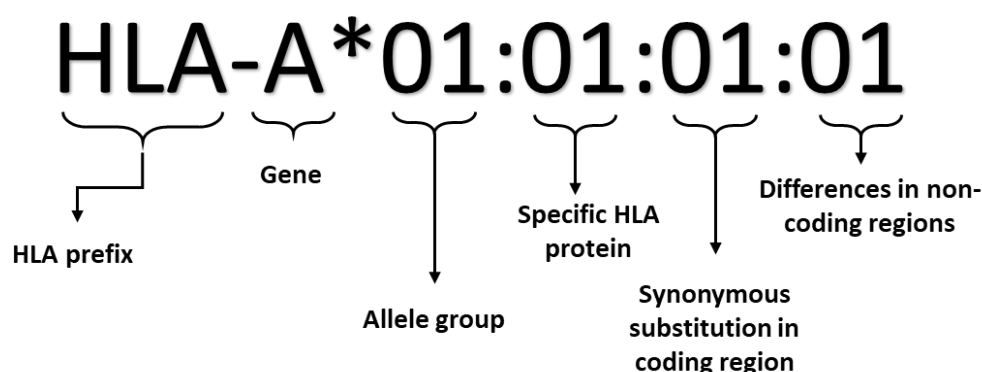


Figure 3. System for nomenclature of HLA alleles.

Platelet refractoriness caused by anti-HLA antibodies is a serious condition associated with increased mortality and risks for delayed therapeutic interventions[80]. Anti-HLA antibodies involved in platelet refractoriness are primarily composed of IgG and are directed toward HLA-A and -B[81]. Exposure to foreign HLA antigens at pregnancy, via transfusion of different blood components, and/or organ transplantation are the primary reasons for anti-HLA antibody development.

At least three possible mechanisms for anti-HLA antibody-dependent platelet destruction have been proposed, graphically envisaged in figure 4. It has been shown that antibody-coating of platelets leads to complement opsonization and formation of membrane attack complexes (MACs), leading to reduced platelet survival *in vitro* as well as in animal transfusion models[82, 83]. Fc-Receptor (FcR)-mediated phagocytosis is also proposed as an important clearance mechanism, with opsonization of HLA-mismatched platelets by anti-HLA antibodies targeting them to the spleen for phagocytosis by macrophages. It has also been shown that anti-HLA antibodies induce Fc γ RIIa-dependent platelet activation and

enhanced phagocytosis[81, 84]. Lastly, NK cells are part of the immune cross talk in antiviral and antitumor responses and from our group suggest that antibody-coated platelets, in a similar way as reported for cancer cells, can activate NK cell degranulation (Meinke et al, unpublished). Whether or not NK cells play a role in platelet refractoriness remains to be addressed[85].

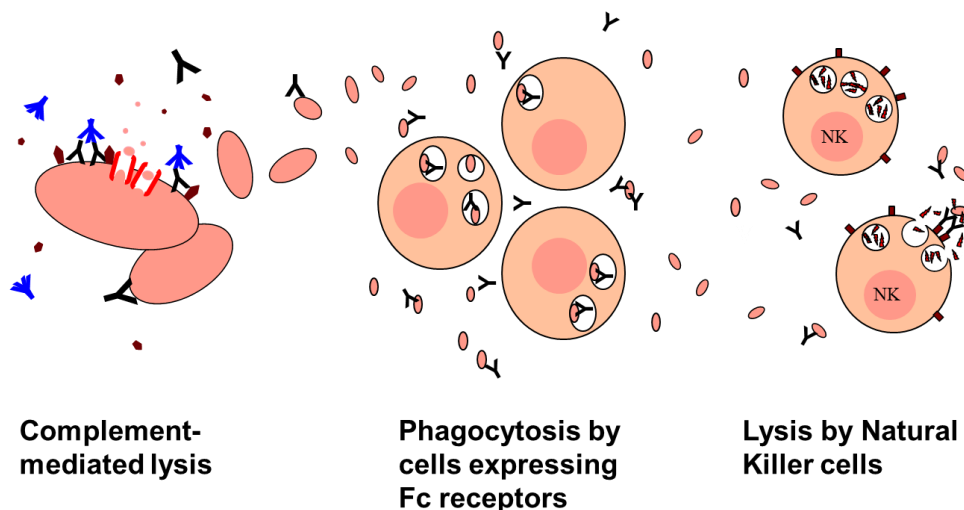


Figure 4. Schematic overview of proposed mechanisms for platelet destruction by anti-HLA antibodies. Figure by Stephan Meinke published with permission.

Most patients with this type of platelet refractoriness are women, which has been explained by the frequent occurrence of HLA immunization during pregnancy. It has been estimated in different studies that 10-50% of women with a history of previous pregnancies are immunized against HLA[86-88]. There is also a risk of immunization against foreign HLA class I, following transfusion with blood products as well as organ or tissue transplantation. Consequently, a majority of platelet refractory patients with anti-HLA antibodies are female patients with hematological diagnoses, most of them acute leukemia or MDS[76, 89].

From a clinical perspective HLA alloimmunization clearly leads to rapid clearance of platelets in some patients, while in others transfusion failure is rarer. It has been shown that patient anti-HLA antibodies bind to donor platelets resulting in IgG-opsonized platelets which are rapidly cleared from the circulation[81]. The efficacy of anti-HLA antibody-induced platelet clearance differs, and several parameters may contribute to this. Firstly, HLA expression levels on platelets may vary between individuals. It has been shown that platelets with consistently low HLA-B8, B12 or B35 displayed a strongly reduced antibody-mediated internalization by macrophages[90]. The amount and affinity of antibodies also seem to be of importance. In the TRAP study, low levels of anti-HLA antibodies were not associated with platelet refractoriness, contrary to high titers of antibodies that were clearly related to refractoriness[89].

Universal leukodepletion was advocated in many countries around 20 years ago. This intervention has significantly lowered the risk of harm from platelet transfusion, including immunization against HLA. It has been shown that alloimmunization was significantly

reduced, from 19% to 7%, in Canada after the introduction of leukodepletion. Similar reduction was seen for alloimmune platelet refractoriness, from 14% to 4% [91]. The PLADO trial showed similar numbers with 5 % of the patients developing platelet refractoriness [86].

As alloimmunization from blood products now rarely occurs, previous pregnancies are currently the leading cause of platelet refractoriness. The broad HLA alloimmunization, beyond paternal HLA specificities, seen after pregnancy has not yet been fully understood. One possible explanation could be that pregnancy results in antibodies specific for epitopes that are common across several HLA specificities [92].

1.6.1 HLA selected platelets

Strategies to select platelets for refractory patients include platelets from HLA-matched donors, avoidance of known anti-HLA antibody specificities, and platelet cross-matching which gives a better outcome compared to transfusion with standard platelets [75, 93].

For selection of platelets from HLA-matched donors, the recipient and the platelet donor are matched for HLA-A and B, as these are most involved anti-HLA antibodies causing platelet refractoriness [94]. For this type of matching, the HLA class I type of patient and donors need to be known. The degree of match can be determined based upon a classification system described in detail in Table 1. Briefly, a grade A match is defined as all donor HLA antigens, two A- and two B-alleles, being identical to the recipient. In paper II, this was referred to as a complete HLA match.

HLA matching can also be done according to the CREG (cross-reactive epitope group) classification system [95]. It has been shown that many epitopes, the part of an antigen that is recognized by antibodies, are shared among several HLA molecules [96]. Anti-HLA antibodies can be directed against these shared epitopes, resulting in cross-reactivity with numerous HLA alleles. As an example, the 9 HLA-A alleles in the A1 CREG share one common epitope. As a consequence, the immune system of a patient with HLA-A11 will not recognize HLA-A80 as foreign, since HLA-A11 and -A80 belong to the same CREG.

HLAMatchmaker was introduced as a computer-based matching algorithm. It originally considered linear sequences of three amino acids (triplets) in antibody-accessible positions on HLA molecules. The triplets could be considered key elements of epitopes that can induce the formation of antibodies [97]. This was later developed into the eplet version, taking into consideration the three-dimensional structure of HLA molecules. Triplets and eplets may be identical, but eplets may also consist of amino acids positioned with some distance that cluster together on the molecular surface [98].

Table 1. HLA-Matched Platelet Classification

Grade	Description
A	All 4 donor HLA antigens are identical to the recipient
B1U	Only 3 antigens are detected in the donor (homozygous at 1 HLA allele); all 3 detected antigens are identical to the recipient
B1X	2 HLA antigens are identical to the recipient and 1 HLA antigen is cross-reactive
B2U	Only 2 antigens are detected in the donor (homozygous at 2 HLA alleles); both detected antigens are identical to the recipient
B2UX	Only 3 HLA antigens are detected in the donor (homozygous at 1 allele); 2 antigens are identical with the recipient and 1 antigen is cross-reactive
B2X	2 antigens are identical to the recipient and 2 antigens are cross-reactive
C	1 antigen of donor is not present in the recipient and are not cross-reactive with the recipient
D	2 antigens of the donor are not present in the recipient and are not cross-reactive with the recipient
R	Random donor

Adapted from [95].

The practice to use platelets from HLA-matched donors is advocated throughout the world[93, 99], but suffers from several problems, of which shortage of optimally matched donors is the most important. Lack of donors poses a particular problem because it necessitates transfusion of partially mismatched platelets, with the risk for enhanced clearance of the platelets by pre-existing anti-HLA antibodies. This problem is particularly present in smaller centers in which the number of HLA-typed donors is limited, but also larger centers with pooled resources have problems offering all patients an optimally matched platelet product[76].

In patients tested for anti-HLA antibodies, with known specificities, choosing antigen-negative platelets, platelets that lack the antigens to which the patient has formed antibodies, is an option[100]. In paper II we referred to this as having no donor specific antibodies (DSAs). This way of choosing platelets for refractory patients can greatly expand the number of compatible donors. This was exquisitely shown in a publication where 29 alloimmunized patients were matched for grade A, grade BU and antigen negative. With this strategy a mean

of 6 grade A-matched donors were found, as compared to a mean of 1426 antigen-negative, from a total pool of 7247 HLA-typed donors[101].

Another alternative is cross-matching in which neither the HLA type of the patient or the donor is necessary. Methods used include solid-phase red cell adherence (SPRCA), modified antigen capture ELISA (MACE) and flow cytometry[100]. These methods only detect antibodies in the sera of patients directed against the antigens of the tested platelet product, but not all antibodies in the patient. The biggest advantage of cross-matching is that it is done on platelets already available in store at the blood bank, and it does not require a large register of HLA typed donors. However, it is not optimal in patients that are broadly HLA-immunized due to the low probability of finding a compatible platelet unit with this method.

1.6.2 HLA deficient platelets

The use of platelets ‘stripped’ of HLA antigens, by chloroquine treatment, for platelet transfusions to alloimmunized patients was suggested in 1984[102]. However, the study had not assessed platelet function after chloroquine treatment and the authors doubted whether the treated platelets would be effective in hemostasis.

Acid treatment as a method to eliminate HLA class I molecules from the surface of viable cells was reported in 1987 by a Japanese research group[103]. They found that a brief incubation at low pH (pH 3) significantly reduced expression of HLA-A, B, C and β 2-microglobulins on the cell surface of human mononuclear cells and MHC class I of mouse lymphocytes. A few years later a study on acid-treated platelets was published[104]. They could show a platelet viability of 83% after acid treatment. Platelet function studies were performed and showed that acid-treated platelets aggregated almost to the extent of PBS-treated platelets. Furthermore, sera from immunized patients with multispecific anti-HLA antibodies reacted with PBS-treated platelets, but not with acid-treated platelets. The platelets showed no morphological changes with the methods used, and platelet-specific glycoproteins were not affected.

It has been suggested that the acid treatment results in removal of complete HLA molecules[105]. However, our data in paper IV and other studies support the proposed mechanism that low pH leads to dissociation of β 2m with the free heavy chain firmly remaining in the platelet membrane[106].

Results from the first transfusion with acid treated platelets in one patient and two healthy volunteers was published in 1991[107]. In the two healthy volunteers, platelet recovery was similar for acid-treated platelets and untreated control platelets, but platelet survival time decreased after acid treatment. In the alloimmunized patient the first transfusion of acid-treated platelets yielded a CCI of $11.3 \times 10^9/L$ while the second transfusion only gave a moderate CCI. The following few studies on acid treated platelets for transfusions showed mixed results[108-110]. With the introduction of leukoreduced blood products from the late 1990s and the following reduction in the frequency of alloimmunization, the interest in HLA-depleted platelets faded.

1.7 EX VIVO PRODUCTION OF HUMAN PLATELETS

Ex vivo production of platelets has been viewed as a method of resolving both shortage of transfusion products and certain safety issues, such as refractoriness. Induced pluripotent stem cells (iPSCs) are cells with pluripotent and self-renewing capacities that has been used to manufacture many different cell types ex vivo, including platelets. In relation to refractoriness, such platelets could be obtained from HLA typed iPSCs to match the refractory patient or be made HLA-depleted by manipulation of HLAs and HPAs. As mentioned before, demand is also expected to increase within 10 years, and ex vivo platelets would be a valuable addition to the standard collection procedures. However, so far, the challenge has been to make ex vivo platelets at large scale production. It was recently reported that over 100 billion platelets were produced, with *in vitro* and *in vivo* evaluation showing functionality comparable to donor-derived platelets[111]. The amount is still not sufficient though, considering that one regular platelet concentrate contains around 300 billion platelets.

2 STATE OF THE ART PRIOR TO THIS THESIS

Even though several methods have gained ground in assessing platelet function and bleeding risk in hematological patients in recent years, platelet count remains the trigger for platelet transfusion. There is a strong agreement that platelet count is poorly correlated with bleeding risk[112, 113]. Better instruments to predict both bleeding risk and the demand for platelet transfusions in the individual patient would be highly valuable.

Platelet refractory patients are relatively few, but they require extensive resources during their thrombocytopenic episodes. Several methods for platelet transfusion matching are used with their different advantages and disadvantages. To evaluate clinical outcome of HLA matched platelet transfusions is of utmost importance, especially considering the costs regarding both money, time, inconvenience for donors and psychological aspects for the patients.

HLA deficient platelets were transfused to a handful of patients in the 1990s and showed varying results, as mentioned in the introduction. At that time, the preclinical testing was limited and there was a great lack of knowledge regarding immune system responses to these platelets. We therefore pursued this method in a series of experiments in a modern setting, with the aim to have a better understanding and characterizing of the acid treatment and the effect on the final HLA deficient platelets as well as immune system responses. A clinical study with transfusion of HLA deficient platelets is also planned.

3 RESEARCH AIMS

The overall aim of this thesis was to gain a better knowledge of factors affecting transfusion outcome. Due to the time limitations of a thesis work more specific aims were necessary.

The specific aims of the studies were:

- I. To evaluate indications and outcome of prophylactic platelet transfusions in hematologic patients, including platelet count increments and effect on hemostasis. Furthermore, we aimed to evaluate platelet function in the platelet concentrate as well as in the recipient, both before and after platelet transfusion.

- II. To characterize the responses to HLA- or cross-matched platelet transfusions, with regard to platelet count and clinical effects as well as antibody patterns, in a retrospective study of a cohort of platelet refractory hematologic patients with detected anti-HLA antibodies at Karolinska University Hospital.

- III. To evaluate function of, and biological changes in, acid-treated platelets (HLA deficient platelets) in order to further work toward a new transfusion principle to platelet refractory hematologic patients.

4 MATERIALS AND METHODS

4.1 ETHICS

4.1.1 Paper I

The Regional Review Board of Ethics in Stockholm approved this prospective study (DNR 2015/1413-311). Informed consent for blood sampling and data collection was obtained from each patient before sampling. The amount of blood sampled from each patient was calculated to be sufficient for the analyses, yet not more than the minimal amount required.

4.1.2 Paper II

The Regional Review Board of Ethics in Stockholm approved this retrospective study (DNR 2015/1413-311). Many of the patients included were diseased at the start of the study and therefore informed consent was not obtained. Data published were generally on group level and unique patients could not be identified. Where individual data were presented, the patients had been given a reference number. Only the responsible researchers have access to the identification list.

4.1.3 Paper III

In this study we only used platelets remaining from production of platelet concentrates. These platelets are otherwise discarded. Since they consist of a pool of platelets from eight blood donors' individual platelets cannot be traced or identified. The Regional Review Board of Ethics in Stockholm approved the study (DNR 2011/1824-311).

4.1.4 Paper IV

In this study we the same type of platelets as in Paper III. The study also included transfusion of HLA deficient platelets to immunocompromised mice. This part of the study was approved by Jordbruksverket (DNR S32-15 plus amendment 42-15).

4.2 STUDY DESIGN

4.2.1 Paper I

Paper I was a prospective observational study of prophylactic platelet transfusions to 40 hematological patients. The patients were admitted to the hematology ward in Karolinska University Hospital and were planned to receive a prophylactic platelet transfusion on the day of inclusion in the study. Blood sampling was done at three time-points: before, 1 hour after and 18-24 hours after transfusion. Outcome of transfusion, hemostasis and platelet function were evaluated at all time-points. Methods used were blood cell counting, flow cytometry and ROTEM (rotational thromboelastometry).

4.2.2 Paper II

Paper II was a retrospective study evaluating 32 platelet refractory hematology patients who received 142 matched platelet units between 2007 and 2016. Four matching strategies were compared:

- 1) Genomic HLA typing with low resolution (giving the allele group, but not specific HLA proteins, see figure 3), performed using polymerase chain reaction–sequence-specific oligonucleotide probing
- 2) Serologic “eplet score” calculated using HLAMatchmaker.
- 3) Cross-matching using lymphocyte cytotoxicity.
- 4) Matching based on donor-specific antibody (DSA) specificity, determined using Luminex.

4.2.3 Paper III

This was a laboratory study evaluating the effect of acid treatment on platelets compared to control-treated or untreated platelets. Kinetics of acid stripping, viability, phenotypic alterations, and sensitivity to complement-mediated lysis and phagocytosis were determined by flow cytometry. Platelet function was evaluated using a multiplate analyzer.

4.2.4 Paper IV

This was a laboratory study of acid treatment in whole platelet transfusion units according to our scaled-up protocol. HLA removal, viability and function were assessed and compared to platelets treated in small scale. HLA re-association during storage of acid-treated platelets was evaluated. We tested the platelets ability to support endothelial integrity by measuring trans endothelial electrical resistance (TEER) across a cell monolayer. Finally, we tested the survival of acid-treated platelets in vivo using a mouse model for platelet refractoriness.

4.3 LABORATORY METHODS

4.3.1 Blood counts

In paper I blood counts was measured on a Sysmex automated analyzer. In paper II blood counts were retrieved from the patients’ electronic medical records in Take Care (the e-health system at Karolinska University Hospital).

4.3.2 Flow cytometry

Flow cytometry was in different ways used in all papers included in this thesis. The technology of flow cytometry provides fast analysis of single cells in solution[114]. Flow cytometers use lasers as light sources to produce both scattered and fluorescent light signals when a cell passes the lasers. The light signals are read by detectors such as photodiodes or photomultiplier tubes and are then converted into electronic signals that are analyzed by a computer. Fluorochrome-labeled antibodies are often used to mark the receptor or molecule

of interest. In the flow cytometer, cell populations can be analyzed and/or purified based on their fluorescent or light scattering characteristics.

Whole blood was used in the flow cytometry analyses in paper I, to not lose platelets or contribute to activation during preparation. This led to a large number of red cells in the samples and too many events per unit of time with normal scatter threshold. Therefore, expression of CD42a was set as the trigger in the flow cytometer settings, i.e. only cells above a set level of CD42a expression is saved by the computer. This allows for analyzing the samples in a reasonable time, given that platelets are a small part of the total number of cells in the samples. Using this setting roughly reduced analyzing time from 30-60 minutes per sample to 5-10 minutes per sample.

4.3.1.1 Resting and activated platelets

Resting platelets express low levels of P-selectin on the surface and GPIIb-IIIa is in its low affinity state. PAC-1 is a specific monoclonal antibody that binds to GPIIb-IIIa in its high affinity state, occurring after platelet activation. PAC-1-negative platelets are hence considered as resting platelets. PAC-1 positive platelets are then logically an indication of platelet activation[115]. P-selectin is present in α -granules of resting platelets and is translocated to the plasma membrane after activation, resulting in the surface expression of approximately 13,000 P-selectin molecules[1]. These can be identified by a monoclonal CD62P antibody.

4.3.3 Rotational thromboelastometry (ROTEM)

Rotational thromboelastometry (ROTEM ®, TEM International GmbH, Munich, Germany) is a viscoelastic hemostasis analysis. During clot formation fibrin networks help to increase the viscosity of the blood. ROTEM uses a mechanical rotating sensor that detects the viscoelastic changes in the clot. As the clot grows, rotation slows down. The ROTEM instrument graphically visualizes the stages of clot formation by a thromboelastometric curve (temogram), displayed on the screen together with calculated ROTEM parameters.

Citrated whole blood is used for the assays. The blood is recalcified and clot formation is activated by different additives. The five principal assays used with the ROTEM instrument are INTEM, HEPTEM, EXTEM, FIBTEM and APTEM[116]. In our studies in paper I and IV, results from the EXTEM assay were reported. EXTEM uses tissue factor as an activator of the extrinsic pathway and reflects the clot strength dependent on platelet and fibrin interactions. The INTEM assay was performed in paper I, but not reported due to inconsistent results. INTEM uses ellagic acid and phospholipids as activators of clot formation via the intrinsic pathway, simulating contact activation. As in EXTEM, clot firmness reflects both platelet and fibrin contribution to the clot.

Coagulation time (CT), in seconds, is the time from start of the test until a clot of 2 mm has formed. CT reflects thrombin generation which mainly depends on the enzymatic activity of coagulation factors, concentration of anticoagulant substances and fibrin split products. CT is

usually achieved within 1 min in the EXTEM assay. Clot formation time (CFT), in seconds, indicates the time it takes the clot to extend from 2 to 20 mm. CFT mainly depends on thrombin generation, platelet count and platelet function, as well as fibrinogen concentration and fibrin polymerization. One of the most important ROTEM® parameters is maximum clot firmness (MCF) measured in millimeters. MCF is defined as the maximum amplitude of clot firmness reached during the test, in other words the maximum size of the clot. MCF reflects the mechanical strength of the clot and mainly depends on platelet count and platelet function, fibrin concentration and fibrin polymerization, factor XIII activity, and colloids. When faster decision-making is wanted such as in patients with severe bleeding, the amplitude of clot firmness at 5 or 10 min after CT (A5 or A10, respectively) can be used. A5 and A10 correlate very well with the MCF and allow for decision-making within 10–15 min after starting the test. EXTEM and INTEM A5 and A10 correlate with fibrinogen concentration and platelet count[117]. CT, CFT, A10 and MCF is shown on a temogram in figure 5.

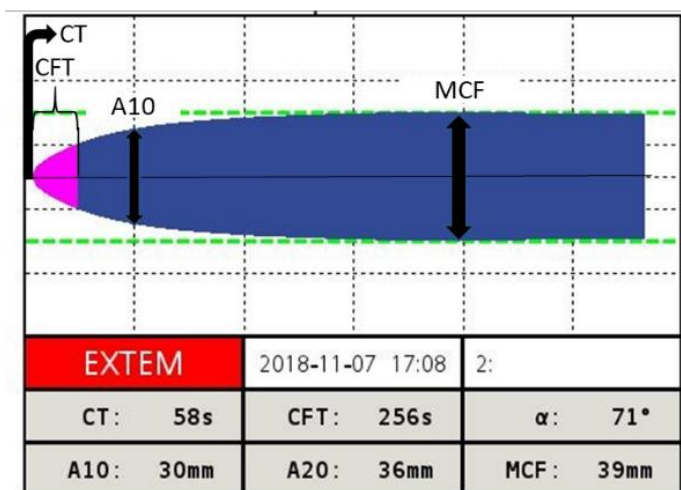


Figure 5. Temogram from a thrombocytopenic patient.

Thromboelastography is useful for evaluating the overall interaction between platelet GPIIb/IIIa receptors and fibrinogen, as activated platelets provide ample binding sites (GPIIb/IIIa) for fibrinogen[116].

4.3.4 Multiplate

Multiplate (multiple electrode impedance platelet aggregometer, Roche Diagnostics) is an instrument for analyzing platelet aggregation in whole blood. The system consists of a five-channel computerized device, hence “multiple electrode”. It uses disposable test cells that contains two electrodes. Platelet aggregation on the electrodes, induced by addition of agonist, causes an increase in impedance (i.e., electrical resistance) that is shown as a curve on the computer screen. The maximal platelet aggregation and aggregation velocity are converted into arbitrary aggregation units[118]. Multiplate analyses are included in paper III. We also used Multiplate to investigate aggregation in the patient samples before and after transfusion in paper I. However, Multiplate is not recommended in patients with platelet

counts below the reference values[119]. We could see small changes after transfusion, but as predicted there were no significant differences, and the results are not included in the manuscript.

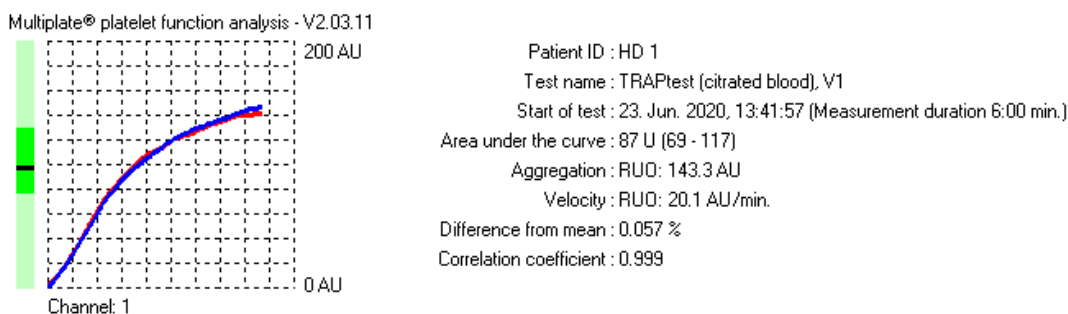


Figure 6. Multiplate graphics from analysis of a healthy donor. The most important parameter for aggregation is the area under the aggregation curve (AUC).

4.4 ACID TREATMENT OF PLATELETS

HLA deficient platelets are obtained by a brief incubation at low pH using treatment with citric acid. This removes the antigenic structure of donor HLA Class I from platelets by denaturation of the trimolecular HLA complexes, resulting in dissociation of $\beta 2m$ while the free heavy chain remains in the platelet membrane and can be detected by antibodies specific for HLA class I free heavy chain.

In more detail, platelets from concentrates were pelleted, cooled on ice, and resuspended in ice-cold citric acid buffer (equal volumes of 263 mmol/L citric acid and 123 mmol/L Na_2HPO_4 , resulting in pH 2.9 to 3.0) or ice-cold storage solution for platelets (SSP+, MacoPharma; control). Treatment was stopped by adding a 20-fold excess volume of ice-cold SSP+. Platelets were pelleted and resuspended in their original supernatant and counted using a cell counter (CASY TT, Roche Diagnostics, Rotkreuz, Switzerland; 60-mm capillary). Untreated samples were adjusted to match the concentrations of the treated samples.

4.5 MOUSE MODEL

To assess survival of the acid-treated platelets in vivo in paper IV, a mouse model of platelet transfusion was set up. We chose NSG-SGM3 mice as recipient mice, as these mice carry a combined genetic background from NOD (non-obese diabetic) mice, SCID (severe combined immunodeficiency) mice and mice deficient in the common γ chain of several cytokine receptors. They have all the phagocytes that are thought to take part in the clearance of platelets from the circulation but lack lymphocytes that might react to human antigens[120]. To provide a permissive environment for transplantation of HSCs, they are also transgenic for human SCF, GM-CSF and IL-3[120]. Acid-treated, control-treated, and untreated human platelets were labelled with different fluorescent dyes and mixed in equal parts before injection into the tail vein of the mice, mimicking a platelet transfusion. One hour after

injection, blood was sampled from the mice and analyzed by flow-cytometry. The human platelets were identified by expression of human CD61 and the distribution of the three differently labelled populations was compared to the composition of the injected mixture. The flow cytometer CytoFLEX (Beckman Coulter) allows for determination of the number of platelets per sample volume. Thus, we could calculate how many of the injected platelets that still circulated in the assumed total blood volume of 1.5 ml.

We also assessed survival of the acid-treated platelets *in vivo* in the presence of anti-HLA antibodies. At one hour after platelet injection the concentration of acid-treated, control-treated, and untreated human platelets were assessed. Next, mouse anti-human anti-HLA antibodies were installed by intraperitoneal injection. Survival of injected platelet concentrations were again measured in blood samples acquired at two and four hours.

4.6 ADDITIONAL METHODS

Additional methods used in the different studies, but not described in this chapter, are described in detail in the methods part of each paper.

4.7 STATISTICS

Statistical analysis was performed with computer software (Prism 6.0 or 7.0, GraphPad Software, Inc., San Diego, CA or SPSS, IBM Corp.).

In paper I and II we used nonparametric statistical methods owing to nonnormality of the data, i.e. Mann–Whitney U test for comparing two groups, Kruskal–Wallis for three or more groups and Spearman rank correlation for correlations.

In paper III the statistical significance of differences between the results for untreated, control-treated, and acid-treated PLTs was determined using one way analysis of variance (ANOVA) with $\alpha = 5\%$ followed by Tukey's multiple comparisons test.

In paper IV we used Wilcoxon matched-pairs signed rank test for the ROTEM analyses, One-way ANOVA with Tukey's multiple comparison test for TEER and 2-way ANOVA with Holm-Sidak's multiple comparisons test for the *in vivo* analyses in the mouse model.

5 SELECTED RESULTS AND DISCUSSION

5.1 PLATELET FUNCTION IN THROMBOCYTOPENIC PATIENTS (PAPER I)

In this prospective study, we evaluated the effect of platelet transfusions in 40 hematology patients in routine clinical care, measuring platelet count increment, clot formation and response to agonist activation. AML was the most common diagnosis. Platelet count increment correlated with improved clot formation. However, the response was surprisingly variable between patients, and 34 % of the patients did not respond to the transfusion. Short time since the last platelet transfusion and extended storage time of the platelet product were linked to poor transfusion outcome, while patient sex, CRP levels and the number of previous drug cycles did not affect transfusion efficacy. In responder patients, efficient clot formation was predicted by good platelet responsiveness to agonist stimulation, but transfusion did not, however, restore poor platelet function in the AML patients to any way near healthy controls.

Already in the 1960s it was shown that bleeding risk was increased in thrombocytopenia but that bleeding risk was low in platelet counts of more than $20 \times 10^9/L$ [121]. Two more recent studies have shown that bleeding risk is increased at platelet counts below $80 \times 10^9/L$ but platelet counts have no clear correlation to bleeding at counts above $10 \times 10^9/L$ [112, 113], indicating that function of the platelets may play a role. The need for an approach to assess platelet function is evident, and what inspired us to do this study from the beginning. Our results show that platelet count is correlated to clot formation and platelet activation, but results were varying and can probably in some cases be depending on variations in the method itself. We could not correlate any of our findings to clinical bleeding in this study due to few bleeding events, however, based on other recently published studies flow cytometry analyses and different viscoelastic methods can to some extent predict bleeding risk[58, 122, 123].

Rotem is not routinely used in thrombocytopenic patients. There is no consensus on cut-off levels or which parameters to assess in the relevance of predicting bleeding. However, some studies have attempted to address this question. In ITP patients with a platelet count of less than $10 \times 10^9/L$ the mean EXTEM MCF was the only parameter significantly higher in non-bleeding patients compared to patients with bleeding symptoms, 46 mm vs 32 mm[124]. MCF was also positively correlated to platelet count. Statistically they found a platelet count of $31 \times 10^9/L$ to be critical for affecting MCF. We did not perform similar statistics in our study but observed that a platelet count of more than $20 \times 10^9/L$ often resulted in an MCF within the reference values (49-71mm). It seems likely that the underlying disorder is of importance as well as the platelet count per se, and that correlation between platelet count and MCF will probably be both highly individual as well as depending on the diagnosis. Focus should probably be directed at to what extent and at what values MCF can be used as a predictor of bleeding risk.

Rotem analyses and flow cytometry have been evaluated in thrombocytopenic patients as methods to assess platelet function, but rarely together in a study of the effect of platelet

transfusions. With our study, one step is taken in the direction of a better understanding of the correlation between flow cytometry platelet function assays and clot characteristics.

Thrombin has been shown to be important in the first steps of clot formation and formation of the compact core of the clot. ADP on the other hand seems important for attracting platelets to form the more porous outer shell[17]. From the results in our and other studies of hematological patients with thrombocytopenia it seems possible that the impaired response to TRAP (thrombin-simulating activation) which is more contact-dependent, would result in impaired formation of the core of the clot, but less so the formation of the outer shell. This could perhaps explain that these patients rarely bleed despite their low platelet counts, adhesion is not depending on thrombin and a loose clot might still be sufficient in small vessel and tissue injuries but not for larger injuries or surgical procedures. In extension, this could also provide an explanation for embolic disease in some hematological diagnoses even at very low platelet counts. Also, inactive platelets interact with immobilized fibrinogen and the very first part of clot formation is hence independent of platelet activation[125].

It is worth noticing that it is not clarified how well whole blood aggregometry assays such as Rotem, or flow cytometry assays, reflect hemostasis in vivo. These methods clearly provide more detailed information compared to classical tests, for example the activated partial thromboplastin time (APTT). However, several components of in vivo clot formation processes, such as structure of the endothelium or dynamics of blood flow, are not included. A test or model including all the components of in vivo hemostasis is probably more likely to be found in science fiction than in clinical practice. Keeping the limitations of the different methods in mind when translating results into bleeding risk in the individual patient is of great importance.

One parameter that has been correlated to bleeding, but that was not included in our study, is immature platelet fraction (IPF). It has been suggested that these young platelets, measurable through special staining of their high concentration of RNA, have better hemostatic properties than older platelets[122, 126, 127]. Up until now IPF has not been a routinely available measurement, but at least in Karolinska University hospital IPF can now be ordered with a normal blood count. Besides IPF, size has also been implicated to matter, with larger platelets having more hemostatic activity[128]. None of these measurements were included in our study, but patients with bone marrow failure have previously been shown to have a low IPF in contrast to patients with immune thrombocytopenia (ITP)[127]. TPO agonists could theoretically lower bleeding risk in the cases where it increases IPF. A recent study showed that bleeding events were fewer with eltrombopag, a commercially available TPO agonist, compared to placebo in MDS and AML patients not receiving disease specific treatment[129].

Different bleeding risks at similar PLT counts in different diagnosis have been shown[130] and one can speculate that the difference to some degree is dependent on platelet activation ability and other factors that are progressively being acknowledged to be correlated to bleeding. Platelet function does not seem to be generally impaired in ITP but has been shown

to be impaired in those with a severe bleeding phenotype[131, 132]. In our study platelet function was clearly impaired in the patients compared to healthy controls, supporting previous studies showing affected platelet function in AML patients[133-135]. The impaired platelet function compared to healthy individuals was also demonstrated at similar platelet counts by our flow cytometry assays of activation of platelets in constructed thrombocytopenic blood. The mechanism for platelet impairment in for example AML still needs to be further addressed. It has previously been shown that the megakaryocytes in the bone marrow of AML and MDS patients show hypogranulation and dysplastic features [136-138]. Qualitative platelet abnormalities, such as macrothrombocytosis and platelet density distribution, were also demonstrated in AML more than 40 years ago[139]. Different chemotherapeutic agents have also been shown to affect both megakaryocytes and platelets. For example, different anthracyclins, common cytostatic components of AML treatment, enhanced platelet expression of GPIIb/IIIa and CD62P, indicating enhanced platelet activation by these drugs.

Clearly no single factor can be pinpointed as predictive for platelet function and bleeding risk, we need a way to weigh all the factors together to reach a personalized transfusion strategy. For many of the assays it is also not known how well they reflect platelet function *in vivo* and we need to reach further understanding of the correlation between *in vitro* testing and *in vivo* function.

5.2 HLA MATCHED PLATELET TRANSFUSIONS (PAPER II)

Paper II is a retrospective study that evaluated HLA matched platelet transfusions to platelet refractory hematology patients. We found that selection of platelets with either a complete HLA match or an acceptable HLA mismatch based on genomic typing and DSA information, each was highly predictable for a successful transfusion response. For HLA-mismatched transfusions, the eplet score correlated with CCI and the fraction of successful transfusions. However, DSA matching gave an even higher correlation to a good transfusion outcome than Eplet score. Cytotoxic crossmatching was least predictive. For transfusions with one or more DSAs, the antibody reaction strength correlated with the 1-hour CCI, but many transfusions were successful despite the presence of DSAs.

Several aspects need to be considered when choosing a matching strategy in a hospital or blood bank service, such as the cost of the matching procedure itself, HLA typing of patients and donors, availability etc. HLA matching by different classification systems or using HLAMatchmaker all require that the HLA type of both patient and donor is known, and for the antigen negative approach, single anti-HLA antibodies in the patient needs to be specified. HLA typing is done on patients eligible for ASCT but is not routine in other hematology patients, neither is antibody screening. Furthermore, a large donor register is necessary for these approaches and it also postulates that the donors are willing to come to the blood bank for apheresis, sometimes at short notice. Some studies have calculated the number of donors needed to find a well-matched donor to a certain percentage of patients. For example, one study reported that 18.000 donors might be needed in European populations

to find five completely matched donors for 80 % of all patients[140]. This is considerably larger than the current donor register at Karolinska University Hospital and an expansion towards those numbers would come at a high cost.

One of the matching strategies evaluated in our study was epitope matching, where a serologic “eplet score” is calculated using HLAMatchmaker. The patients in our study were only HLA typed at low resolution, while the program require input of matching at high resolution. We therefore used the four-digit allele converter program and the ethnicity of the patient to calculate the most likely allele at the four-digit level, not only a drawback of our study but of the matching strategy itself in hospitals where low-resolution testing is standard procedure in platelet refractory patients. Eplet matching was recently evaluated in a RCT cross-over study compared with regular HLA matching. This study found it favorable to use eplet matching[141]. Eplet score was in our study, as mentioned, correlated to CCI and the fraction of successful transfusions but DSA showed even better correlation to outcome. Using DSA as matching strategy will also result in more potential donors, an important aspect not least in patients with a rare HLA type.

Using DSA as a matching strategy on the other hand, would result in transfusion of platelets that are likely fully or partly mismatched but that the patient has not developed antibodies against yet. In this scenario the risk of further alloimmunization would have to be considered. To my knowledge, no clinical studies addressing this specific question has been published, perhaps not a surprise since it could be ethically questionable to put patients at risk for broader alloimmunization than necessary. Alloimmunization is also troublesome for patients eligible for ASCT or future organ transplantation, decreasing the likelihood of finding a well-matched donor.

Another method for matching that has been reported in two studies is choosing platelets with low expression of HLA class I[90, 142]. The first study published in 1989 could show a successful transfusion in 69% of events despite a HLA mismatch[142]. The second study only assessed antibody-mediated phagocytosis and showed that platelets with low expression of HLA-B8, -B12, and -B35 showed minimal antibody-mediated internalization by macrophages as compared to platelets with high expression of the same HLA-antigens[90]. In theory, with such platelets, only HLA-A would have to be considered in the matching process.

No matter which of the methods we use, we need to gain knowledge on which HLA antigens that are important in matching. Are all antibodies a barrier to a successful transfusion or are some more relevant *in vivo*? Are there certain HLA alleles that are more immunogenic and more easily leads to alloimmunization? One possible study design for increasing the understanding could be to purify antibodies to different HLA alleles and study them in an animal transfusion model.

Some recently published studies have started to gain knowledge on the variability in response to platelet transfusions in patients with anti-HLA antibodies. It has been shown that a subset

of anti-HLA IgG-antibodies induces Fc γ RIIa-dependent platelet activation and enhanced phagocytosis[81]. The authors discuss that affinity of the antibodies are partly responsible for the different abilities to activate platelets. They also hypothesize that the specific binding site is of importance for whether the Fc tail of an anti-HLA antibody can bind and crosslink with Fc γ RIIa. The same group showed in later publication that a subset of anti-HLA IgG induced complement activation via the classical pathway, resulting in permeabilization of platelet membranes and increased calcium influx[83]. Interestingly, a very recent publication tried eculizumab, a complement C5 inhibitor, in 10 refractory hematology patients to overcome platelet refractoriness. They found that treatment with Eculizumab resulted in successful transfusions in 40% of the patients, and the effect seemed to last for the two-week study period[143]. Eculizumab is presently not usable in routine care due to the high cost of the drug. It could however be a possibility in the bleeding refractory patient not responding to platelet transfusions or other hemostatic treatment. Linking the research mentioned above together, it could be possible that only some subsets of anti-HLA IgG induce complement activation and patients with these antibodies would then have a benefit of Eculizumab. This would have to be clarified before recommending this treatment in clinical practice.

Further clarification of the characteristics of anti-HLA antibodies and their receptors on platelets would surely bring valuable understanding on which HLA antigens are important in matching. As an example, antibodies causing fetal neonatal alloimmune thrombocytopenia (FNAIT) have been found to have a decreased Fc fucosylation. The low levels of Fc fucosylation led to higher affinity to the receptor Fc γ RIIIa/b and enhanced phagocytosis. No such decrease was found in patients with refractory thrombocytopenia due to anti-HLA antibodies[144], but continued investigation would be of interest.

5.3 HLA DEFICIENT PLATELETS (PAPERS III AND IV)

In Paper III we evaluated the function of HLA deficient platelets after acid treatment. In summary we found that acid-treated platelets were viable and that they upregulated activation markers normally. The HLA deficient platelets also aggregated to a similar extent as untreated platelets in response to stimulation with three natural agonists. Acid treatment removed 70 to 90% of HLA Class I complexes from the platelet surface, which led to complete protection from HLA antibody-mediated complement lysis and reduced monocyte-mediated phagocytosis in the presence of anti-HLA *in vitro*.

In Paper IV we further evaluated the function of HLA deficient platelets by extending the acid treatment protocol to whole transfusion units (platelet concentrates) and investigating the survival of transfused platelets in a mouse model. We found that recovery, HLA reduction, and viability in the acid-treated platelet bags were comparable to the small-scale treatment protocol. The acid-treated platelets contributed normally to clot formation as assessed by Rotem. The HLA deficient platelets also contributed to endothelial integrity. There were no signs of HLA re-association during storage of acid-treated platelets. Acid-treated platelets showed a lower *in vivo* recovery than untreated platelets in the mouse model. They were

protected from rejection by single HLA allele-specific antibodies, but still cleared by the clone W6/32.

The current approach in platelet refractory patients is HLA matching. As discussed in Paper II this is an effective approach only in a certain percentage of cases. Alternatives to HLA matching would be welcome both regarding patient safety, quality of life and healthcare costs. The production of HLA deficient platelets is an attractive approach as it can be readily available within 2-3 hours, using platelets that are already in store at the blood bank, however the evidence of its efficiency is lacking. The previous few transfusions reported showed mixed results, as discussed in the introduction of this thesis. The study presented in paper III was purely pre-clinical and did not test the effect of HLA deficient platelet transfusions in patients, however it was the first study to test whether acid treatment protects platelets from complement activation and phagocytosis. This approach was of importance to evaluate the chance of clinical success in a larger patient population with such a product.

A drawback of our study is that the HLA genotype of platelet donors was not known. On the other hand, the transfusion products were all buffy coat platelets from 6-8 donors and a variety of HLA alleles were undoubtedly present. As discussed in the previous part on paper II, platelets are known to have a variant expression of HLA and platelets with a low expression would likely not induce strong binding of anti-HLA antibodies and subsequent phagocytosis.

Using an animal model has clear limitations, and the use of human platelets in a genetically modified mouse calls for cautious conclusions. In our mouse model, the acid-treated platelets were cleared faster from the circulation in the mouse model than untreated or control-treated platelets. The reason for this is so far unknown but it is reasonable to assume that platelet changes due to the acid treatment makes them more prone to clearing. This would need to be further investigated, both as an attempt to improve acid treatment before a clinical trial but also to gain increased knowledge of platelet biology. Interestingly, in paper III the acid treatment per se did not make the platelets prone to phagocytosis by monocytes, neither did they activate the complement cascade in the presence of anti-HLA antibodies in contrast to both untreated and control-treated platelets, however this was only tested *in vitro*. Cold storage of platelets results in complex storage lesions[145]. Chilled platelets are also cleared faster from the circulation but it was recently published that they provided adequate hemostasis when given to bleeding patients[62]. It has been shown that such platelets cluster their VWF receptors, leading to the recognition of these platelets by hepatic macrophage complement type 3 (CR3) receptors[67]. We cannot exclude that the short part of the acid treatment that is performed on ice would induce cold-related damage, but there are no publications supporting that this would be the case. The control-treated platelets also did not clear faster, further supporting that other factors are responsible for activation and clearance. It has been shown that loss of HLA might make cells more susceptible to NK cell killing. However, in an ongoing study by our group (not published) HLA deficient platelets were not

killed by NK cells. We believe this is due to platelets not having activating receptors for NK cells.

Proteomic studies could be one way to further dissect the effect of cold- and acid-treatment. For example, as mentioned in the introduction, platelets lose sialic acid in the aging process and this leads to phagocytosis. This is something that we have not studied in the acid treated platelets so far. Modifications could likely be done to improve our acid treatment protocol, and better understanding of the molecular changes induced by the acid treatment would be the first step.

6 CONCLUSIONS

In paper I we studied platelet function in patients with severe thrombocytopenia and examined the effect of a platelet transfusion. We found that platelet function and clot formation improved after transfusion, and that this is likely due to the better function of the transfused platelets compared to the patients' own platelets. The patients' platelets were impaired in function compared to healthy controls. This is, to my knowledge, one of the few studies linking flow cytometry and Rotem assays in thrombocytopenic patients. We found that poor platelet function measured by flow cytometry was correlated to impaired clot formation, and our study could be a part of continuous work towards new standards for assessing platelet function and the need for platelet transfusions.

In paper II we evaluated different strategies for HLA-matching of platelet transfusions to platelet refractory patients. We found that matching by DSA was strongly correlated to transfusion outcome, and that transfusions with no DSAs or a complete HLA-A and -B match were highly successful. Matching by DSA is recommended, but the risk of further alloimmunisation should be considered.

In paper III and IV we evaluated the effect of acid treatment on platelets. Our results take acid-treated platelets closer to being tested in patients. However, possible solutions for the reduced in vivo recovery and the remaining susceptibility to clearance by broadly specific antibodies need further investigation.

7 FUTURE PROSPECTS

Platelet transfusions represent an important part of supportive care for patients with hematologic diagnoses and severe thrombocytopenia. Platelet transfusions come with risks and it is a clinical challenge to transfuse the right patient at the right time. Continuous research is necessary to optimize transfusion strategies further. At present it is understood that many of the prophylactically transfused patients would never have had a serious bleeding. More precise methods to assess whether a patient suffers from a severe coagulopathy or not could contribute to reduce the transfusion frequency. This would be beneficial for the individual patient as well as lowering healthcare costs. Development of a clinical scoring system including for example platelet count, flow cytometry and a platelet aggregometry test such as ROTEM could be one possibility. The great challenge is to find the clinically most valid measurements, and a large multicenter study would probably be of immense value. In the future we will certainly be able to better predict both bleeding risk and transfusion outcome in hematological patients.

Paper I was very much a hypothesis generating study and left us with more questions than we began with. Our results imply that functional recovery of transfused platelets is of importance for overall hemostasis, but we could not with certainty differ between autologous and allogeneic platelets in our study. In future studies we plan to adopt the technique of transfusing platelets mismatched for HLA or HPA, allowing for discrimination between patient platelets and transfused platelets.

Our studies on constructed thrombocytopenic blood could also be further developed with almost endless possibilities. This *in vitro* assay could be used to assess the effect of anemia and leukopenia on clot formation and platelet function. Several different platelet agonists can be tested, one by one or in combination. The latter approach is attractive, considering that agonists rarely exist alone *in vivo* but rather simultaneously at different concentrations. Platelet activation *in vitro* by co-stimulation of two or more agonists has not been extensively investigated so far. In further experiments blocking antibodies could also be used, to dissect the role of each of the agonists and to simulate poor granule release function for example.

Further, including other hematological diagnoses such as lymphoma would likely shed more light on the question and could be performed based on the results from our and other studies. Considering that there seem to be no significant difference between prophylactic transfusions or no-prophylaxis in grade 3 and 4 bleedings in patients with lymphoma undergoing autologous stem cell transplantation, it would be very interesting to see if this could partly be explained by a better platelet function.

Besides the above-mentioned aspects, it would be of interest to further investigate the reason for platelet function impairment in AML patients and how this impairment resolves during the treatment course. Both bone marrow environment, effect of cytostatic drugs and the effect of thrombocytopenia could possibly contribute. Platelet function should be studied during the

disease course as well as after full remission. In such a study it would be of great interest to include IPF and size (mean platelet volume) in relation to platelet activity and hemostasis.

In a perfect world there would be a registry with a donor for each platelet refractory patient, no matter how rare or complex their HLA type is. However, even in that perfect world there would still be acute situations where transfusions need to be given as soon as possible.

Therefore, continued work on improving the protocol for acid treatment of platelets should be pursued before continuing with a controlled clinical study in humans. Increased knowledge of the effect of acid treatment could also lead to greater understanding of platelet biology and the mechanism behind refractoriness. Clearance mechanisms for the acid treated platelets need to be further addressed in new animal studies using the mouse model constructed for paper IV. For example, it would be of interest to study where the platelets end up immediately after transfusion and where they can be found a few hours later.

In giving the right patient a platelet transfusion at the right time, the next challenge is predicting the response or choosing the right platelet product. Much greater knowledge is needed of the immunological mechanisms behind platelet clearance and function of the allogeneic platelets after transfusion. We could show that the ability of the platelets in the concentrate to respond to stimulation with TRAP correlated to the activation of the platelets in the patient one hour after transfusion. Further knowledge of different characteristics in the platelet concentrate and the in vivo effect will certainly be beneficial for the patients.

Finally, attempts have been made to make platelets from different kind of progenitor cells. So far this has not been successful to the extent that it could replace platelets from blood donors. If one gets to speculate about the future this would perhaps be the most exciting breakthrough and an endless possibility to tailor platelets to every patient is need of them.

8 POPULÄRVETENSKAPLIG SAMMANFATTNING

I människan är benmärgen kroppens blodbildande organ d.v.s. kroppens blodfabrik som tillverkar bland annat de röda och vita blodkropparna samt blodplättarna. Blodplättar (trombocyter) har som funktion att levera blodet och på så vis stoppa upp blödningar. Hematologi betyder läran om blodet och inom den medicinska specialiteten hematologi arbetar man med att utreda och behandla blodsjukdomar. Exempel på allvarliga blodsjukdomar är akuta former av blodcancer (leukemi) samt Myelodysplastiskt syndrom (MDS). Patienter med dessa sjukdomar kan ha för lågt antal blodplättar i blodet såväl av sjukdomen i sig som ger dålig benmärgsfunktion men också av den kraftfulla cytostatikabehandlingen som ges för att behandla sjukdomen. Att ha för låga nivåer av blodplättar innebär en ökad risk för blödningar, ibland livshotande, och blodtransfusioner med blodplättar kan vara livsavgörande för dessa patienter. Blodtransfusioner med blodplättar är dock inte helt utan risk då allergiska transfusionsreaktioner förekommer. Tidigare var förorening med bakterier i blodplättspåsen under lagringstiden från blodgivning till blodtransfusion ett återkommande problem, men med nya metoder för bakterieavdödning har denna risk minskat.

Effekten av en transfusion med blodplättar mäts ofta utifrån om antalet blodplättar i blodet har ökat efter transfusionen. Denna ökning tolkas som ett mått på hur många av de transfunderade blodplättarna som har överlevt och återfinns i blodbanan vid tidpunkten för blodprovstagningen. När transfusioner med blodplättar ges till patienter med blodsjukdomar är det svårt att förutsäga om transfusionen kommer att ha en bra effekt. Ordet refraktär betyder oemottaglig eller okänslig. Refraktäritet kallas tillstånd där en patient vid upprepade tillfällen får en väldigt liten eller ingen ökning av antalet blodplättar efter en transfusion. Tillstånd som blödning, feber och infektion bidrar till en sämre överlevnad av de transfunderade blodplättarna och kan bidra till refraktäritet.

Ett annat tillstånd som kan orsaka refraktäritet är om patienten har bildat antikroppar mot HLA klass I (human leukocyte antigen). HLA är strukturer (molekyler) som finns på ytan på olika celler, inklusive blodplättar, och som är viktiga för immunförsvaret. Vi har alla vår egen individuella uppsättning av HLA, där man ärver hälften från sin mamma och den andra hälften från sin pappa, och det finns mer än 9000 olika varianter. Vid graviditet, blodtransfusioner och benmärgs- och organtransplantationer kommer vårt immunförsvaret i kontakt med främmande celler med främmande HLA-molekyler på ytan och då bildas ibland antikroppar mot dessa främmande HLA. Om en patient som har antikroppar mot en viss HLA-typ får en transfusion med blodplättar från en givare med just den HLA-typ som patienten har antikroppar mot finns en risk att de transfunderade blodplättarna attackeras av patientens immunförsvaret och förstörs. I dessa fall får patienten en dålig effekt av sin transfusion och i vissa fall även en transfusionsreaktion, som kan yttra sig som feber och frossa eller i allvarliga fall blodtrycksfall och allergisk chock. Patienter med refraktäritet och antikroppar mot HLA kan få transfusioner med blodplättar som är matchade för att passa med patientens HLA-typ. Ett annat alternativ är att avlägsna HLA-molekyler från ytan på de

blodplättar som skall transfunderas genom att pH-värdet runt blodplättarna sänks under kort tid med en så kallad syrabehandling. HLA-molekylerna förlorar då sin struktur och vår hypotes är att detta leder till att blodplättarna undkommer HLA-antikropparna och överlever bättre efter transfusion. Syrabehandlade blodplättar användes till transfusioner till enstaka patienter på 90-talet men därefter infördes filtrering av blodprodukter vilket minskade antalet patienter som utvecklade antikroppar mot HLA.

Vid benmärgssjukdom är det ej helt känt hur patienternas egna trombocyter eller hur transfunderade blodplättar fungerar i patienten efter en transfusion. För att mäta blodplättarnas funktion (trombocytfunktionen) mäter man deras förmåga att svara på olika signaler i kroppen och bidra till koagelbildning för att stoppa blödning. Trombocytfunktionen kan undersökas med olika metoder. Med flödescytometri kan man undersöka celler i en vätska med hjälp av laserljus, man kan då bland annat analysera hur blodplättarna ser ut på ytan med och utan olika stimulerande ämnen. ROTEM (rotational thromboelastometry) är ett instrument som ger en övergripande bild av hur blodet koagulerar i ett blodprov.

I denna avhandling studeras trombocytfunktion i patienter före och efter transfusion med blodplättar samt hur väl refraktära patienter svarar på HLA-matchade transfusioner. Vidare undersöktes funktionen av syrabehandlade blodplättar med olika laboriemetoder och i en musmodell. **I det första delarbetet** undersöktes blodplättar i blodprov från 40 hematologiska patienter före transfusion, en timme efter transfusion och 18 till 24 timmar efter transfusion. Vi undersökte antalet blodplättar, koagelbildning och hur blodplättarna svarade på stimulerande ämnen. Ökningen av antalet blodplättar var kopplad till förbättrad koagelbildning, men hur patienter svarade på transfusionen varierade avsevärt och 34% fick ett dåligt svar på transfusionen. I patienter som svarade bra på transfusionen var en effektiv koagelbildning korrelerad till bra svar på stimulerande ämnen men transfusionen förbättrade inte trombocytfunktionen till samma nivå som hos friska blodgivare.

I det andra delarbetet undersöktes hur väl refraktära patienter svarade på HLA-matchade transfusioner med blodplättar. Vi fann att blodplättar som var helt matchade, där givare och patient har exakt samma HLA klass I typ, i stor utsträckning förutsådde ett bra transfusionssvar. En acceptabel ofullständig matchning, där patienten ej hade antikroppar mot det eller de HLA-molekyler som skiljde mellan givare och patient, kunde i ungefär samma utsträckning förutsäga ett bra transfusionssvar. Många transfusioner var också lyckade trots att patienten hade antikroppar mot givarens HLA.

I det tredje delarbetet undersöktes funktionen av syrabehandlade blodplättar. Vi fann att syrabehandlingen inte påtagligt försämrar trombocyternas funktion och att syrabehandlade blodplättar kan undkomma immunförsvarets attack och därmed undgå förstörelse. Syrabehandling tog bort 70 till 90% av HLA klass I molekyler på blodplättarnas yta.

I det fjärde delarbetet undersöktes funktionen av syrabehandlade blodplättar ytterligare och ett protokoll utvecklades för att syrabehandla blodplättar i en hel transfusionspåse för att möjliggöra en senare studie i refraktära patienter. Vi undersökte också hur de syrabehandlade

blodplättarna överlevde i möss. Vi fann att blodplättarnas återhämtning, minskning av HLA-molekyler och viabilitet (livskraftighet) var jämförbart i de syrabehandlade transfusionspåsarna och i mindre skala med en liten mängd syrabehandlade blodplättar. De syrabehandlade blodplättarna bidrog på ett normalt sätt till koagelbildning i ROTEM instrumentet. I musmodellen visade de syrabehandlade blodplättarna en kortare överlevnad jämfört med obehandlade blodplättar. De var skyddade från att förstöras i närvaro av antikroppar som bara binder till specifika undergrupper av HLA men inte vid närvaro av HLA-antikroppar som binder till de flesta HLA-molekyler.

Sammanfattningsvis presenterar avhandlingen resultat och slutsatser som har lett till ändrade rutiner på Blodcentralen på Karolinska Universitetssjukhuset avseende hur blodplättar väljs ut för transfusion till refraktära patienter. Resultatet i det första delarbetet kommer att användas för fördjupade analyser av blodplättars funktion i relation till transfusion och även i relation till olika hematologiska diagnoser, behandlingar och sjukdomsförlopp. Resultaten i delarbete tre och fyra har tagit syrabehandlade blodplättar några steg närmare en framtida forskningsstudie på människor med transfusion av syrabehandlade blodplättar till refraktära patienter.

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