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CHRONIC BONE MARROW FAILURE AND TRANSFUSION PATTERNS: EPIDEMIOLOGICAL STUDIES OF BLOOD TRANSFUSIONS AND OUTCOMES IN PATIENTS WITH MYELODYSPLASTIC SYNDROMES

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Chronic bone marrow failure and transfusion patterns: Epidemiological studies of blood transfusions and outcomes in patients with myelodysplastic syndromes
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To my beloved family

POPULÄRVETENSKAPLIG SAMMANFATTNING

Myelodysplastiskt syndrom (MDS) är ett samlingsnamn för en grupp elakartade sjukdomar i benmärgen. En rad förändringar på gennivå leder till att blodcellerna inte utvecklas som de ska vilket leder till blodbrist, blödningsbenägenhet och ökad känslighet för infektioner. Sjukdomsförloppet varierar från stabil blodbrist över årtionden till snabb progress mot akut blodcancer (leukemi). MDS drabbar främst äldre åldersgrupper och medianåldern vid insjuknandet är strax under 75 år. En benmärgstransplantation ger en möjlighet till bot och både patienter med högrisk- och lågrisk MDS blir tilltänka för denna behandling. Det är dock en mycket intensiv behandling med risk för både ökad sjuklighet och död varför en del patienter med hög ålder eller andra samtidiga sjukdomar inte passar för denna behandling, där försöker man istället genom andra mediciner att öka blodvärden och livskvalitet. Under given behandling eller om sjukdomen inte svarar på given behandling, så är det många patienter som har ett tillfälligt eller kroniskt behov av blodtransfusioner av röda blodkroppar och blodplättar, för att lindra symtom på blodbrist och förhindra blödningar.

Samtidigt som blodtransfusioner är livräddande, lindrar symtom av blodbrist och ökar livskvaliteten så har man också observerat att patienter med MDS som har ett upprepat behov av blodtransfusioner både har sämre prognos och livskvalitet jämfört med de patienter som inte behöver blodtransfusioner. Även om inte hela orsaken är fastställd har publikationer visat att den ökade sjukligheten mest troligt är en konsekvens av en mer allvarlig sjukdom i kombination med den påfrestning för kroppen som blodbrist innebär.

Trots pågående forskning inom detta område, saknas det ännu vetenskapliga studier som belyser effekten av transfusioner över tid, hos patienter med MDS. Det övergripande syftet med denna avhandling var att fördjupa kunskapen kring transfusionsbehandling till patienter med MDS för att erhålla säkrare och mer effektiv transfusionsbehandling. Mer specifikt ämnade vi att bättre förstå hur mycket blodtransfusioner som patienter med MDS och andra blodcancerformer behöver (studie I) och undersöka vilka faktorer hos patienten och sjukdomen som driver transfusionsbehovet (studie II). Vidare ville vi undersöka hur lagring av röda blodkroppar påverkar effekten av blodtransfusionen (studie III) samt hur många patienter som bildar antikroppar mot transfunderade blodkroppar och vad det har för betydelse (studie IV). Samtliga studier är gjorda på historiska data. Utförandet av samtliga studier är godkända av Etikprövningsmyndigheten, Stockholm, Sverige.

I studie I, fokuserade vi på patienter med elakartad blodsjukdom från hela Sverige och alla åldrar. Vi beskrev hur många blodtransfusioner med röda blodkroppar, blodplättar och blodplasma som patienterna behöver de första två åren efter diagnos och vad det kostar. Vi fann stora skillnader mellan olika diagnosgrupper och åldersgrupper. MDS och akut blodcancer var bland de sjukdomar som fick flest antal transfusioner och därmed också stod för de högsta kostnaderna.

I studie II, ämnade vi att förstå vilka patient- och sjukdomsspecifika faktorer som driver transfusionsbehovet av röda blodkroppar och blodplättar hos patienter med MDS. Ett andra

syfte var att förstå hur frekvensen av blodtransfusioner påverkar överlevnad. Vi kunde identifiera flera faktorer som var förknippade med ökad transfusionsfrekvens, däribland manligt kön och vissa grupper av mutationer. Vidare, så fann vi att tätare intervall mellan transfusioner påverkar överlevnaden negativt.

I studie III, undersökte vi hur lagring av röda blodkroppar påverkar effekten av blodprodukten hos patienten med MDS. Detta gjorde vi genom att studera ökningen av blodvärdet efter blodtransfusionen, hos blodprodukter som hade lagrats olika länge innan transfusion. Vi fann att längre lagringstid ger minskad ökning av blodvärdet. Dessa resultat höll sig stabila även när vi tog hänsyn till andra variabler som kan påverka resultaten.

I studie IV, studerade vi riskfaktorer för att utveckla antikroppar mot de transfunderade röda blodcellerna (alloantikroppar). Ett andra syfte var att undersöka om alloantikroppar medförde ett ökat behov av blodtransfusioner och om det påverkade stegringen av blodvärdet. Oberoende riskfaktorer för utveckling av dessa alloantikroppar var kvinnligt kön och en annan typ av antikropp mot den röda blodkroppen. Efter bildningen av alloantikroppar fann vi ett ökat behov av blodtransfusioner och en minskad stegring av blodvärdet.

Sammanfattningsvis så är behovet av blodtransfusioner stort hos patienter med MDS. Det är viktigt att karaktärisera transfusionsmönster, kostnader och hitta vilka variabler som är kopplade till ett ökat transfusionsbehov då kunskap om dessa faktorer kan hjälpa till i valet av behandling för MDS-sjukdomen. Resultaten från denna avhandling bidrar till kunskap om faktorer där vi har möjlighet att påverka för att optimera effekten av blodtransfusioner, till patienter med kronisk anemi på grund av MDS. Detta kan ha betydelse både på individnivå genom en mer effektiv behandling samt ur ett större perspektiv med minskade behandlingskostnader.

ABSTRACT

Myelodysplastic syndromes (MDS) encompass a diverse group of clonal hematological malignancies characterized by dysplasia and ineffective hematopoiesis with an increased risk of leukemic evolution. It is a disease of the elderly with a median age of nearly 75 years. Anemia is the most common cytopenia and a majority of the patients have a temporary or chronic need for red blood cell (RBC) transfusions, either during treatment or at loss of response to treatment. Recognizing the importance of RBC transfusions, the transfusion burden is likewise associated with a reduced overall and progression-free survival and with other unwanted effects, such as alloimmunization and impaired quality of life. This thesis aimed to expand the knowledge on transfusion patterns primarily in patients with MDS, but also to investigate transfusion patterns in hematological malignancies overall. Specific goals were to characterize transfusion patterns, identify clinical and patient-specific parameters associated with transfusion intensity and to investigate variables that might affect the efficacy of the RBC transfusion, such as RBC storage time and alloimmunization.

In study I, we presented a nation-wide overview of transfusion patterns in patients diagnosed with a hematological malignancy of myeloid, lymphoid or plasma cell origin, during the first two years following diagnosis. Great variations in the transfusion patterns were observed between hematological diagnoses with regard to transfusion incidence, median number of transfused units and direct costs. Patients with acute leukemia and MDS received the highest cumulative number of transfusions and thereby accounted for the highest costs. Conversely, patients with chronic lymphoid leukemia, Hodgkin's lymphoma or follicular lymphoma received the lowest cumulative number of transfusions. The transfusion incidence was highest immediately after diagnosis in patients with acute leukemia and in patients undergoing allogeneic stem cell transplantation.

In study II, we aimed to identify clinical and patient-specific parameters associated with transfusion intensity of RBC and platelet transfusions, in patients with MDS. Independent predictors of RBC and platelet transfusion intensity were male sex and mutations in genes encoding histone modulation, signaling and transcriptional regulation. We observed that transfusion intensity was significantly associated with poor survival.

In study III, we investigated if duration of RBC storage affected the hemoglobin increment following RBC transfusions in a cohort of MDS patients. A longer duration of RBC storage was associated with a smaller increment of the hemoglobin level after transfusion, per RBC unit, compared to units stored less than five days. The estimates proved stable when adjusting for age and sex and in five different sensitivity analyses.

In study IV, we analyzed risk factors of alloimmunization and potential clinical changes following alloimmunization, such as transfusion requirements and the post-transfusion hemoglobin increment, in an MDS cohort. Female sex and a positive direct antiglobulin test were significantly associated with alloimmunization. Following alloimmunization, we

observed an increase of the average transfusion intensity and estimated lower post-transfusion hemoglobin increments per RBC unit.

In conclusion, characterization of transfusion patterns and identification of variables associated with transfusion intensity are of great importance and could guide therapeutic options and optimize transfusion therapy to patients with a chronic bone marrow failure due to MDS.

LIST OF SCIENTIFIC PAPERS

I. Blood use in hematologic malignancies: a nationwide overview in Sweden between 2000 and 2010
    Zhao J., **Rydén J**., Wikman A., Norda R., Stanworth J. S., Hjalgrim H., Edgren G. (2018) *Transfusion*, doi: 10.1111/trf.14440

II. Male sex and the pattern of recurrent myeloid mutations are strong independent predictors of blood transfusion intensity in patients with myelodysplastic syndromes
    Rydén J., Edgren G., Karimi M., Walldin G., Tobiasson M., Wikman A., Hellström-Lindberg E., Höglund P. (2019) *Leukemia*, doi:10.1038/s41375-018-0256-0

III. A longer duration of red blood cell storage is associated with a lower hemoglobin increase after blood transfusion: a cohort study
    Rydén J., Clements M., Hellström-Lindberg E., Höglund P., Edgren G. (2019) *Transfusion*, doi:10.1111/trf.15215

IV. Red blood cell immunization in patients with myelodysplastic syndromes: a retrospective analysis between 2003 and 2017 of associated risk factors and transfusion patterns
    Rydén J., Wikman A., Clements M., Hellström-Lindberg E., Edgren G., Höglund P.
    Manuscript

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LIST OF ABBREVIATIONS

AML	Acute myeloid leukemia
ALL	Acute lymphocytic leukemia
ANC	Absolute neutrophil count
CHIP	Clonal hematopoiesis of indeterminate potential
CLL	Chronic lymphoid leukemia
CLP	Common lymphoid progenitor
CML	Chronic myeloid leukemia
CMP	Common myeloid progenitor
DLBCL	Diffuse large B-cell lymphoma
ESA	Erythropoietin stimulating agent
ETP	Earliest thymic progenitor
FFP	Fresh frozen plasma
FL	Follicular lymphoma
GCS-F	Granulocyte colony stimulating factor
GMP	Granulocyte-monocyte progenitor
HMA	Hypomethylating agent
HSC	Hematopoietic stem cell
HTB	High transfusion burden
IPSS	International prognostic scoring system
IPSS-R	International prognostic scoring system-Revised
IST	Immunosuppressive therapy
LMPP	Lympho-primed multipotent progenitors
LTB	Low transfusion burden
MDS	Myelodysplastic syndromes
MEP	Megakaryocyte-erythroid progenitor
MHC	Major histocompatibility complex
MM	Multiple myeloma
MPN	Myeloproliferative neoplasm
MPP	Multipotent progenitor
NK	Natural killer

NTD	Non-transfused
PFS	Progression free survival
PLT	Platelet
RBC	Red blood cell
RCT	Randomized clinical trial
Rh	Rhesus
SCT	Stem cell transplantation
TD	Transfusion dependency
TID	Transfusion independency
VAF	Variant allele frequency
WHO	World health organization

1 BACKGROUND

1.1 INTRODUCTION

Patients with hematological malignancies commonly develop a temporary or chronic need of blood transfusions (1) due to cytopenias related to disease infiltration of the bone-marrow, hemolysis (2) or chemotherapy (3). Transfusions of blood products are common also within other disease entities, with an estimated amount of 85 million red blood cell (RBC) units being transfused yearly, worldwide (4). Even though blood transfusions are generally considered safe, they are not completely without risks. Current transfusion-related concerns primarily include non-infectious complications such as hemolytic or allergic reactions, transfusion-associated overload, transfusion-related lung-injury and immunomodulation (5). For patients with a chronic transfusion need, concerns of iron overload and alloimmunization are more evident (6-8). For myelodysplastic syndromes (MDS), the transfusion need has been associated with an impairment of the overall and progression-free survival and quality of life (9). With regard to possible risks and the potentially limited resource based on voluntary donors, it is of great importance that transfusions are given with optimal effect. This thesis focus on RBC transfusions to patients with MDS, but will also present transfusion patterns of RBCs, platelets and plasma in other hematological malignancies (paper I) and assess which clinical and patient-specific parameters that are associated with transfusion intensity and its association with survival in patients with MDS (paper II). Further studies investigate RBC transfusion-related aspects that might influence the efficacy of the blood unit and transfusion burden, and include analysis of RBC storage time (paper III) and RBC alloimmunization (paper IV), in patients with MDS.

1.2 HEMATOPOIESIS

Hematopoiesis is a process by which all hematopoietic cells are produced and is mainly taking place in the adult bone marrow. Human fetal hematopoiesis originates in the yolk sac and successively continues in the fetal liver and bone marrow (10). Hematopoiesis is classically described as a hierarchical structure originating from the self-renewing and pluripotent hematopoietic stem cells (HSCs) with the capacity of differentiating to progenitor cells for all blood cell lineages (11-13). HSCs are classified into long-term HSCs (LT-HSCs) which differentiate into short-term HSCs (ST-HSCs) (14). By the time the ST-HSCs has differentiated into to the multipotent progenitor (MPP), the self-renewal capacity has ceased (14). The first branch point separates hematopoietic cells into two major lineages of hematopoietic cells, that is, to the common myeloid progenitor (CMP) and common lymphoid progenitor (CLP) which successively lose their multilineage potential and differentiate into unilineage committed precursors. The former being responsible for the development of erythrocytes, megakaryocytes, granulocytes and macrophages/monocytes and the second giving rise to T-lymphocytes, B-lymphocytes, plasma cells and natural killer (NK) cells (Figure I) (11, 15).
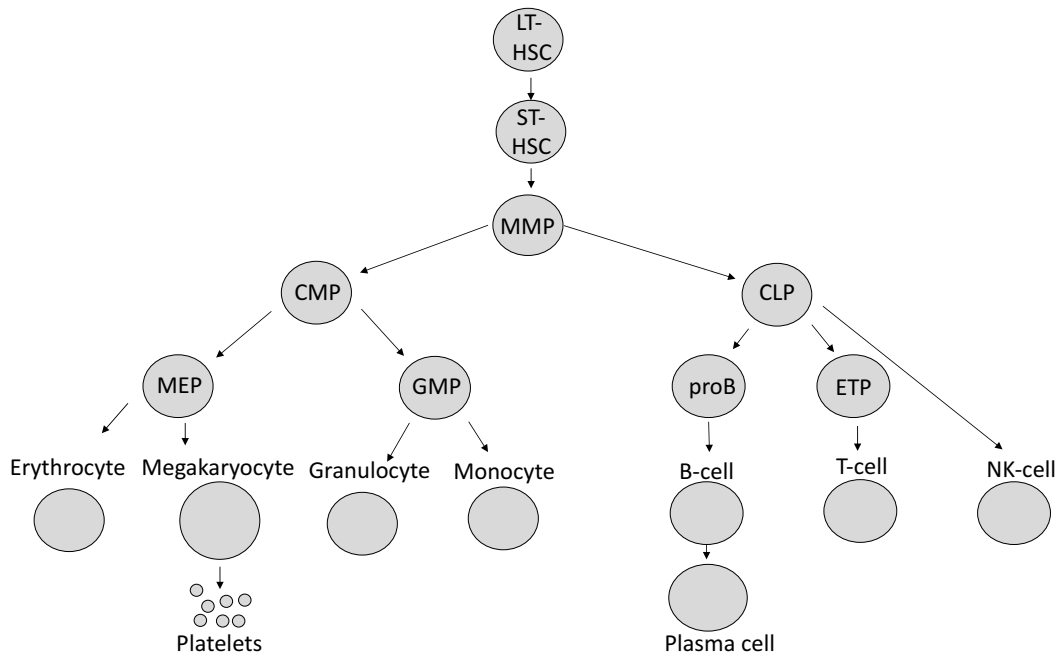
Figure I. A schematic model of the classical hierarchy of adult human hematopoiesis. Long-term hematopoietic stem cell (LT-HSC), short-term hematopoietic stem cell (ST-HSC), multipotent progenitor (MPP), common myeloid progenitor (CMP, common lymphoid progenitor (CLP), megakaryocyte-erythroid progenitor (MEP), granulocyte-monocyte progenitor (GMP), B-cell progenitor (proB), earliest thymic progenitor (ETP), natural killer cells (NK-cells). The figure was adapted from Meyer 2017 (16).

The hematopoiesis is the best characterized stem cell system, yet it is still incompletely understood and research continuously add knowledge to the field. Evidence suggest that the hematopoiesis is a more complicated system than the classical hierarchical tree is indicating. Recent findings on cells derived from mouse and human, imply that lymphoid and myeloid lineages are associated further down the hierarchy through the lympho-primed multipotent progenitors (LMPPs) (17-19) and that differentiation may be a continuous process rather than stepwise (20).

1.3 MYELODYSPLASTIC SYNDROMES

The myelodysplastic syndromes (MDSs) are a group of clonal stem cell disorders, characterized by dysplasia and ineffective hematopoiesis, resulting in unilineage or multilineage cytopenia in peripheral blood (21, 22). MDS is often described as heterogeneous due to diversity on molecular level, clinical appearance and prognosis. It is a disease of the elderly and the number of patients with newly diagnosed MDS has increased over the years and will continue to grow, due to longer life expectancy and a growing elderly population in combination with better diagnostics (23, 24). Among available therapeutic options, allogeneic stem cell transplantation (SCT) is the only possible cure and is considered for applicable patients of both higher-risk MDS and lower-risk MDS patients that present with unfavorable prognostic variables (25). Other therapeutic options aim to improve cytopenias and quality of

life. During treatment or disease progression, many patients with MDS have a temporary or chronic need for supportive care with blood transfusions (26-29).

1.3.1 Epidemiology

The MDSs are among the most commonly diagnosed myeloid malignancies (30) with a median age at diagnosis between 70 and 75 (29, 31). The crude incidence rate of these conditions in the general population is about 3.5-4 cases per 100 000 persons-years but increases with age in similarity with most hematological malignancies with a few exceptions of acute lymphocytic leukemia and Hodgkin's lymphoma with incidence peaking in ages 0-14 years and 15-44 years, respectively (32, 33). Passing the age of 60, the incidence rate increases, to 7.1 per 100,000 person-years for ages 60-69 years and upwards 56.8 per 100,000 person-years for those 80 years or older (34, 35) or estimated as 20 to 50 cases per 100,000 persons per year for ages over 60 years (21, 36). The incidence rate in Sweden is similar, with 4 cases per 100,000 persons-years with around 300 newly diagnosed cases yearly (31). There is a slight male predominance and the incidence rates are higher in males by a factor of 1.8 (35) and with male proportion of approximately 60% (31).

1.3.2 Biological background and disease evolution

1.3.2.1 Clonal hematopoiesis

The hematopoietic system generates more than 3.5×10^{11} cells per day (37) including around 2 million erythrocytes per second in healthy adults (38). It is reported that mutations per HSC are acquired in an average rate of 1.3 +/- 0.2 mutations per decade (39). While most mutations occur in non-coding regions, mutations occasionally affect regions of the genome responsible for cell fate and give advantage for clonal expansion, in both MDS and acute myeloid leukemia (AML). The recent advances in medical technology with next-generation sequencing and high-resolution single nucleotide polymorphism-array enables detection of mutations that are recurrently mutated in myeloid malignancies (40, 41).

Acquired somatic mutations in genes, that we know are associated with MDS, in peripheral blood cells can be found in healthy adults without signs of a myeloid malignancy and are referred to as clonal hematopoiesis of indeterminate potential (CHIP). Involved mutations must have a variant allele frequency (VAF) of at least 2% (42). Commonly involved mutations are transcriptional regulator mutations, for example ASXL1, TET2 and DNMT3A. Although mutations are considered part of normal aging, CHIP is associated with a 0.5-1% annual risk of further development into a myeloid malignancy (43). The incidence of CHIP increases with age and is detected in approximately 10% of patients 70 years or older (44-46) although a study using whole genome sequencing observed that 50% of patients over 85 years harbored clonal hematopoiesis (47).

1.3.2.2 Secondary and therapy-related MDS

The majority of the MDS cases are classified as primary or *de novo* MDS, but approximately 15-20% are classified as therapy-related MDS (tMDS) or secondary MDS (sMDS) (48-50).

By definition, tMDS has developed due to exposure to chemotherapy or irradiation for a prior disease. Secondary MDS is a broader definition and include all cases where there is a known risk factor and by definition also including cases with tMDS (50). It is of great importance to separate *de novo* MDS from tMDS due to the inferior overall survival in tMDS. The median overall survival is reported to be approximately half as long in tMDS compared to *de novo* MDS (48, 50). Although rare, there are also inherited forms of myeloid malignancies classified in the category of 'myeloid neoplasms with germline predisposition' (22). Since the discovery of the heterozygous germline RUNX1 mutation in the late 1990's, and development of the next generation sequencing technique, around 20 different loci have been associated with familial myeloid diseases of both MDS and AML. The most frequently discussed mutations are GATA2, CEBPA, DDX41, ETV6, TERC, TERT, ANKRD26 and TP53 (51, 52).

1.3.2.3 *Cytogenetics and chromosomal aberrations*

The pathophysiology of MDS is a multistep process involving a variety of genetic alterations, where cytogenetic changes may be present with or without gene mutations (53). Chromosomal abnormalities are found in approximately 50% of patients with *de novo* MDS but the corresponding proportion in sMDS or tMDS is substantially higher (21, 54). Chromosomal aberrations most often involve gain or loss of chromosomal materials, referred to as 'chromosomal and copy-number abnormalities'. The most common chromosomal aberrations are complex karyotypes, del(7q), del(5q), +8, del(20q), inv(3)/t(3q)/del(3q), del(12p), del(11q), del(17p) and +19, although the order of frequency can differ slightly depending on MDS cohort (55, 56) (Figure II). In addition, -Y and -7 are included in one of the most commonly adapted risk score, the revised International Prognostic Scoring System (IPSS-R). Targeted gene sequencing detects mutations in 80-90% of MDS cases (55, 57). Somatic point mutations recurrently mutated in MDS include genes involved in epigenetic regulation (chromatin/histone modification; ASXL1, EZH2, MLL2 and DNA methylation; TET2, DNMT3A, IDH1, IDH2), RNA splicing (SF3B1, U2AF1, U2AF2, SRSF2, ZRSR2, SF3A1), but also genes involved in transcriptional regulation (RUNX1, ETV6, BCOR, GATA, CEBPA) and signaling (CBL, JAK2, NRAS, KRAS, FLT3) (40, 55, 56) (Figure II). The number of driver mutations in each MDS individual vary but the typical patient has a median of 2-3 mutations (56, 58). In these cases, it is of value to quantify the VAF to gain knowledge about clonal evolution and about the hierarchy of the mutations. The mutation with highest VAF is thought to be the disease driving mutation and the mutation/s with smaller VAF are considered to be acquired during disease progression (56).
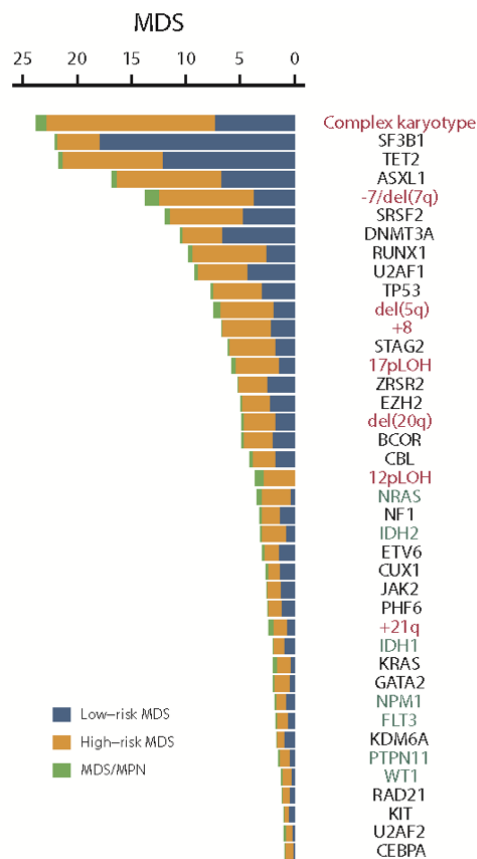
Figure II. Frequency and specificity of chromosomal aberrations and myeloid driver mutations. *Reprinted with permission from American Society of Hematology (ASH): Blood, Genetics of MDS, S. Ogawa, copyright 2019.*

1.3.3 Clinical presentation

The clinical presentation and the natural course of the disease vary between subgroups of MDS and individuals. However, the typical patient presents with persistent peripheral cytopenia of unclear etiology. Major clinical challenges in MDS are morbidities caused by cytopenias and the risk of leukemic evolution. The three possible cytopenias in MDS are anemia, thrombocytopenia and leukopenia/neutropenia and are categorized into unilineage or multilineage. Table I presents normal ranges of peripheral blood counts. Pancytopenia refers to the simultaneous, and often severe, manifestation of anemia, leukopenia and thrombocytopenia (59).

Table I. Mean normal ranges of peripheral blood counts

	Normal range (95%, CI)
Hemoglobin level (g/L)	120-155/130-175*
Platelet count (x10^9/L)	150-350
Leukocyte count (x10^9/L)	4-11
Absolute Neutrophil count (x10^9/L)	1.5-8

*males

1.3.3.1 Anemia

The red blood cell (RBC) also known as the erythrocyte, contains the hemoglobin molecule with its four folded globin chains. One RBC contains 300 millions of hemoglobin molecules which means that one erythrocyte binds and transports 1.2 billion oxygen molecules. Anemia is the most common cytopenia in MDS and up to 90% of the MDS patients are anemic already at the time of diagnosis (6). Anemia in MDS is significantly associated with a higher risk of hospitalization, hazard ratio (HR) 1.80 (95% confidence interval, CI 1.61-2.01) and receiving RBC transfusions, HR 2.28 (95% CI, 2.01-2.59) (60). Moderate to severe anemia of less than 90 g/L in males and less than 80 g/L in females has shown to be associated with reduced overall survival, HR 5.56, $p=0.08$ and HR 5.35, $p=0.026$, for males and females respectively (61). Symptoms of anemia relate to the inability to carry sufficient amount of oxygen throughout the body. Patients with anemia might experience fatigue, vertigo, headache, tachycardia, dyspnea and chest pain among other symptoms, and varies with the severity of the anemia and the rate by which the anemia has developed.

1.3.3.2 Thrombocytopenia

The main function of platelets (PLTs), also referred to as thrombocytes, are adhesion and aggregation to prevent and stop bleeding. A PLT level below 100 $x10^9$/L is defined as thrombocytopenia in MDS and occurs in approximately half of the patients over the course of their disease (40-65%) (62, 63). Mild hemorrhagic symptoms may occur at levels below 50 $x10^9$/L primarily by easy bruising, epistaxis or sore oral mucous membrane. Platelet counts less than 10-20 $x10^9$/L are associated with a risk of severe bleeding, that is internal, cerebral or massive bleedings. Early papers also found evidence of platelet dysfunction as an alternative cause of bleeding in MDS (64-66).

1.3.3.3 Neutropenia

Neutrophils are granulocytes and are important in both innate and adaptive immunity. As indicated by the name, granulocytes contain granules with cytokines and chemokines that are released during phagocytosis of microorganisms. This is followed by release of substances that attract monocytes who differentiate into macrophages in the tissue. Neutropenia occurs in about 50% of the MDS patients, with higher incidence in advanced stages (67). Mild neutropenia with an absolute neutrophil count (ANC) of 1-1.5 $x10^9$/L is normally not associated with an impairment of host defense. Moderate neutropenia (ANC 0.5-$<1x10^9$/L) is associated with an increased risk of infections if the immune system is affected by other mechanisms as well. ANC below 0.5 $x10^9$/L is associated with an increased risk of bacterial infections, and especially agranulocytosis with values below 0.2 $x10^9$/L entails a risk of severe bacterial and opportunistic infections.

1.3.4 Classification

Because of the heterogeneity of the MDS population, the disorders are classified into subtypes with clinically relevant disease features to facilitate choice of treatment and

prognostic information. The first morphological classification was the French-American-British (FAB) classification, introduced 1982 and included morphological features and the bone marrow blast percentage (68). In year 2001, the World Health Organization (WHO) introduced a new classification system of myeloid neoplasms to improve its prognostic value, incorporating the grade of myelodysplasia, cytogenetics and blast percentage. The 2001 classification also redefined the border between MDS and AML, from 30% blasts to 20% (69). Due to the rapidly emerging genetic information, the 2008 revision of the WHO classification integrated gene mutations that were recognized as important diagnostic and prognostic markers (70). Subtypes of the 2008 WHO classification of MDS in adults are presented in Table II, and included a change of the nomenclature to myeloproliferative neoplasm (MPN) from the previous myeloproliferative disorders (MPD). Following the 2008 revision, newer techniques with gene expression analysis and next generation sequencing have provided genetic information that has strongly improved the diagnostic criteria and the prognostic relevance. The 2016 revision focused on incorporating these new data and refined the 2008 classification rather than creating new classifications (22) (Table II).

Table II. Table over the 2008 and 2016 revision of the WHO classification of myelodysplastic syndromes in adults

2008 WHO classification	2016 WHO classification
Refractory cytopenia with unilineage dysplasia (RCUD) Refractory anemia (RA) Refractory neutropenia (RN) Refractory thrombocytopenia (RT)	MDS with singel lineage dysplasia (MDS-SLD)
Refractory cytopenia with multilineage dysplasia (RCMD)	MDS with multilineage dysplasia (MDS-MLD)
	MDS with ring sideroblasts
Refractory anemia with ring sideroblast (RARS)	MDS with ring sideroblasts, single lineage dysplasia (MDS-RS-SLD)
Refractory cytopenia with multilineage dysplasia with ring sideroblasts (RCMD-RS)	MDS with ring sideroblasts, multi lineage dysplasia (MDS-RS-MLD)
Myelodysplastic syndrome with isolated del(5q)	MDS with isolated del 5q
	MDS with excess of blasts
Refractory anemia with excess of blasts (RAEB-1)	MDS with excess of blasts-1 (MDS-EB-1)
Refractory anemia with excess of blasts (RAEB-2)	MDS with excess of blasts-2 (MDS-EB-2)
Myelodysplastic syndrome unclassified (MDS-UNS)	Myelodysplastic syndrome unclassified (MDS-UNS)
Myelodysplastic syndrome/myeloproliferative neoplasm (MDS-MPN) Chronic myeloid leukemia (CMML) Atypical chronic myeloid leukemia, BCR-ABL-negative (aCML) Myelodysplastic/myeloproliferative neoplasm, unclassifiable (MDS/MPN)	Myelodysplastic syndrome/myeloproliferative neoplasm (MDS-MPN) Chronic myeloid leukemia (CMML) Atypical chronic myeloid leukemia, BCR-ABL-negative (aCML) Myelodysplastic/myeloproliferative neoplasm, unclassifiable (MDS/MPN)
Refractory anemia with ring sideroblast with ring siderobla(RARS-T)	MDS-MPN with ring sideroblasts and thrombocytosis

1.3.5 Prognosis

Risk stratification is crucial in order to estimate survival, assess the risk of progression to AML, and evaluate treatment. Two of the most accepted and widespread current prognostic scores, are the IPSS-R (71) and the WHO-classification Prognostic Scoring System (WPSS) (9, 72). IPSS-R was published 2012 and is a development of the IPSS which was introduced 1997 (73). IPSS-R is refined with variables acknowledging levels of cytopenias (rather than number of cytopenias, 0-1, 2-3), cytogenetic categories with 5 levels (instead of 3) and bone marrow blasts percentage, in addition, weighing negative cytogenetics higher than percentage of bone marrow blasts. The previous IPSS identified 4 different risk groups, but IPSS-R can discriminate 5 different risk groups with significantly different prognosis: very low, low, intermediate, high and very high. Limitations of IPSS-R involve that calculation is not recommended in tMDS or CMML with LPK exceeding 12×10^9/L and is only validated for

risk stratification at diagnosis and not during follow-up (73, 74). The WPSS risk score was validated year 2007 and identifies 5 diverse prognostic groups. The risk score integrates transfusion requirements instead of presence of cytopenias and has the advantage of being validated during follow-up and not only at diagnosis. Median survival according to the IPSS-R ranges from 8.8 years in patients with 'very low' risk, to 0.8 years in 'very high' risk. Figure III, shows estimated survival probability stratified by IPSS-R, using patient data from study IV. Important prognostic information is also provided by the WHO classification with estimates of the AML transformation risk, ranging from 0% in patients with RA-RS according to the 2008 WHO classification up to 32.2% in RAEB-2.
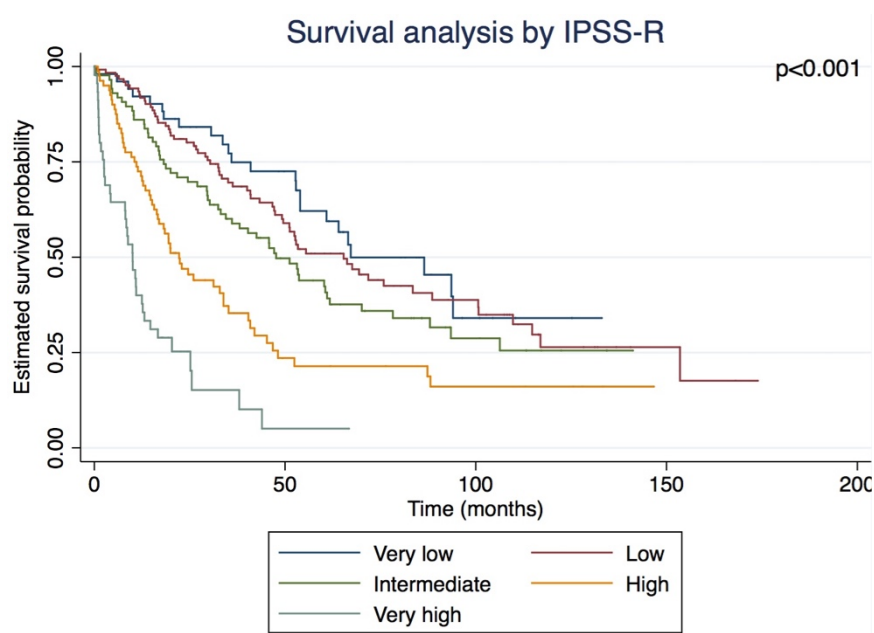

Figure III. Estimated survival probability stratified by the revised International Prognostic Scoring System (IPSS-R). Survival curves were visualized using Kaplan-Meier survival plots and difference between groups was analyzed using the log-rank test.

Of the cytogenetic abnormalities, only isolated del(5q) defines a specific MDS subtype. This subtype is often associated with a favorable prognosis, although there are exceptions which entail unfavorable prognosis caused by a larger deletion of 5q or coexisting TP53 mutations (75-77). Other specific abnormalities, as those incorporated in the IPSS-R, give important information and are closely related to prognosis (22). Mutational status adds additionally to already established risk scores. Both number of mutations and type of somatic mutation are guiding for prognosis and significantly correlated with overall survival (55, 57). Low number of driver mutations is correlated with lower-risk MDS, as are mutation of SF3B1, which is predictive for a favorable prognosis (57). On the contrary, a high number of driver mutations is correlated with higher-risk MDS, and mutations of TP53, even in combination with mutation of SF3B1 is predictive for a poorer prognosis. Other specific mutations associated with adverse outcome are ASXL1, EZH2, DNMT3A, ETV6 and other splice factor mutations like U2AF1 and ZRSF2 (56, 78). Both NRAS and FLT3 mutations are often observed as late

events and each is associated with AML transformation (79). The coexistence of mutations and how they correlate in between add to the complexity of interpretation (40, 57).

1.3.6 Therapeutic options

The introduction of reduced intensity conditioning has increased the number of patients with MDS that are considered for allogeneic SCT (25, 80, 81), which is the only possible cure. In addition, hypomethylating agents (HMAs) as a bridge to allogeneic SCT has also increased the availability of allogeneic SCT for some patients. Other available therapeutic options aim to improve cytopenias and prolong time until disease progression. Therapeutic regimens for lower-risk MDS with symptomatic cytopenia include immunomodulatory agents (lenalidomide) specially for del(5q)-syndrome, growth factors (erythropoietin stimulating agents; ESAs in combination with/or granulocyte stimulating factors; GCS-F) and immunosuppressive therapy (IST). Starting treatment with ESAs when the hemoglobin level falls below 100 g/L has shown to significantly delay the onset of a permanent transfusion need. This recommendation is to a high degree followed in Sweden (82). Recently, luspatercept has shown promising results in a phase 3 trial, with significantly higher probability of transfusion independence compared to placebo, in patients with lower-risk MDS with ring sideroblasts with a regular RBC transfusion need (83). Higher-risk MDS may benefit of more intensive treatment regimens primarily with HMAs: azacitidine or decitabine but induction chemotherapy may also be an option (53, 74, 84, 85). Comparing the two available HMAs, only treatment with azacitidine has shown survival benefits compared to best supportive care (81, 86). However, lower-risk MDS with unfavorable prognostic features may benefit of low-dose decitabine (87). A recent study validated this finding and observed better overall and progression-free survival (PFS) in lower-risk MDS with unfavorable features treated with low-dose decitabine compared to azacitidine (88). In spite of available treatment options, many patients require transfusions of RBCs and/or PLTs during the course of their disease and may develop a chronic need for supportive care with RBC transfusions.

1.4 BLOOD COMPONENTS

Donated whole blood is collected in citrate-phosphate-dextrose-containing blood bags, separated after centrifuging into the different blood products platelets and leukocytes (buffy coat), RBCs, and plasma.

1.4.1 Red blood cell units

RBC units are prepared from whole blood by removing plasma and most of the leukocytes by centrifugation. The RBC fraction is filtered to remove remaining leukocytes and then suspended in a nutrient additive solution, which is most often a saline-adenine-glucose-mannitol (SAGM) solution. This processing allows for up to 42 days of RBC storage at a temperature of 2-5°C (89). The maximum limit of 42 days is decided based on the degree of hemolysis and remaining RBCs 24 hours after transfusion (90). One unit of RBC contains approximately 200-250 mg of iron (91) and is estimated to increase the hemoglobin level around 10 g/L (92).

1.4.1.1 Storage of red blood cells

The possibility to store RBC units is essential to ensure availability of blood to meet expectations for both elective and acute clinical needs. Both interest and concerns of potential changes of the RBCs during storage have resulted in numerous publications of both in vitro and observational character. The observed changes are referred to as 'storage lesions', and involve biomechanical, morphological and structural changes (93-95). Many observational studies have tried to assess the association between storage time and adverse events. Some have suggested evidence for different types of adverse effects of longer duration of storage (96-98) while more recent randomized clinical trials (RCTs) (99-102), a large meta-analysis (103) and a binational cohort study (104) could not observe any association between storage time and risk for adverse outcomes. In contrast to the many studies done trying to assess the safety issue, not many studies cover the efficacy issue. As indirect measure of efficacy, two of the RCTs found no difference in number of transfused RBC units (100, 101) and one RCT studied the lactate decrease following transfusion and oxygen delivery in RBC with different duration of storage (102).

1.4.2 Platelet concentrates

Platelet concentrates (PCs) are prepared from whole blood into single-unit preparations using either a platelet rich plasma or pooled buffy coats derived from four to six whole blood donations, or they can be collected by apheresis (105). The PC contains about 3×10^{11} PLTs per unit (106). PCs cannot be stored refrigerated but only at room temperature, which shortens storage time to 5-7 days primarily because of risk for contamination of the product. The high temperature of storage also accelerates the storage lesion and it has been shown that storage time has a negative effect on platelet quality and transfusion outcome (107, 108).

1.4.3 Plasma products

Fresh frozen plasma (FFP) is the most common plasma product. It is prepared from whole blood or through apheresis, the two products are considered equally efficient in the recipient (109), and it is frozen usually within 8 hours of donation. FFP contains all coagulation factors such as fibrinogen, albumin, protein S, protein C, antithrombin and tissue factor pathway inhibitor, but is free from PLTs, RBCs and leukocytes. Before use, FFP is thawed at 30-37 degrees Celsius in a water bath (110).

1.5 BLOOD TRANSFUSION THERAPY

1.5.1 Overview

Blood transfusion therapy is a common method to treat symptomatic anemia in medical, surgical and intensive care patients and around 85 million RBC units are transfused worldwide per year (4). The need for RBC transfusions is foremost depending on the individual patient's physical condition and symptoms. However, in clinical practice, the hemoglobin concentration threshold is a complementary guideline for RBC transfusion and the recommendation is generally to adhere to a restrictive transfusion strategy and transfuse

when hemoglobin concentration drops below 70 g/L (4). Postoperative surgical patients or patients with cardiovascular disease or severe sepsis may benefit of a more liberal transfusion strategy and the level is usually set higher (4, 111). In contrast to the anemic patient in the acute setting and for the general hospitalized patient, the optimal hemoglobin threshold for patients with chronic anemia due to MDS is not established (112). Transfusions must always be evaluated for their benefits versus possible risks. Whereas the risk of transfusion-related infections has decreased due to improved screening for transfusion-associated infections, awareness of non-infectious transfusion-related complications have increased. Transfusion-related complications are categorized into acute or delayed and the most common include febrile reactions, circulatory overload, hemolytic transfusion reactions, transfusion-related acute lung-injury, transfusion-related immunomodulation, transfusion-associated graft-versus-host-disease and allergic reactions (5).

PLT transfusions are categorized into therapeutic or prophylactic (113) and are used in thrombocytopenic patients or in patients with a PLT dysfunction. These indications are mainly observed in hematological patients and it is reported that up to 67% of all PCs are transfused to patients diagnosed with a hematologic malignancy (106, 114, 115).

Plasma contains all the coagulation factors and is therefore useful to correct deficiencies of clotting factors and is mainly given to patients with an active bleeding (109).

1.5.2 In MDS

Supportive care, with administration of blood transfusions and antibiotics, is the mainstay in the management of cytopenias in MDS. During evaluation of ongoing treatment with ESAs or azacitidine, the threshold for RBC transfusions is usually below 80 g/L. However, when the patient become refractory to treatment, the transfusion threshold is established based on individual symptoms and comorbidities at many centers, and the majority of patients are kept at hemoglobin levels higher than 90-100 g/L (116). This strategy is also recommended by the European Leukemia Network in order to alleviate symptoms of anemia and improve quality of life (21, 117, 118).

Over the course of the disease, it is reported that up to 94% of patients receive one or more RBC transfusion and 31-52% receive more than one PLT transfusion (26-28). Over half of the patients (51%) with lower-risk MDS become transfusion dependent (TD) (29). In the overall MDS population, the proportion of TD patients during the first and second year following diagnosis are 37% and 74%, respectively. By the third year, 90% of the patients required regular RBC transfusions (27, 28). The median number of RBC transfusions with interquartile ranges (IQR) is not commonly reported, but numbers suggest a median of 8 RBC units (range 0-186) during the MDS phase and higher if the disease progress to AML (119). A recent clinical trial investigated the effect of restrictive (hemoglobin threshold of 80 g/L) versus liberal (hemoglobin threshold of 105 g/L) transfusion indications, and found that patients with a goal of higher hemoglobin level had improved quality of life but also higher overall transfusion requirements (120).

One unit of prophylactic PLT transfusion is recommended to patients without significant bleeding during active chemotherapy to keep the PLT count at ≥ 10 x10^9/L (106). The prophylactic threshold can be increased in patients with a higher risk of severe bleeding.

1.5.2.1 Transfusions and prognosis

TD in MDS is associated with a significantly reduced overall and leukemia-free survival and impaired quality of life compared to non-TD MDS (9, 27, 121-123). Estimated survival by transfusion status at diagnosis is visualized in Figure IV. The impaired outcome is not fully understood but it has been hypothesized that iron overload in combination with bone marrow dysfunction and comorbidities related to severe anemia are contributing factors (27). However, the association between serum ferritin levels and prognosis has been investigated in two studies that could not confirm any significant association (124, 125). A meta-analysis tried to assess the influence of disease severity by adding an interaction term in the analysis but found no significant interaction (126). A recent study found that also a low transfusion burden of <3 RBC units per 16 weeks was associated with inferior PFS in patients with lower-risk MDS (127).
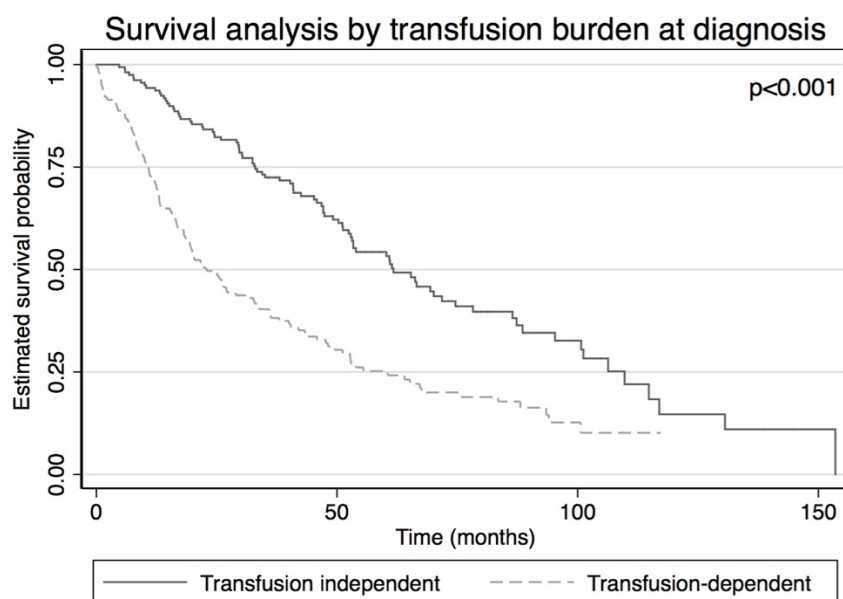

Figure IV. Estimated survival by transfusion status at diagnosis. Patients were considered transfusion-dependent if they required ≥ 1 RBC unit during the 2 months period before and after the diagnosis date. Survival curves were visualized using Kaplan-Meier survival plots and difference between groups was analyzed using the log-rank test.

1.5.2.2 Definitions of transfusion dependency in MDS

The term TD is used with a variety of definitions, although some are more frequently used. A regular transfusion need according to the WPSS is at least 1 RBC transfusion every 8 weeks over a period of 4 months (9). Somewhat higher transfusion burden for TD patients was defined in the treatment response criteria by the International Working Group (IWG) which

categorizes patients into TD or transfusion independent (TID), with TD patients receiving ≥4 RBC units over a period of 8 weeks with a pre-transfusion hemoglobin level of <90 g/L (128, 129). Similar definitions have been adopted, identifying TD-patients who required ≥2 RBC units over a period of 28 days (130-132). Proposal of the revised IWG 2018, categorized patients into three groups, also taking into account patients with a low transfusion burden. The three categories are non-transfused (NTD), low transfusion burden (LTB) receiving 3-7 RBC units within 16 weeks, and high transfusion burden (HTB) ≥8 RBC units within 16 weeks (133).

1.6 IMMUNOHEMATOLOGY AND RED BLOOD CELL ANTIGENS

At present, a total of 38 blood group systems and 360 blood group antigens have been recognized. The majority of the RBC antigens (89.4%) are found within 36 blood groups systems (134). The first blood group system ever described was the ABO-system, discovered by Dr. Karl Landsteiner from Austria, in the year 1901, a discovery for which he received the 1930 Nobel Prize in Physiology or Medicine. Still, more than a century later, ABO remains our clinically most important blood group. Also our second most clinically important blood groups system, the Rhesus (Rh) system, was initially discovered by Karl Landsteiner. Together with Alexander Wiener, he described an antigen similar to Rhesus macaque blood cells antigen, hence its name. In parallel work by Philip Levine and Rufus Stetson, the clinical significance of RhD was elucidated (135) and the unique identity of the Rh blood group system could later be identified. The Rh system contains several additional antigens, of which RhC/c and RhE/e are the next most important after RhD. The description of RhD was of major clinical importance and shed light on risks for immunizations during pregnancy. The development of anti-RhD prophylaxis decades later has now reduced pregnancy immunization in RhD-negative mothers dramatically, thus reducing number of infant deaths (136).

RBC antigens are attached to the RBC membrane and are either made up from carbohydrates or proteins. The antigens of the ABO blood group are carbohydrates, while most other blood groups, including the Rh system, consist of protein polymorphisms (137). Immunity to ABO antigens are different from that of most other blood groups. For reasons not completely understood, antibodies against A and B antigens are spontaneously formed in individuals lacking the corresponding antigens, the so called "Landsteiner's rule". Those spontaneously occurring antibodies, which are often of IgM type, immediately agglutinate and destroy antigen-expressing RBCs, which is the reason blood transfusion across a major ABO barrier (for example blood group A blood erroneously transfused to an individual of blood group O) is life-threatening. In contrast to the natural ABO antibodies, antibodies to most other blood groups (including antibodies against Rh antigens) are classical immune antibodies, meaning that they are formed in antigen-negative individuals after exposure of antigen-positive RBCs. Different blood group antigens have different immunogenicity. For example, in the Rh system, RhD is the most immunogenic, followed by RhE. Among other blood group

antigens, the Kell antigen is also considered highly immunogenic. This is one of the reason anti-RhE and anti-K are commonly found alloantibodies in immunized individuals.

1.6.1 Pathophysiology of alloimmunization – an overview

Formation of alloantibodies can occur when a recipient is exposed to a foreign RBC antigen, in reality after RBC transfusion or during pregnancy, referred to as alloimmunization. Considering the large variety of blood group antigens, each transfusion recipient is exposed to numerous foreign RBC antigens with each transfusion. Published papers of alloimmunization in overall recipients of RBC transfusions, describe a risk of 2-9% (138, 139). In brief, the pathophysiology in alloimmunization involves antigen recognition, a process by which the antigen-presenting cell (dendritic cells, macrophages or B lymphocytes) takes up the foreign erythrocyte antigen (via pinocytosis, phagocytosis or antibody-mediated uptake), breaks it down into small pieces and presents peptides from the foreign antigen by the major histocompatibility complex class II (MHC class II) molecules. Antigen-loaded MHC class II molecules are then recognized by the T cell receptor on specific CD4+ T-lymphocyte of the T helper type, which subsequently 'helps' B lymphocytes that a B cell receptor specific for the same antigen to proliferate and differentiate into antibody-producing plasma cells. Most alloantibodies of this type can be found in the circulation for very long times (often decades), and even if antibody titers can decline, memory B cells persist and are rapidly activated to produce large amounts of new antibodies quickly after encountering the same antigen again. It is therefore of outmost importance to screen patients for the presence of previously formed alloantibodies before choosing blood for transfusion (140).

1.6.2 Pre-transfusion testing and antibody detection

A blood transfusion is a transplantation of blood cells, and in analogy with all organ transplantations (with a few exceptions), compatibility between recipient and donor is of outmost importance. For RBC units, upfront selection of ABO and RhD compatibility is always performed. Following this, pre-transfusion testing to identify possible pre-existing alloantibodies is done. This pre-transfusion testing often includes automated type-and screen using test erythrocytes with known alloantigenic setups. If the antibody screening test was negative, ABO and RhD identical units are given without further matching, except for patients with hemoglobinopathies who most often are subject of extended matching to avoid alloimmunization (140-142). If the RBC antibody screen in plasma is positive, additional tests with extended RBC panels take place to identify the specificity of the antibody/ies using indirect antiglobulin techniques. RBC units negative for the antigens corresponding to the detected antibodies are then selected for transfusion, provided that a direct crossmatch turns out negative (143).

1.6.3 Immunological mechanisms following blood transfusions in MDS

Previously published data on alloimmunization and refractoriness in MDS report a RBC alloimmunization incidence of 12-15% in TD MDS (7), and platelet HLA-antibodies in 7% in patients with hematological malignancies (144). Results suggest that development of

alloantibodies is taking place within the period of the first 20 transfused RBC units (145). Formed alloantibodies are commonly directed to antigens of the Rh and Kell systems which are consistent with observations in other heavily transfused hematological diagnoses, such as in sickle cell disease and thalassemia (146, 147). Irrespective of the type of antibodies, they mediate destruction of transfused cells. The most well-known destruction mechanism is monocyte-dependent phagocytosis in the spleen, but complement-mediated lysis in the blood might also take place. A hitherto poorly explored possibility is that NK cells can kill antibody-coated erythrocytes and platelets via antibody-dependent cellular cytotoxicity. One published paper observed increased RBC transfusion requirements following alloimmunization (145). The general recommendation during pre-transfusion testing of patients with chronic bone marrow failure due to MDS, does not involve a strategy of upfront typing and matching. One guideline suggests grade 2 C evidence for considering extended RBC phenotyping for MDS patients with a regular RBC transfusion need (118).

2 RESEARCH AIMS

2.1 OVERALL AIM

The overall aim of the thesis was to improve knowledge on transfusion therapy to patients with chronic bone marrow failure due to MDS, with an emphasis to optimize transfusion therapy to these patients in the future.

2.2 SPECIFIC AIMS

The specific aims for the papers in this thesis were:

I. To describe transfusion patterns in patients with hematological malignances during the first two years following the diagnosis. The second aim was to repeat the analyses for patients who were treated with allogeneic stem cell transplantation.

II. To identify clinical and patient-specific characteristics associated with RBC and PLT transfusion intensity in patients with MDS. The secondary aim was to study the association between transfusion patterns and survival.

III. To investigate how duration of RBC storage might affect the transfusion efficacy with regard to hemoglobin increments post-transfusion, in a cohort of MDS patients.

IV. The primary aim was to assess the risk factors of alloimmunization in a cohort of MDS patients. Secondary aims were to investigate clinical changes after alloimmunization, in terms of transfusion intensity and the post-transfusion hemoglobin increment.

3 MATERIALS AND METHODS

The methods and statistical analyses used in studies I-IV are described in detail in the respective papers. Hence, this section will describe a summary of the most important methods and epidemiological concepts.

3.1 OVERVIEW

Studies I-IV are classified as retrospective observational studies covering different aspects of transfusion therapy to patients with hematological malignancies. Data were collected retrospectively from different sources and linked as appropriate for each study. Study I was a population-based, nationwide descriptive cohort study of transfusion patterns in patients with incident cases of hematological malignancies in Sweden of all ages. Studies II-IV were single-center cohort studies where we followed a well-characterized adult cohort of MDS patients from the MDS Biobank and Register at the Karolinska University hospital, Stockholm, Sweden with different analytic approaches with regard to transfusion therapy.

Table III. Overview of methodology paper I-IV.

	Study I	Study II	Study III	Study IV
Study design	Observational	Cohort	Cohort	Cohort
Study description	Describe transfusion patterns	Identification of parameters associated with transfusion intensity	Determine the effect of RBC storage on hemoglobin increment	Risk factors of alloimmunization and clinical consequences
Study population	Nation-wide, hematological malignancies in Sweden, all ages	Single-center: Adult patients with MDS in Stockholm	Single-center: Adult patients with MDS in Stockholm	Single-center: Adult patients with MDS in Stockholm
Time period	2000-2010	2003-2013	2003-2014	2003-2017
Number of patients	28,693	309	255 patients	455
Number of transfusions or episodes	541,441 mixed units	11,350 RBC units, 1,956 PLT units	3,399 episodes	25,079 RBC units
Data sources	Swedish Cancer Register, SCANDAT2	MDS Register at Karolinska University Hospital, ProSang	MDS register at Karolinska University Hospital, ProSang, Hemoglobin	MDS register at Karolinska University Hospital, ProSang, hemoglobin
Data collection	Retrospective	Retrospective	Retrospective	Retrospective
Main statistical methods	Descriptive, stratification	Poisson regression, Cox regression	Mixed effect linear regression model	Cox regression, non-parametric test
Exposure	Hematological malignancy	MDS disease-and patient characteristics, transfusion intensity	RBC storage time	Alloantibodies
Outcome	Transfusion burden, costs	Transfusion intensity, survival	Hemoglobin increment post-transfusion	Transfusion intensity

3.2 SETTING

3.2.1 Citizens of Sweden

All studies (I-IV) were performed in Sweden where each citizen is assigned a unique 10-digit identification number at birth or at immigration, the personal identification number, which enabled data collection and linking of different data sources.

3.2.2 Regional MDS cohort

Management and therapeutic options of the study population in study II-IV are based on the Nordic guidelines (148) which are in line with the European recommendations for treatment of MDS (21). Swedish MDS patients who have failed therapies for anemia are commonly transfused aiming for a hemoglobin level >95-100 g/L. This strategy is supported by a Nordic MDS Group study showing that a higher hemoglobin threshold is associated with a significantly better quality of life, but not with a higher transfusion intensity over time (117).

3.2.3 Red blood cell units in the Stockholm County

RBC units are leukocyte-reduced with in-line filters since 1999. The volume is approximately 260 +/- 15 mL, with a hematocrit of 60-65%. Patients that undergo allogeneic SCT receive irradiated blood units in addition to leuko-reduced. In study I, we performed additional separate analyses for patients who were treated with allogeneic SCT. In study II and IV, patients were followed only up until allogeneic SCT, and in study III, we excluded transfusion episodes of irradiated blood units in a sensitivity analysis.

3.3 STUDY POPULATIONS

For the study population in study I, we identified all patients in the Swedish Cancer Register that had been diagnosed with an incident hematological malignancy between year 2000 and 2010 in Sweden, of all ages. If a patient had two hematological malignancies registered during the study period, we only included the first registered hematological malignancy. Patients (N=28,693) were categorized into nine groups of diagnoses, including acute lymphoblastic leukemia (ALL), AML, chronic myeloid leukemia (CML), chronic lymphoid leukemia (CLL), multiple myeloma (MM), Hodgkin's lymphoma, diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL) and myelodysplastic syndromes (MDS). Patient data and clinical data were retrieved from the Swedish cancer register and linked to the binational transfusion database, the Scandinavian Donations and Transfusions (SCANDAT2) database. Transfusion patterns during the first two years after diagnosis were described. Patients were followed until date of death, emigration, or end of follow-up December 31, 2012.

For study populations in studies II-IV, we identified consecutively sampled adult patients (≥18 years) in the MDS register and biobank at the Hematology Center, Karolinska University Hospital, Stockholm, Sweden with a diagnosis of MDS or MDS/MPN overlap disorders according to the 2001 WHO classification and the revised 2008 WHO

classification. In study II, we identified 309 eligible patients with a date of diagnosis between January 1st 2003 and December 1st 2013. Their transfusion history was retrieved from the local transfusion database, ProSang, in Stockholm. Patients were followed until date of death, allogeneic SCT or December 1st 2013, whichever occurred first. In study III, we included the same 309 patients as in paper II, but excluded patients who never received any RBC transfusions during the study period. Available hemoglobin measurements from three laboratories were linked to patient and transfusion data. The final study population consisted of 255 patients with 3,399 transfusion episodes. Each transfusion episode had hemoglobin measurements within pre-specified intervals before and after each transfusion. In study IV, we identified patients with a date of diagnosis between January 1st, 2003 and up until July 1st, 2017 and retrieved their transfusion history and parameters of immunohematology from the ProSang database. Patients who had not received any RBC transfusion or patients that had alloantibodies detected before the first registered RBC transfusion were excluded, leaving 455 eligible patients for inclusion.

3.4 DATA SOURCES

3.4.1 National Registers

3.4.1.1 Swedish Cancer Register

The Swedish Cancer Register was founded in 1958 and records information on all incident cancer cases in Sweden. Both clinicians and pathologist are obliged to report new cases of cancer, which can be based on clinical data, morphological data or laboratory parameters. The overall completeness is considered high (149). Data include age, sex, personal identification number, date of diagnosis, ICD codes and histological type, stage and reporting hospital (150). Extracted data for study I, included date of diagnosis and type of hematological malignancy, classified using the International Classification of Diseases, Revision 10 and SNOMED codes.

3.4.1.2 Scandinavian Donations and Transfusions (SCANDAT2) Database

In study I, patient data from the Swedish Cancer Register was linked to the Scandinavian Donations and Transfusions (SCANDAT2) database. SCANDAT2 contains anonymized information on practically all blood donors, blood transfusions and recipients who has ever been registered at any of the regional blood bank databases in Sweden and Denmark since the start of the computerized registration 1968 and 1981, respectively. Coverage has gradually increased due to the introduction of computerized systems in blood banks and health regions, with complete coverage in Sweden since 1996 and since 2002 in Denmark. The SCANDAT2 version, contains computerized information of blood donations and blood transfusions from Sweden and Denmark until at least 2010, but in most cases throughout 2012. Data include complete follow-up with regard to cancer, hospital care and cause of death. Availability of this data allows for analysis of short and long-term health effects in both donors and recipients, including possible transfusion-transmitted diseases (151). In paper I, all

transfusions given to the study cohort of Swedish patients with hematological malignancies between year 2000 and 2012 were included.

3.4.2 Regional Registers

3.4.2.1 MDS Biobank and Register at the Karolinska University Hospital, Stockholm, Sweden

The register enrolls consecutive patients with MDS and MDS/MPN. Over the years, the coverage has improved with more registered number of patients per year. In studies II-IV we included patients from the beginning of year 2003 to ensure several consecutively sampled patients per year. Data are informative on disease characteristics, cytogenetics and mutations, date of diagnosis and death, IPSS and IPSS-R, WHO classification, blood values at diagnosis, marrow blast cell count and history of MDS specific therapies. The register is continuously updated with new data when applicable, for example new bone marrow examinations, MDS therapies, date of allogeneic stem cell transplantation and death.

Targeted gene sequencing

Diagnostic samples from all patients in the MDS Biobank and Register at the Karolinska University Hospital have previously been sequenced for mutations in genes recurrently mutated in MDS, using Haloplex® technology for 42 or 72 genes. A panel of genes, selected on the basis of known association with the pathogenesis of myeloid diseases, had previously been analyzed using the Illumina HiSeq 2000 system at the Sci-Life laboratory (Uppsala, Sweden) (152). Figure V, shows each mutation and number of patients with the respective mutation, using data from study II.
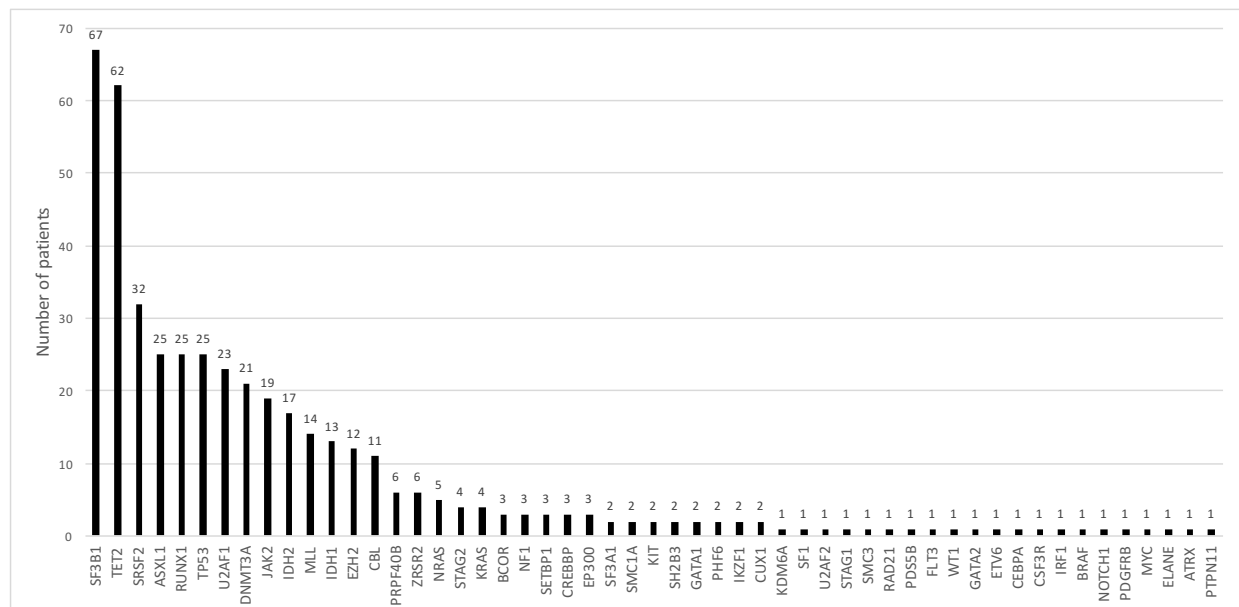

Figure V. Number of patients with mutations recurrently mutated in MDS. The figure is derived from patient data in study II.

3.4.2.2 ProSang

In studies II-IV, MDS data was linked to the local transfusion medicine database, ProSang (CSAM e-Health Company, Oslo, Norway) that records data of all transfusions administered at in and out-patient care facilities in the Stockholm county. Data is computerized and includes information regarding type of blood component, date of collection, reservation time for transfusion and blood group serology analyses performed for each patient, including alloantibodies and autoantibodies. ProSang data also include donor parameters which were not included in any of the work for this thesis.

3.4.2.3 Laboratory data sources for hemoglobin measurements

Study III was depending on hemoglobin data for evaluation of the post-transfusion hemoglobin increment. Data were retrieved from the Laboratory Information System (FlexlabTM; Tieto, Helsingfors, Finland) at the Clinical Chemistry Laboratory, Karolinska University Hospital and from two private contractors in Stockholm: Aleris Medilab and Unilabs AB to include as many hemoglobin measurements as possible, taken during the study period. Data on hemoglobin measurements were retrieved until May 8th 2014. This data set was also used in a subgroup-analysis in study IV.

3.5 STUDY DESIGN

3.5.1 Retrospective observational studies (study I-IV)

All four studies were cohort studies. In a cohort study, a defined study population (cohort) is followed over time to evaluate if a specific exposure is associated with one or more specific outcomes. The outcome cannot have happened before start of follow-up. A cohort study can be either prospective or retrospective and terminology is not used fully stringent but is often called prospective when data collection or start of the study precedes the occurrence of the outcome, or retrospective, when data collection or start of the study is initiated after the outcome has occurred. Patients are followed until event, death or end of follow-up, and their time from start until end of follow-up is each individual's risk time (153).

Study I, was performed as a descriptive nation-wide cohort study where we described transfusion patterns in patients diagnosed with a hematological malignancy in Sweden between year 2000 and 2010. Generally, in a descriptive cohort study, no hypothesis is tested but instead, the results often generate hypothesizes. Study II, was a retrospective, single-center register-based cohort study investigating predictors of RBC and PLT transfusion intensity and the association between transfusion intensity and survival. Study III, was a retrospective, single-center cohort study, investigating how duration of RBC storage affect transfusion efficacy by estimating the hemoglobin increment per RBC unit after a transfusion episode, stratified by different storage categories. Study IV, was a retrospective, single-center cohort study investigating risk factors of alloimmunization and how alloimmunization changes clinical parameters such as transfusion need and hemoglobin increments post-transfusion.

3.6 STATISTICAL APPROACHES

Statistical analyses were performed using SAS, Version 9.4, SAR Institute, Cary, NC (study I and data preparation of time-dependent models study II), and Stata, Version 13.1, StataCorp (for data preparation and analyses in study II-IV). A two-sided p-value below 0.05 was considered statistically significant.

3.6.1 Descriptive analyses (study I-IV)

Summary statistics for continuous variables were presented as proportions, medians with interquartile ranges (IQRs) and means with standard deviations (SDs). Differences between groups were analyzed using Pearson's chi-square test and Fischer's exact test for categorical variables, depending on sample size. Quantile regression was used to test differences between medians.

3.6.2 Poisson regression (study II)

A Poisson regression model uses count data as the dependent variable and the counts are expected to approximately follow a Poisson distribution. Independent variables are categorical or continuous and the model makes it possible to analyze which explanatory variables that are statistically associated with the dependent variable.

In study II, we analyzed predictors of transfusion intensity by using a time-dependent Poisson regression model. Number of transfusions was the dependent variable and we included the logarithm of follow-up time as an offset. The Poisson regression estimated the incidence rate ratio (IRR) of transfusion intensity per person-year with 95% confidence interval (CI) and the association with a number of considered parameters. First, univariate analyses were performed for each considered variable, including sex, age at diagnosis (categorized as <65, 65-74, >74 years), IPSS-R (very low, low, intermediate, high, very high), WHO classification, bone marrow cellularity at diagnosis (categorized as <30, 30-50, >50%), and mutation status as a series of binary variables comparing carriers of that mutation to all non-carriers. Mutational status was also presented as number of mutations (categorized as no mutation or SF3B1 mutation, 1-2 mutations but not SF3B1, more than three mutations but not SF3B1. RBC and PLT transfusions were analyzed in separate models. In the multivariate model, we included all parameters except WHO classification and number of mutations to avoid overlapping with IPSS-R and groups of mutations. Both univariate and multivariate analyses were performed separately for RBC and PLT transfusion intensity.

3.6.3 Cox proportional hazards regression (studies II, IV)

The Cox proportional hazards regression is a well-recognized technique to model survival data. The model assesses the relationship between several explanatory variables (risk factors) and time to event (often death, relapse or another event) and estimates the risk of event (hazard) with CIs. When two groups are compared with regard to their hazards, the reported measure is the HR.

The analyses of predictors of patient survival (study II) were performed using a time-dependent Cox proportional hazards model, estimating HR of death, with 95% CIs. Patients were censored at the date of allogeneic SCT. Similar analyses of AML progression could not be done due to few cases of AML progression in the cohort during the study period. In addition to number of RBC and PLT transfusions during the past year, these analyses included age, sex and the risk score IPSS-R.

In study IV, we applied the Cox proportional hazards regression to assess potential risk factors of alloimmunization. We used both univariate and multivariate Cox regression for several considered covariates including sex, age (categorized as <65, 65-74, >74), IPSS-R (categorized as lower-risk MDS if IPSS-R were very low, low or intermediate, otherwise categorized as higher-risk MDS), WHO classification (selectively categorized into patients with and without ring sideroblasts), bone marrow cellularity (categorized as hypoplastic ≤25%, normocellular 26-69% or hypercellular ≥70%), RhD status, mutational status as binary variables comparing carriers of that mutation to all non-carriers (categorized into mutations involved in chromatin modification, DNA methylation, splicing factors, cohesion factors, signaling factors, transcription factors, TP53 and others, see supplementary Table I for details), number of mutations (categorized 0 or SF3B1 without high-risk factor TP53 mutation or complex karyotype, 1-2 or ≥3), number of cumulative RBC transfusions (categorized as low transfusion burden <10 RBC units or high transfusion burden ≥10 RBC units), DAT-positivity and a binary variable indicating an RBC transfusion before the MDS diagnosis. Kaplan-Meier survival estimation was used for visualization, and comparison between groups was performed using the log-rank test.

3.6.4 Mixed effect linear regression (study III)

Mixed effect linear regression is a development of the common linear regression, to allow both fixed and random effects to occur. Mixed effect models allow the analysis of repeated measures data, allowing participants to change exposure status over time.

In study III, we applied a mixed effect linear regression model to estimate the effect of RBC storage time on the post-transfusion hemoglobin increment per RBC unit. The hemoglobin increment per RBC unit was the outcome of interest and was modelled by multiplying the covariates by the number of RBC units. The RBC storage time was included as fixed effects and categorized as <5 (as reference category), 5-9, 10-19, 20-29 or ≥30 days storage. A random intercept was included to acknowledge that the baseline hemoglobin might vary between patients. Analyses were adjusted for age and sex as well as time from transfusion to subsequent hemoglobin measurement. This time interval could be up to 28 days and was included as a restricted cubic spline with the placement of four knots at the quantiles. The estimates were further modeled to get the estimates of the mean hemoglobin increase with 95% CI. This method was also adapted in a subgroup analysis in study IV.

3.6.5 Wilcoxon signed-rank test (study IV)

Wilcoxon signed-rank test is a non-parametric test that compares two sets of numbers within the same participant or group. The test is appropriate when data is not normally distributed and it would not be correct to use the corresponding t-test for normally distributed data. The Wilcoxon signed-rank test was used in study IV to compare the average transfusion intensity before and after alloimmunization, within groups.

3.6.6 Missing data

Within our data, we observed low numbers of missing data among several covariates which was always reported. We did not use multiple imputation in any of the studies.

3.7 EPIDEMIOLOGICAL CONCEPTS

Observational studies are important and complement both basic research and clinical trials. The methods enable studies of large data sets, estimate the strength of an association, describe incidence and prevalence and generate hypothesizes, to mention a few things. A well-designed cohort study has the possibility to provide strong scientific evidence, however, in observational studies one must always consider potential influence on the results by random and systematic errors. Depending on the type and extent of these errors, they can affect precision, internal validity and external validity. The 'ideal' study has high precision and high internal validity, meaning that we trust our results that they are not estimated by chance and that the association between the exposure and outcome are not affected by systematic errors.

3.7.1 Random error

Random error refers to the possibility of the results being influenced by chance and is also known as variability. It is inherent in all measurements, but the aim is to reduce the extent to which a random error affects the estimate. The width of CIs and the level of the probability value (p-value) help to evaluate the degree of statistical uncertainty of an estimate. With a CI of 95% which is the most commonly set CI, we can be 95% confident that the true estimate is within the range of the two values that defines the CI in the absence of systematic errors. The most common threshold for the p-value is 0.05. Meaning, if the p-value is <0.05 we have evidence to reject the null hypothesis and that the probability of observing an effect as large as the one observed is <5%. A larger group of participants in the study population generally results in higher precision with more narrow CIs and lower *p*-values.

3.7.2 Systematic errors

Systematic errors are deviations that are not driven by chance and can result in an incorrect association. It could result in either higher or lower estimates than the true association between exposure and outcome. They are commonly categorized into selection bias, information bias and confounding.

Selection bias refer to bias due to inaccurate inclusion or (loss to) follow-up, making the study population unrepresentative of the population that we want to make inference on. For

example, in studies II-IV, patients were excluded only they had a date of diagnosis before January 1st, 2003, to minimize the risk of selection bias due to the smaller number of newly registered patients per year before 2003.

Information bias is also referred to as misclassification. It refers to an incorrect measure of exposure, outcome or covariate(s). This type of bias is sometimes classified into non-differential and differential. In the former, the frequency of error is similar in the groups that are compared and results in diluted estimates, biased 'towards the null'. In the differential misclassification on the other hand, the degree of misclassification differs between the groups that are being compared, which may lead to an association biased in any direction. For example, in study IV, we don't have information on autoantibodies detected using direct antiglobulin test (DAT) on all patients. This is a limitation of the transfusion data and could result in a misclassification by two reasons. First, by the fact that we might have missed patients who would have had a positive DAT if the test had been taken. In the setting when we compared the frequency of DAT-positivity between the sexes, the misclassification was considered non-differential given that DAT is tested equally between the sexes. However, DAT is also taken on the initiative of the blood bank during identification of suspected RBC antibodies which might result in differential misclassification. This is further elaborated on in paper IV.

A confounding factor is a factor that is associated with both the exposure and outcome variable, but must not be in the causal pathway between the two. If confounding occurs, the estimated effect is not reliable. Two confounding factors that are usually taken under consideration in epidemiological studies are sex and age which was also accounted for in studies II-IV. To handle confounding, we used methods of adjusting (study II-IV) and stratification (I, III-IV).

3.8 ETHICAL CONSIDERATIONS

The conduct of all studies was approved by the regional ethics review board in Stockholm, Sweden; 2014/2090-32 for paper I and 2013/1448-31/1 for paper II-IV.

The research was performed in line with national and international ethical standards, with the four ethical principles in mind: i) respect for autonomy and protection of persons with impaired autonomy, ii) beneficence, meaning trying to maximize the benefits iii) non-maleficence, do no harm and iiii) justice, to act on the basis of fair judgment (154).

Handling sensitive data such as personal registration numbers and clinical data, involves a certain intrusion of privacy. However, the intrusion was considered relatively limited, since the personal identification numbers were only use on initial stages and the analyses were performed on anonymized data. Further, results were presented at group level and cannot be traced back to individuals.

All studies (I-IV) were based on historical data and due to the malignant diseases, many patients have already gone ad mortem at the study initiation, and hence it would be

impossible to assess informed consent from all individuals in each study population. Further, most study participants would unfortunately not live long enough to take advantage of the potential results from these papers. However, the results might be of importance for future patients with hematological diseases, and this knowledge might be valuable on an individual level to those patients who are still alive.

If the results contribute to increased knowledge with potential of improved transfusion therapy to patients with a chronic bone marrow failure, it could be of importance not only to individual patients, but for the health care system with reduced costs of laboratory testing, patient visits and number of transfusions.

4 RESULTS

4.1 STUDY I

In this retrospective, population-based observational study we included 28,693 patients of all ages with an incident hematological malignancy diagnosed between year 2000 and 2010 and described their transfusion patterns the first two years following diagnosis.

The cohort received in total 541,441 number of transfusions of which RBC units constituted the largest share of 375,171 units, followed by 137,870 PLT units and 24,211 plasma transfusions. Patients with ALL had the highest proportion of patients requiring any transfusion (98.3%) and in contrast, follicular lymphoma was the group with the lowest proportion of patients requiring any transfusion (36.4%). The median number of blood transfusion of any type ranged from 40 (IQR, 16-78) in the AML group to 0 (IQR, 0-8) in patients with CLL, Hodgkin's lymphoma (IQR, 0-5) and follicular lymphoma (IQR, 0-4). Studying RBC transfusions specifically, we found the highest median number of transfused RBC units to patients with AML 25 (IQR, 11-44) followed my MDS 20 (IQR, 5-50).

The transfusion incidence, measured as number of blood components per person-year, was highest immediately after diagnosis in patients with ALL (Figure VI) and AML. This was followed by a rapid decline with some minor peaks. A similar transfusion incidence was observed in patients with Hodgkin's lymphoma, DLBCL and MM. For CLL, CML, follicular lymphoma and MDS, the transfusion incidence was more stable throughout the observation period of two years. We observed that the proportion of patients that received transfusions was generally higher in the youngest age group (0-18 years), except for in Hodgkin's lymphoma where patients older than 65 years had the highest proportion of transfused blood products. Patients younger than 65 years with acute leukemia received almost twice the number of RBC and PLT units compared to patients older than 65 years. In MDS, patients between 19-65 years received a mean of approximately 10 more RBC units and 5 more PLT units compared to patients older than 65 years (Figure VII).

Of 629 patients who received allogeneic SCT, a majority (N=604, 96%) was younger than 66 years. Diagnoses prior to allogeneic SCT included ALL, AML, MM and MDS. We found that the mean transfusion incidence was highest immediately after SCT, then followed by a sharp decline as observed in patients with other groups of aggressive hematological malignancies. Younger patients received in general more PLT transfusions compared to patients in the age bracket of 19-65 years.

Direct transfusion costs were highest among patients with ALL, AML or MDS, with an average range between 150,000 to 200,000 SEK (17,857-23,809 USD) during the study period. Patients with follicular lymphoma and CLL were found in the opposite end with mean transfusion costs of approximately 6000 SEK (714 USD). In similarity with previous analyses, we found major differences between age groups.
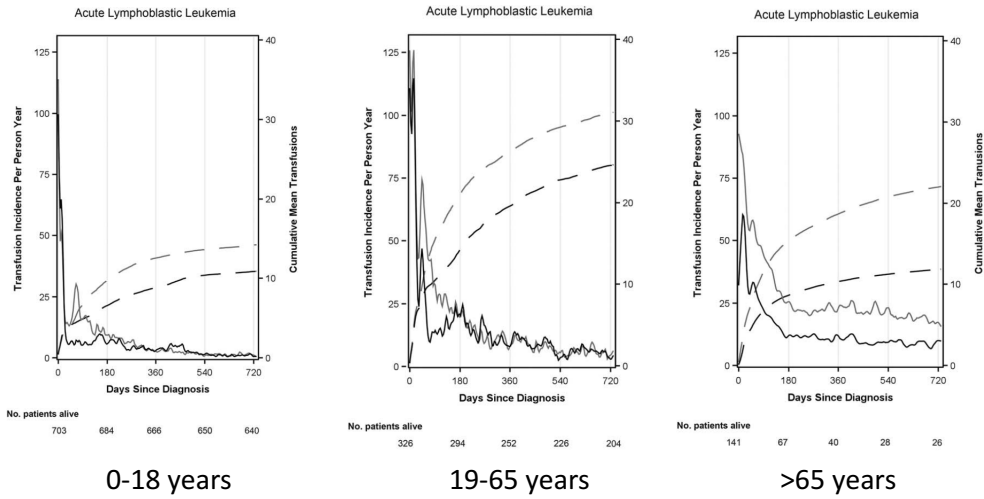
0-18 years   **19-65 years**   **>65 years**

Transfusion incidence (—, RBCs; —, PLTs) and cumulative mean of PLTs (– – –) and RBCs (– – –) measured over time since
diagnosis (0-18 years old at diagnosis). Groups with fewer than 25 patients have been omitted.

Figure VI. Transfusion incidence of RBCs and PLTs and cumulative mean of RBCs and PLTs in ALL.
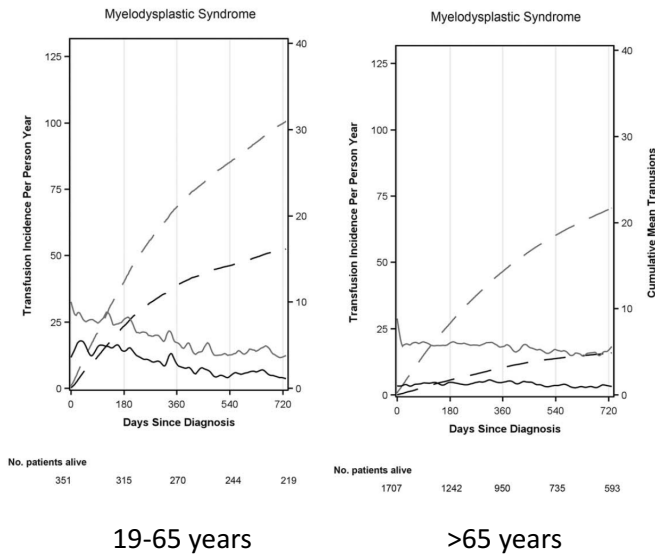
19-65 years   **>65 years**

Transfusion incidence (—, RBCs; —, PLTs) and cumulative mean of PLTs (– – –) and RBCs (– – –) measured over time since diagnosis.

Figure VII. Transfusion incidence of RBCs and PLTs and cumulative mean of RBCs and PLTs in MDS.

4.2   STUDY II

We included 309 patients with MDS or MDS/MPN and retrieved their transfusion history to investigate predictors of transfusion intensity and to study the association between transfusion intensity and prognosis.

The cohort was followed for 777 person-years and received in total 11,350 RBC transfusions and 1,956 PLT transfusions. Using a Poisson regression model, we estimated the association between patient and disease-specific variables with transfusion intensity expressed as IRR. We found several covariates that were independently associated with transfusion intensity. For RBC transfusion intensity, we found that very high IPSS-R (IRR 2.7, 95% CI 2.6-3.0), high IPSS-R (IRR 2.0, 95% CI 1.9-2.1), male sex (IRR 1.7, 95% CI 1.6-1.8), histone modulator mutations (IRR 1.8, 95% CI 1.7-1.9), signaling factor mutations (IRR 1.5, 95% CI 1.4-1.6) and transcription factor mutations (IRR 1.2, 95% CI 1.1-1.3) were independent predictors of higher RBC transfusion intensity. Variables associated with higher PLT transfusion intensity were to a large extent similar to the predictors of RBC transfusion intensity, but with higher estimates of the IRR for male sex, histone modulator mutations, transcription factor mutations and signaling factor mutations (Figure VIII).
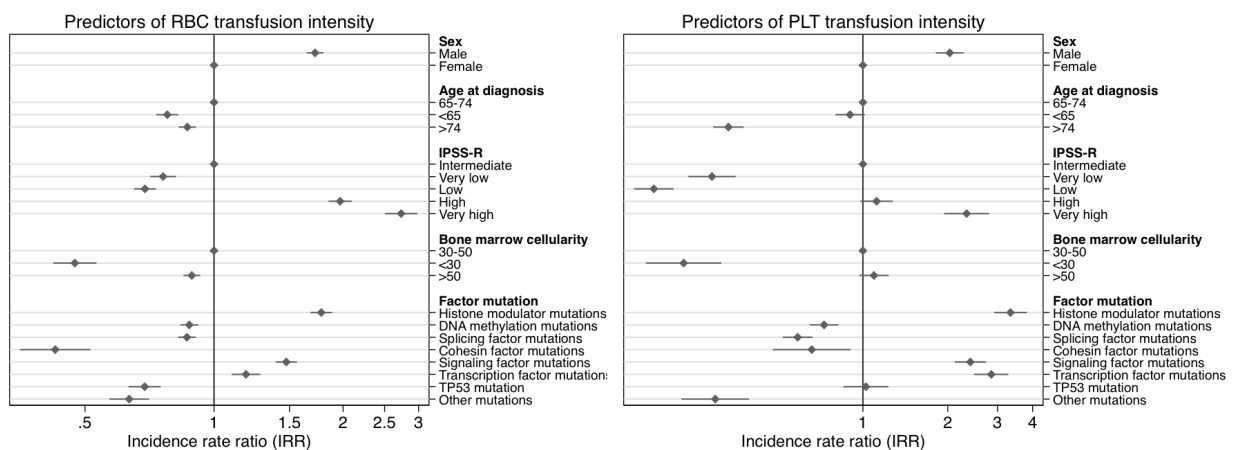

Figure VIII. Forest plot of predictors of RBC and PLT transfusion intensity. Point estimates and confidence intervals were retrieved from the multivariate Poisson regression analyses.

Some variables were associated with a lower RBC transfusion intensity. These included cohesin complex mutations, hypocellular bone marrow <30% cellularity, 'other mutations', low IPSS-R, age <65 years, very low IPSS-R, splice factor mutations, DNA methylation mutations and age >74 years. In similarity, independent predictors of lower PLT transfusion intensity were age >74 years, very low and low IPSS-R, hypocellular bone marrow and mutations in splicing factors, DNA methylation and cohesion complex (Figure VIII).

Studying the transfusion intensity the past year, we found that the numbers of RBC and PLT transfusions were significantly and independently associated with an increased risk of death, these findings remained significant even after adjusting for age, sex and IPSS-R (p<0.05).

4.3 STUDY III

The objective in paper III, was to study the effect of RBC storage time on the hemoglobin increment in transfused MDS patients. Data included 255 patients who had 3,399 registered transfusion occasions that qualified for inclusion of having both pre and post-transfusion hemoglobin measurements taken within 2 days before and 28 days following the RBC transfusion, to enable evaluation of the post-transfusion hemoglobin increment.

We observed that longer duration of RBC storage was associated with a modest but statistically significant decrease of the post-transfusion hemoglobin increment. Compared to recipients of units stored <5 days, receipt of blood units stored 5-9, 10-19, 20-29, or ≥30 days resulted in gradually lower hemoglobin increases, per RBC unit (Table IV).

We did five sensitivity analyses trying to assess potential influence of confounding factors. In the first analysis, we excluded transfusion episodes where the follow-up hemoglobin measurement was taken within 48 hours. In the second we included only chronically transfused patients, defined as having received ≥1 RBC unit per month over a period of 4 months. The following three analyses separately excluded patients with ≥1 alloantibody, transfusion episodes with ABO-nonidentical units and irradiated RBC units. Generally, results were similar to the original model with a trend of successively lower hemoglobin increment with longer duration of RBC storage and with statistical significance in all sensitivity analyses for storage time of or exceeding 20 days.

Table IV. Effect of storage duration on the post-transfusion hemoglobin increase (g/L).

Average storage time (days)	Estimated mean hemoglobin increase per unit (95% CI)	Estimated difference from Model 1 (95% CI)*	Estimated difference from Model 2 (95% CI) †
<5	8.05 (7.39–8.71)	0 (ref)	0 (ref)
5–9	7.22 (6.71–7.74)	-0.83 (-1.41, -0.24)	-0.79 (-1.37, -0.20)
10–19	7.13 (6.62–7.63)	-0.92 (-1.51, -0.34)	-0.85 (-1.44, -0.27)
20–29	6.72 (6.12–7.32)	-1.33 (-2.02, -0.65)	-1.17 (-1.85, -0.49)
≥30	6.54 (5.67–7.42)	-1.51 (-2.43, -0.58)	-1.27 (-2.20, -0.35)

*Model 1 included the full cohort; †Model 2 adjusted for age and sex.

4.4 STUDY IV

In a cohort of 455 consecutively sampled patients with MDS who had received at least one unit of RBCs, we estimated the risk of alloimmunization to 12.5% (95% CI, 9.79-15.90). A majority of the formed alloantibodies belonged to the Rh or Kell blood group systems (N=49, 86.0%). We evaluated possible risk factors of alloimmunization using Cox regression and found that female sex and DAT-positivity were independently associated with alloimmunization with hazard ratios of 2.00 (95% CI, 1.15-3.47) and 6.19 (95% CI, 3.53-10.88), respectively. No association between RBC immunization and investigated disease characteristics was found.

The median number of transfused RBC units per person during the entire observation period was 56 in the alloimmunized group (IQR 32-111) versus 28 (IQR 12-62) in the non-alloimmunized group (p=0.019). We compared the average number of RBC units per month, in the 6 months period before and 6 months period after alloimmunization and observed that the transfusion intensity increased in patients with alloimmunization, from a mean value of 1.3 (IQR 0.33-2.00) RBC units/month before alloimmunization to a mean value of 2.0 (IQR, 0.66-3.50) RBC units/month (p=0.0006). Next, we evaluated if DAT-positivity influenced the transfusion intensity and stratified the cohort into patients with both alloimmunization and DAT-positivity, alloimmunization alone and DAT-positivity alone. We observed an absolute increase of 1.5 units/month in patients with a combination of alloimmunization plus DAT-positivity and with DAT-positivity alone, (p=0.0012 and p=0.0002, respectively). In patients with alloimmunization alone we found a small absolute increase but without statistical significance.

To evaluate whether the increased transfusion intensity was also reflected by a smaller hemoglobin increase after alloimmunization, we estimated the difference of the 'post-transfusion hemoglobin increment' before and after alloimmunization. We observed a lower hemoglobin increment after alloimmunization of 1.35 g/L (95% CI, 0.50-2.20) per RBC unit (p=0.002) compared to before alloimmunization, adjusted for sex and DAT-positivity.

Table V. Median number of RBC units during follow-up and average number of RBC units/month, within the 6 months period before and after date of immunization respectively

	Number of patients	N (%) males	Median number of RBC units during follow-up (IQR)	Average number of RBC units/month, pre-immunization period	Average number of RBC units/month, post-immunization period	p-value
Allo+	51	20 (39.2)	62 (32-117)	1.3 (0.33-2.00)	2 (0.66-3.50)	0.0006
Allo+ DAT+	31	16 (51.6)	78 (44-129)	1.3 (0.33-2.00)	2.8 (0.83-4.00)	0.0012
Allo+ (DAT- (presumably))	20	4 (20.0)	44 (17-112)	1.0 (0.33-1.92)	1.3 (0.67-3.00)	0.1587
Allo- DAT+	35	22 (62.9)	37 (20-107)	0.2 (0.00-0.67)	1.7 (0.67-3.50)	0.0002

5 DISCUSSION

5.1 GENERAL DISCUSSION

Strengths of the papers in this thesis involve the population-based material used in study I and the consecutively sampled patients from the regional MDS database in studies II-IV. Patients in the regional MDS database are well-characterized with regard to patient and disease-specific variables. Baseline patient characteristics were validated with national and international MDS data. This thesis focus on several important aspects of transfusion therapy in patients with hematological malignancies (study I) with a special focus on MDS (study II-IV). In study I, we observed that the transfusion burden among patients with MDS and acute leukemias are substantial. In all groups of diagnoses, we found that RBC transfusions were the most commonly transfused blood product. Results from study I, provide new knowledge and understanding of both transfusion patterns and costs in patients with hematological malignancies. Given the high quantity of transfused blood products to these specific patient groups, even modest improvements of the transfusion efficacy could have impact on the total transfusion burden and transfusion-related costs. In study II, we focused on the MDS disease and explored factors associated with transfusion intensity. We identified patient and disease-specific predictors associated with both higher and lower transfusion intensity, of RBC and PLT transfusions. Further, we found a strong association between transfusion intensity and survival which highlights the importance of transfusion intensity in addition to TD as a binary variable. The relationship between blood transfusions and impaired prognosis is not considered causal, but most likely reflects a multifactorial association and we should continue to use RBC transfusions when there is indication. With focus on the efficacy of the RBC unit in study III, we observed that longer RBC storage time impaired the transfusion outcome with regard to the post-transfusion hemoglobin increment. Lastly, that immunological consequences after RBC transfusions are of considerable risk in the MDS population, and observed important clinical changes after alloimmunization, such as higher transfusion intensity and lower post-transfusion hemoglobin increments. This warrants for future prospective studies to evaluate the clinical relevance of these finding, and further discussion about prophylactic antigen matching in patients with MDS.

In addition to the main results, the studies also generated several questions and hypothesizes for further follow-up studies in the important field of transfusion therapy in patients with hematological diseases. Future follow-up studies should evaluate transfusion patterns during the full disease course, and not limit the analysis to two years as in our study. This would be of special importance in hematologic malignancies that not necessarily starts with an aggressive transfusion need, but might gradually develop an increasing transfusion need at loss of response to treatment or during disease progression. In addition, it would be of interest to assess transfusion patterns in patients within the spectrum of benign hematology, such as in patients with hemoglobinopathies who often suffer from consequences of heavy RBC transfusion therapy from young ages. It would also be of both interest and importance to understand the underlying mechanisms of increased transfusion intensity (study II) and to

find out more about the relationship between alloimmunization and autoantibodies. Given the retrospective nature of this thesis, there are also limitations to acknowledge.

5.2 MAIN FINDINGS

Blood transfusion patterns

Paper I, was the first, to the best of our knowledge, published article presenting transfusion patterns in patients with a first-time hematological malignancy, on population-based material. The strengths of the study involve the nationwide material with patients from different centers and diagnosis groups.

In the analyses, we split the cohort into 9 main groups of diagnoses and by age (0-18, 19-65, >65 years). The variation of the natural course of the disease and treatment regimens in the nine groups of hematological malignancies included in study I, are depicted in several ways by the diverse transfusion patterns that we observed in our results. Not surprisingly, the distribution of age within each group shows high variance. For example, ALL is mostly a pediatric cancer and accounts for 75% of acute pediatric leukemias. The acute pediatric leukemias as a group, constitute the leading cancer type in the ages 0 to 14 years (155). In contrast, AML is rarely seen in pediatric patients (156) but is instead more common in adults and elderly, with a median age at diagnosis of 72 years (157). All patients with high risk or high proliferative diseases are considered for intensive treatment protocols with chemotherapy aiming for cure and complete remission. These treatment regimens and allogeneic SCT are associated with several weeks of pancytopenia with a transient need of both RBC and PLT transfusions. This is also reflected in our results, where we observed a high transfusion incidence in aggressive diseases early on after disease onset, probably due to intensive treatment with chemotherapy. Another contribution factor could be peripheral cytopenia due to disease infiltration in the bone marrow. Younger and adult patients are more often treated with intensive regimens including allogeneic SCT compared to elderly who are not treated with intense regimens to the same extent (158). This could at least partly explain the difference in transfusion incidence in between age categories within the same group of diagnosis. Aggressive non-Hodgkin B-cell lymphomas are treated with high dose chemotherapy bi-weekly or every three weeks aiming to cure the disease (159, 160), whereas a low-malignant disease like CLL is often diagnosed en-passent and is not in need for treatment until development of symptoms or laboratory deviations which might take years or even decades (161). Approximately one third of the patients with CLL does never require treatment and this strategy does not affect prognosis (162). The rapid decline of the transfusion intensity that we observed in the most aggressive diseases is most probably a result of patients being cured or due to early mortality. In the diagnosis group of MDS we observed a fairly stable transfusion intensity which could be explained by a case mix of lower and higher-risk MDS that together gave this stable transfusion incidence. Patients with lower-risk MDS and anemia are often treated with ESAs early in the disease course with a median response duration of 23-24 months (163, 164). The initial response is followed by a refractory state with need for transition to supportive care with regular RBC transfusions. Higher-risk

MDS on the other hand, might require more transfusions early on after diagnosis for the same reason as stated above in aggressive diagnoses.

Results can be of importance when comparing therapeutic options for whole disease groups, but limit the possibility of generalizing findings to the individual patient, since we did not take into account patient or disease-specific characteristics. A newly published nation-wide study explored the transfusion patterns and associated costs in MDS, stratified by IPSS-R (165). Patients were followed for four years and the average number of transfused RBC units ranged from 25 in IPSS-R very low to 171 in IPSS-R very high. The corresponding numbers for PLT units were 4 and 66, for very low and very high IPSS-R, respectively. Transfusion-related costs (including blood products, disposables, labor costs and laboratory testing) increased gradually with IPSS-R risk group, ranging from 8,805 USD in IRSS-R very low to 80,106 USD in IPSS-R very high. With the better overall survival in lower-risk MDS compared to higher-risk, it would be interesting to further evaluate transfusion patterns during the complete course of the disease (71).

Predictors of transfusion intensity

In the analyses of predictors of transfusion intensity (study II), we had a high total number of RBC transfusions contributing to a high precision as indicated with generally narrow CIs among the included covariates in the multivariable Poisson regression analysis. We observed several covariates that were significantly associated with transfusion intensity of both RBCs and PLTs. Significant and independent variables associated with higher transfusion intensity of RBCs included male sex, higher-risk MDS and mutational status (mutations involved in histone modulation, signaling and transcriptional regulation). We elaborated about the male significance for transfusion intensity in paper II. This finding correlate well with a higher disease burden and a worse prognosis in males, as earlier reported (166). Indeed, we found a significant difference in the median overall survival and risk of death, unfavorable for the male sex. However, the multivariate analyses showed significant results independent of the IPSS-R risk score. We elaborated on the pre-hemoglobin transfusion level but found no relevant difference. One possible contributing factor, derived from results in study IV, is the potential influence of subclinical hemolysis as we observed a higher proportion in males in patients with DAT-positivity, which could affect the survival of both transfused and autologous RBCs, and thus influence the transfusion intensity. Another possibility derived from a published paper, is that males have a lower hemoglobin increment 24 hours post-transfusion, with smaller increments with higher weight (167), which also could contribute to the higher transfusion intensity observed in males. Although not investigated in the same manner, we did not observe any association with higher blood volume in a separate multivariate analysis.

Considering the important prognostic information about survival and disease progression that are provided by the pattern of recurrent mutations (55, 168, 169) it could be hypothesized that certain gene mutations may predict transfusion need and transfusion intensity. If not directly, it could reflect disease pathogenesis and indirectly affect transfusion needs. The association

of RUNX1, TP53 and NRAS with severe thrombocytopenia has been described earlier (40), but less research have described mutations and their association with transfusion data. We showed that several groups of mutations were significantly associated with higher transfusion intensity. The mutations were grouped according to their mechanism as done in previously published papers. Given that 123 patients had at least 2 different mutations, the groups of mutations were not mutually exclusive in all cases. This is a limitation and a potential source of confounding, which must be kept in mind during interpretation. However, in the groups of mutations where we could find an association, we also run the analyses separately for the most clinically relevant mutations in the respective group. For the histone modulator mutations with the highest observed association with higher transfusion intensity, we found that ASXL1 compared with the rest of the histone modulator mutations, provided similar and significant point estimates. We propose a prospective, experimental study for evaluation of these findings and to understand underlying mechanisms.

Mutation of ASXL1 has been identified as a poor prognostic marker in MDS (40, 55, 57). In study II, we could find an association of ASXL1 and other histone modulator mutations with a higher transfusion intensity, but in the Cox regression, these mutations were no longer associated with poor survival when we adjusted for transfusion intensity, age, sex, and risk score. We interpreted this finding as suggestive of a more pronounced role of ASXL1 in hematopoiesis than disease progression.

Transfusions and survival

Several publications have shown an impaired overall and leukemia-free survival due to TD. One published study observed that when including transfusion intensity, the cumulative number of RBC units was not significant for prognosis in terms of leukemia-free survival (27). The second aim of study II, was to investigated the association between transfusion intensity the past year and survival. We observed how the number of RBC and PLT transfusions the past year were significantly associated with an increased risk of death, with estimates that remained similar even after adjusting for age, sex and IPSS-R ($p<0.05$). We did not adjust for MDS treatment which is a potential limitation. However, the only therapeutic option that is associated with a long-lasting effect on transfusion intensity is allogeneic SCT, and thus we did not followed patients after allogeneic SCT. In the analysis of transfusion intensity the past year and association with survival, there are not many active therapeutic options later on in the disease course other than supportive care, which decreases the possibility of confounding, as elaborated on in paper II. The relationship between RBC transfusion need and the impaired outcome is not considered causal. It is rather complex and multifactorial, and yet unknown or unestablished effects are likely to constitute potential confounding factors.

A recent publication, indicate than in addition to the lower transfusion burden defined in the revised IWG criteria, a low transfusion burden of <3 units over a period of 16 weeks was associated with inferior PFS in patients with lower-risk MDS (127). When patients were compared with regard to their transfusion intensity one year after registration, a significant

difference of the PFS was observed between patients receiving <0.87 transfusions/month and those receiving >0.87 transfusions per month.

Hemoglobin data

Patient with MDS are commonly tested for their hemoglobin level. Hemoglobin measurements were included if being taken either within 2 days before an RBC transfusion or within the 28 days following a transfusion. We included 3,399 full transfusion episodes, where each episode consisted of one or more RBC transfusion with both pre and post-hemoglobin measurements. The numerous complete transfusion occasion is a strength of the study which enabled well powered groups even after stratification by five storage categories.

We added a term in the mixed model to acknowledge the varying time gap between post-transfusion hemoglobin measurement and the previous transfusion. We did not have information on possible bleeding, infection or hemolysis that could have affected the hemoglobin response. We tried to get around this issue by performing several sensitivity analyses. In the first sensitivity analysis, we excluded the full transfusion episode if follow-up hemoglobin was taken close to the transfusion, which could indicate pre-term testing because of bleeding or infection, for example. A fixed setting of appropriate stable patients without ongoing events that could bias the results could validate these findings.

Storage time

In study III, we aimed to investigate if storage time of RBC units affected the efficacy of the RBC transfusion with regard to hemoglobin increment post-transfusion. We chose to study this retrospectively in an MDS cohort who often have their hemoglobin level tested for, as described earlier. In our results, we found that prolonged storage was significantly associated with a lower post-transfusion hemoglobin increase, compared to short-time stored units. This finding has recently been confirmed in a large study of 23,194 transfusion episodes with linked pre and post-transfusion hemoglobin measurements (170). In our study, we observed gradually lower estimates of the hemoglobin increment post-transfusion with longer RBC storage. The Mixed regression model carefully accounted for varying time between the transfusion and the following hemoglobin measurement, patient characteristics and also allowed for different baseline hemoglobin between patients. Even though data were retrospective and we could not adjust for clinical events such as bleeding or hemolysis, the estimates gave proof of both statistical significance and stability, in five performed sensitivity analyses. Even though the effect was modest, with a hemoglobin increment up to 1.51 g/L lower per RBC unit, compared to short term stored RBC units less than 5 days, these findings could be of clinical relevance in some patients, for example in patients with a regular transfusion need.

RBC antibodies

The risk of alloimmunization (study IV) was estimated to 12.5% in the cohort of 455 patients. In the overall transfusion recipient (also including hematologic and oncologic patients) a risk

between 2-9% has been estimated (138, 139). Reported risk estimates of alloimmunization in MDS varies between 12-27% depending the transfusion burden at inclusion (7, 119, 145, 171). In other disease entities with chronic anemia and transfusion-dependency, for example sickle cell disease and thalassemia, the risk of alloimmunization is generally higher, ranging from 5 to 58% (8, 142, 146, 172, 173) with a few reporting even higher risk (65-76%) for sickle cell disease (174, 175). The great variation depends on the extent of RBC antigen matching, geographical area of the study indicating the homo/heterogeneity of RBC antigens between blood donors and recipients, and on chronic or episodic transfusion need.

For patients with hemoglobinopathies, and foremost sickle cell disease, the pre-transfusion testing includes RBC pheno or geno-typing and up-front matching of transfusion units for an extended panel of RBC antigens, beyond ABO and D to minimize the risk of alloimmunization. Several studies have confirmed reduces rates of alloimmunization by this strategy (140-142, 176, 177). Interestingly, despite of CEK-antigen matching (phenotyping), patients might still form Rh antibodies both in the setting where antigen-negative typed patients receive compatible antigen-negative units, and in recipients whose RBCs present a specific antigen and receive compatible units, most often due to Rh allele variants confirmed by genotyping, in patients with sickle cell disease (8, 178, 179). Genotyping was also performed in 43 regularly or episodically transfused MDS patients where mismatch or discrepancy for multiple antigens were observed in 39.5% of the patients, compared to the antigen profile that had been serologically matched for them (180). This highlight the advantage of genotyping recipients with an increased risk of alloimmunization but also donors. Current strategies in Stockholm and for the regional MDS cohort, genotyping is only performed during RBC antibody investigation if the patient is previously multitransfused.

In study IV, 6 patients (1 male, 5 females) had alloantibodies without a previous registered RBC transfusion. This could be explained by an RBC transfusion outside Stockholm or due to primary immunization during pregnancy in females. Since we didn't have information on previous pregnancy and there was a mix of females and males in this group, these patients were excluded from all analyses. This method probably attenuated the results where we investigated the time to first alloantibody between females and males, hypothesizing that female patients with a primary immunization due to previous pregnancy would have detectable alloantibodies earlier than patients that had not been immunized earlier.

The most common antibodies were directed towards Rh and Kell-antigens. The development of alloantibodies depends both on the mismatch of RBC antigen expression between donor and recipient but also on the immunogenicity of the antigen. In this cohort of MDS patients in Sweden, we did not have information about origin of the patients or blood donors, but the clinical experience from these patients, is that many are of Caucasian origin, like many blood donors, still we observed a relatively high rate of alloimmunization.

We performed two multivariate analyses to assess baseline risk factors separately and in another multivariate analysis include risk factors during follow-up. Female sex was the only baseline parameter associated with alloimmunization, confirming other published papers

(139, 181). In the second multivariable analysis, both female sex and a positive DAT test were significantly associated with alloimmunization. The observed risk factors are important to have knowledge on, but in the clinical setting, current information doesn't help us to distinguish which patients within the MDS cohort that are at greatest risk of developing alloantibodies.

The estimates of increased transfusion requirement after alloimmunization were significant. When we investigated the post-transfusion hemoglobin increment before and after alloimmunization, the results indicated a lower hemoglobin increment after alloimmunization, which support the results of an increased transfusion intensity following alloimmunization.

One concern is misclassification of patients without a positive DAT, since autoantibodies, detected with direct antiglobulin test, are not automatically tested some patients could be falsely classified as DAT negative. This is considered a non-differential misclassification with a risk of hiding a potential association. More important, patients with a suspect RBC antibody have DAT routinely tested during antibody identification in the blood bank, this however is a differential misclassification and could lead to inaccurate associations. The performance of a nation-wide study could better evaluate risk factors and potential differences between alloantibodies. To better understand the relationship between alloantibodies and DAT-positivity, and to evaluate the clinical relevance of increased transfusion intensity we propose a prospective study.

Transfusion efficacy

The mean normal lifespan of red blood cells in the human blood is 115 days (182, 183) but may vary between 70-140 days (184). There is a wide heterogeneity even in healthy individuals (185), before senescent cells are being removed by macrophages in the liver and spleen. One interesting aspects of transfusion efficacy is the observed impaired life-span of allogeneic RBCs in the circulation compared to autologous RBCs, although this study was performed in a pediatric setting (186). In addition, donor parameters and characteristics of the blood unit such as donor sex, whole-blood derived blood products or apheresis, RhD status, donor age and duration of storage and irradiated blood units, have shown effect on the transfusion efficacy, measured by hemoglobin increments after transfusion (170).

6 CONCLUSIONS AND CLINICAL IMPLICATIONS

This thesis encompasses a broad focus of both descriptive and analytic transfusion-related research questions mainly in a well-enumerated regional MDS cohort but also touch upon transfusion patterns and associated costs in patients with hematological malignancies overall.

Results from study I, will serve as an important reference for comparison, both with regard to transfusion patterns and costs in patients with hematological malignancies.

In study II, we observed several variables that were associated with an increased transfusion intensity. Prediction of transfusion needs in MDS patients could guide therapeutic decision-making.

In study III, we observed that longer duration of RBC storage was associated with a lower post-transfusion hemoglobin increment. The absolute estimates were modest, but this is an important observation which raises questions about possibly avoiding long-term stored RBC units to patients with a regular transfusion need.

In study IV, we observed an increased transfusion intensity following alloimmunization which warrants for further studies and discussions about upfront typing and matching beyond ABO and RhD, in patients with MDS.

7 POINTS OF PERSPECTIVE

Among generated hypothesizes and the many remaining research questions, I want to elaborate on two further studies aiming to explore the mechanisms of increased transfusion intensity.

In study II, we observed that mutations in genes involved in histone modulation and transcriptional regulation were associated with a higher RBC transfusion intensity. Specific mutations in these groups include ASXL1 and RUNX1, respectively, among a few others. In contrast, mutations in genes involved in the splicing machinery or DNA methylation, for example SF3B1 and TET2, were associated with a lower RBC transfusion intensity. We want to understand the mechanisms of increased transfusion intensity and why certain patient and disease-specific factors (male sex and ASXL1 mutation) give rise to a higher transfusion intensity, and propose that we can address these research questions by performing two separate prospective projects.

I) Hypothesis: Transfused cells are 'cleared' faster from the circulation in patients that harbor mutations in histone modulators, such as ASXL1 or in males. Method: In a clinical transfusion model, where flow cytometry can distinguish between allogeneic and the patients' own RBCs, study the survival of transfused RBCs and compare male sex with female sex, and ASXL1 mutation with SF3B1 mutation. This study could be performed by giving RhD negative units to RhD positive patients. Flow cytometry analyses and hemoglobin measurements would be analyzed on fixed time points before and after each transfusion episode. Another related question is proposed with regard to the proportion of endogenous RBC production versus transfused RBCs.

II) Hypothesis: Erythrocytes in patients with a ASXL1 mutation have more fragile RBCs with a reduced life-span. This might be observed by studying the sensitivity to storage lesions of the endogenous RBCs. Method: In an in-vitro model study if RBCs derived from MDS patients with specific factors (male sex, specific mutations, for example ASXL1) have an increased fragility and reduced life-span compared to erythrocytes derived from MDS patients with female sex and SF3B1 mutation. This could be done by studying the sensitivity to storage lesions of the endogenous RBCs. The method would include sampling of peripheral blood from MDS patients with ASXL1 and SF3B1 mutations as well as from healthy blood donors. The blood sample would be stored under normal blood banking conditions. Standard quality controls once weekly of hemolysis, hemoglobin content, lactate dehydrogenase, 2,3-DPG and ATP. In parallel performance of flow cytometry on samples from MDS patients focusing on the expression of phosphatidylserine and glycophorins, since RBCs from MDS patients have shown alterations of cell phosphatidylserine and glycophorin expression, which could suggest increased erythrocyte clearance by phagocytes and more rapid turnover in vivo.

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