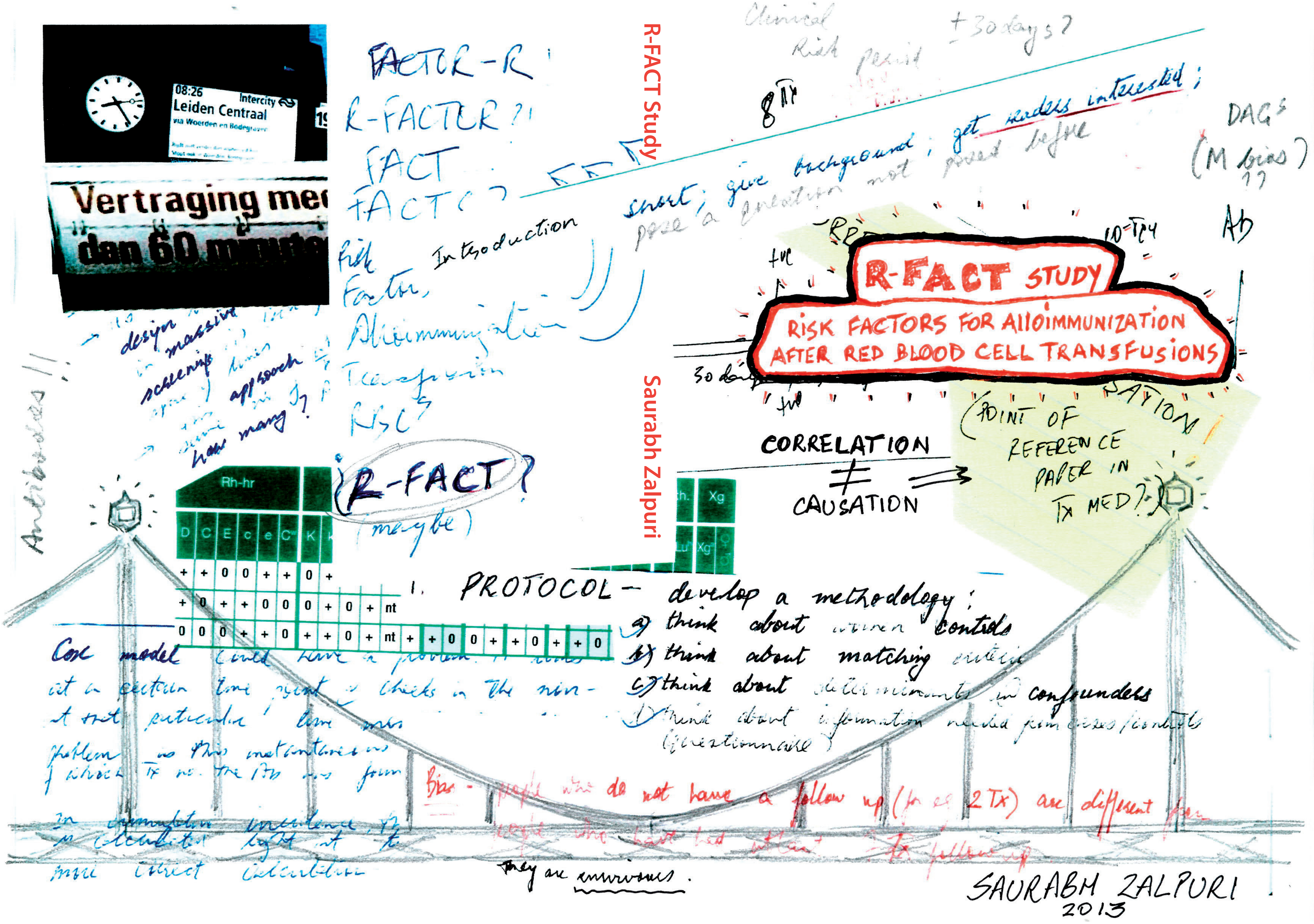

R-FACT Study
Saurabh Zalpuri
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Intercity
Leiden Centraal
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FACTOR-R
R-FACTOR?
FACT
Introduction
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Alloimmunization
R-FACT STUDY
RISK FACTORS FOR AllOIMMUNIZATION AFTER RED BLOOD CELL TRANSFUSIONS
CORRELATION ≠ CAUSATION
(POINT OF REFERENCE PAPER IN Tx MED?)
R-FACT?
(maybe)
Antibodies!!
design a massive screening approach
how many?
Rh-hr
Xg
1. PROTOCOL - develop a methodology:
a) think about controls
b) think about matching
c) think about confounders
d) think about information needed from cases/controls (questionnaire)
Cox model
Bias - people who do not have a follow up (for eg 2 Tx) are different
They are enormous.
SAURABH ZALPURI
2013

R-FACT STUDY:
RISK FACTORS FOR ALLOIMMUNIZATION AFTER RED BLOOD CELL TRANSFUSIONS

Methodology, Risk Factors and Challenges in transfusion medicine research

Saurabh Zalpuri

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R-FACT STUDY:

RISK FACTORS FOR ALLOIMMUNIZATION AFTER RED BLOOD CELL TRANSFUSIONS

Methodology, Risk Factors and Challenges in transfusion medicine research

Proefschrift
ter verkrijging van
de graad van Doctor aan de Universiteit Leiden
op gezag van Rector Magnificus prof. mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
ter verdediging op 11 juni 2013
klokke 11:15 uur
door

Saurabh Zalpuri

geboren te Chandigarh, India

In 1982

PROMOTIECOMMISSIE

Promotors:	Prof. J. J. Zwaginga, Prof. J. Vandenbroucke
Copromotor:	Dr. J. G. van der Bom
Overige leden:	Prof. A. Brand
	Prof. C. E. van der Schoot, Sanquin, Amsterdam
	Dr. J. Zimring, Puget Sound Blood Centre, Seattle

The work described in this thesis was performed at the department of Clinical Transfusion Research, Sanquin Research, Leiden and the Department of Clinical Epidemiology, Leiden University Medical Center, Leiden, the Netherlands. The research was fully funded by Sanquin Blood Supply, division Research as part of a full PhD project.

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CHAPTER 1

INTRODUCTION

Red blood cells are transfused with the intent to improve the oxygen carrying capacity of blood during and after a clinical event which has led to severe bleeding, or in case of other types of anemia leading to cardiovascular symptoms. Over half a million red cell concentrates are transfused annually in the Netherlands (source: Sanquin Blood Supply Annual Report 2011). With improvements in blood donation, storage and transfusion procedures, red cell transfusions have come a long way in terms of safety. Yet, donated transfused blood is a foreign, non-self tissue and therefore carries an intrinsic hazard for the recipient. One of these hazards is antibody formation. The aim of the studies described in this thesis was to examine the effect of transfusion related and patient specific risk factors on the occurrence red cell alloantibody formation (or alloimmunization). Because the design, analysis and interpretation of observational studies in clinical transfusion medicine present specific methodologic challenges, the methods of the studies are extensively discussed in several chapters of this thesis.

RED BLOOD CELL ANTIGENS AND RISK OF ALLOIMMUNIZATION

Red blood cells membranes have embedded carbohydrate, protein and lipid structures whose presence or absence is genetically determined. These so called blood group antigens, are mostly determined by single nucleotide differences in the encoding genes and, which as the name implies, define a person's blood group type. Including the commonly known *ABO* and rhesus *D* blood group systems (or, major blood group antigens), there are 33 recognized blood groups systems (or, minor blood group antigens- for example other rhesus antigens like C, E, c, e; Kell; Duffy; Kidd groups) carrying around 600 antigens (*Blood Transfusion in Clinical Medicine, 11th edition by P.L. Mollison*).

The distribution of minor blood group antigens (the focus of this thesis) varies in different populations: for example rhesus C- 68% of Caucasians, 27% blacks and 93% Asian are positive for this blood group. On the other hand, 29% of Caucasian, 22% of blacks and 39% of Asians are positive for rhesus E blood group type. Kell (K) is found in only 9% of Caucasians, 2% in blacks and up to 25% in Arab population (Reid ME and Lomas-Francis C. The Blood Group Antigen Facts Book. Second ed. 2004, New York: Elsevier Academic Press). Thus, quite a few of these antigens are rare and or have a skewed presence in different ethnic groups.

A non-self (or *allogenic*) blood group antigen comes in contact with the transfusion recipient's immune system mostly via a blood transfusion and pregnancy; the recipient's immune system may react leading to formation of antibodies against these foreign red cell antigens. These antibodies are called *alloantibodies* and the phenomenon- *alloimmunization*. Alloantibodies, which could cause such hemolytic reactions, are also called clinically relevant alloantibodies. Besides alloantibodies against incompatible transfusions and pregnancies, naturally occurring antibodies like IgM (immunoglobin M type) against the A and/ or B antigen occur in the absence of a previous red cell transfusion, previous pregnancy or an organ transplant (likely sources of red cell antigen exposure). Contact with such antigens in the environment that resemble non self red blood cell antigens (antigens located outside the

red cell membrane, or from other foreign substances such as bacteria) are held responsible for the formation of such naturally occurring antibodies.

Alloimmunization has various clinical consequences. Alloimmunization may present itself as an acute (within 24 hours of transfusion) hemolytic reaction or a delayed (more than 24 hours after transfusion) hemolytic transfusion reaction. Symptoms may include fever, rigors, nausea, hypotension, tachycardia, skin flushing, hemoglobinemia, hemoglobinuria and bleeding (*Blood Transfusion in Clinical Medicine, 11th edition by P.L. Mollison*).

In the Netherlands, close to 800 cases of alloimmunization against red cell transfusions are reported yearly, according to the Transfusion Reactions in Patients (TRIP) national hemovigilance office. This reported number could well be an underestimation since the reporting from hospitals is voluntary.

Each antigen differs in its immunogenicity, with the *ABO*, *D* and Kell (*K*) highly immunogenic whereas the majority are not. The ability to detect transfusion related (or from previous pregnancy, previous organ transplant) as well as naturally occurring antibodies leads us to avoid subsequent hemolytic transfusion reactions by selecting donors with red cells lacking the antibody targeted antigen. Determining both the red blood cell type of patient and donor in principle also allows matching of transfused donor blood to patient's blood type, and thus preventing alloimmunization in the first place. For example, the severely immunogenic antigen groups- ABO and rhesus D with significant percentages of the population that differ in their presence are as a standard tested for, both in patients that need blood and their donors. Transfusions are matched for these antigens; in other words patients thus receive red blood cell units that are compatible with their own ABO and D type. This is done not only to avoid a severe intravascular hemolysis mediated by the naturally occurring IgM antibodies, but also to avoid (further) (IgG) immunization against these antigens.

Such preventive measures before an allogeneic red cell transfusion hence consist of routinely matching the recipient's blood group type to the donor red cell unit. While A*BO* and D matching is practiced in all patients, additional matching is more sensible- a) for common, highly immunogenic antigens other than ABO and D like *K* and *e* antigens and b) in special patient populations with chronic requirement of blood transfusions (sickle cell anemia, thalassemia, auto-immune hemolytic anemia, myelodysplastic syndrome- *CBO richtlijn- Bloedtransfusie 2011*) as well as women in the reproductive age. The *Danger Model* theory of immune response[1,2] suggests that the extent of the immune response depends not only on the exposure to a foreign antigen itself, but also on the immune modulating conditions surrounding that exposure- transfusion related and co-existing patient clinical profile. Identification of patients or clinical conditions that are associated with high risks for alloimmunization and subsequently transfusing these high risk patients with more extensive matched blood could be the third most likely and most cost-effective strategy to prevent alloimmunization. A first step for this strategy would be to define the transfusion and clinical risk factors and in this way identify the patients with the highest risk for alloimmunization. In this respect, female sex, diabetes, solid malignancies and progenitor cell transplants

were shown to be associated with a higher risk of clinical alloimmunization[3]; and lympho-proliferative disorders and atherosclerosis with a lower risk of alloimmunization[3].

In studying clinical risk factors for an adverse event, (alloimmunization in this instance) case- control epidemiological study design (with its variants) and cohort design are logical and feasible study designs. A major pitfall of such case control study designs though is improper selection of a control patient population for comparisons with the case patients. It is essential that the control patients are a good representative sample of the population at risk for alloimmunization, or the source population. This less optimal sampling of control patients was a limitation of a study[3] which examined clinical risk factors (or predictors) of alloimmunization.

OUTLINE OF THIS THESIS

The scope of the work described in this thesis is to examine whether potential transfusion related and clinical risk factors modulate the risk of alloimmunization in a general, previously not transfused, non alloimmunized population of transfusion recipients. In these studies we emphasize the methodological aspects of observational research in clinical transfusion medicine.

Briefly, in chapter 2, we presented the design of our case- referent study as a benchmark for the rest of the thesis. We then aimed to study the risk of alloimmunization and the number of transfusions, using a new user cohort study design in chapter 3. The aim of the case- referent study conducted in chapter 4 was to examine storage time of red cells as a risk factor of alloimmunization. In chapter 5, we examined the intensity (or the dose) of red cell transfusions and the risk of alloimmunization. The effect of concomitant immunosuppressive medications as a clinical risk factor for alloimmunization was studied in chapter 6. Finally, in chapter 7, we aimed to highlight the distinction between etiologic and prediction observational research in clinical transfusion medicine.

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CHAPTER 2

RISK FACTORS FOR ALLOIMMUNIZATION AFTER RED BLOOD CELL TRANSFUSIONS (R-FACT): A CASE REFERENT STUDY

BMJ Open. May 2012;2(3)

Protocol Title:

R-FACT STUDY RISK FACTORS FOR ALLOIMMUNIZATION AFTER RED BLOOD CELL TRANSFUSIONS

Project Leaders/ Principal Investigators

1. *Dr. J.J. Zwaginga, M.D*
   Sanquin-LUMC Jon J van Rood Center for Clinical Transfusion Research
2. *Dr. J.G. van der Bom, M.D*
   Sanquin-LUMC Jon J van Rood Center for Clinical Transfusion Research

PhD Fellow

Saurabh Zalpuri, M.D

Principal Collaborators

1. *Prof. Dr. A. Brand, M.D*
   Sanquin Region South- West
2. *Dr. H. Schonewille*
   Sanquin, Region South- West
3. *Prof. Dr. E. Briët*
   Director, Sanquin Blood Bank
4. *Prof. Dr. J.P. Vandenbroucke*
   Department of Clinical Epidemiology

Sponsor

Sanquin Bloodbank
Plesmanlaan 1a, 2333 BZ, Leiden

Abbreviation List

RBC: Red Blood Cell
SNP: Single Nucleotide Polymorphism
HLA: Human Leukocyte Antigen
SES: Socio- Economic Status
EIN: Unit Identification Number
MEC: Medical Ethical Committee
CRF: Case Report Form
LUMC: Leiden University Medical Center

ABSTRACT

Introduction

Individuals exposed to red blood cell (RBC) alloantigens through transfusion, pregnancy, or transplantation may produce antibodies against the alloantigens expressed by RBCs. Although the incidence of these events is debated and ranges between percentages of 1-6% in single transfused patients and up to 30% in poly-transfused patients (e.g. sickle cell disease, thalassemia and myelodysplasia), they can pose serious clinical problems such as delayed haemolytic reactions as well as logistic problems e.g. to obtain timely and properly matched transfusion blood for patients in which new alloantibodies are detected.

Rationale

It is known that the risk of a recipient to develop antibodies depends on dose and route of administration and the immunogenicity of the antigen, as well as on genetic or acquired patient-related factors. The latter factors however are ill defined and therefore we hypothesize that the particular clinical conditions (e.g. used medication, concomitant infection, and cellular immunity) during which transfusions are given may contribute to the risk of immunization.

Research objective

Examine the association between clinical, environmental and genetic characteristics of the recipient of erythrocyte transfusions and the risk against immunization against erythrocyte alloantigens exposed to during that transfusion episode.

Study design

A nationwide multicenter case- cohort study.

Study population

Cases will be defined as first time alloantibody formers during the study period. Controls are defined as transfused individuals matched to cases and that have not formed an alloantibody.

1. INTRODUCTION

Individuals exposed to red blood cell (RBC) alloantigens through transfusion, pregnancy, or transplantation may produce antibodies against the alloantigens expressed by RBCs. Although the incidence of these events is debated and ranges between percentages of 1-6% in single transfused and up to 30% in poly- transfused patients (e.g. sickle cell disease, thalassemia and myelodysplasia[1]), they can pose serious clinical problems such as delayed haemolytic reactions as well as logistic problems e.g. to obtain timely and properly matched transfusion blood for patients in which new alloantibodies are detected. Of course, prevention of alloimmunization by extended matching between donors and all transfused patients (i.e. on the basis of typing patients for the most relevant RBC antigens) would be an ultimate but complicated and costly solution. However, matching of donors only for patients who are defined to have a high alloimmunization risk would be a more feasible step forward. This strategy would be especially valuable because as soon as immunization for one antigen develops, additional immunizations tend to develop more frequently[2,3].

Characterization of patients and clinical conditions with high immunization risk can be derived from studying the possible correlations between the actual immunization and patient related factors (both genetic and acquired) and/ or transfusion associated situations.

Such a study comparing immunized and non-immunized patients with a similar transfusion history will generate relative risk or relative protective factors.

We expect a two-fold impact from our study: a) to identify a set of transfusion recipients who need to be extensively matched, and b) to help understand the mechanisms underlying the development of alloantibodies to erythrocyte transfusion.

2. RATIONALE/ BACKGROUND

Alloantibodies can lead to serious clinical consequences and logistic problems like obtaining properly and timely matched blood for the patients who do develop these antibodies. Prevention of such serious events is possible by extended matching and typing of donor's blood against the patient's for all the possible antigens, but this process is cumbersome and costly. Identifying a high risk group will be a feasible first target and advanced matching a big step forward, and the aim of our study.

It is known that the recipient's formation of antibodies depends on dose and route of administration and the immunogenicity of the antigen, but probably also on genetic or acquired patient-related factors. It has been shown that the number of transfusions also play an important role in alloimmunization against red blood cell, with the risk increasing with the increasing number of transfusions[13]. It is generally recognized that immuno- compromised patients have a lower risk to develop such antibodies.[4] Relatively little is known, however, about other patient related risk factors.[2,3,5-8]

A recent study examined such patient related risk factors in a case-control study among 101 cases developing erythrocyte alloantibodies and 87 controls.[9] In this two-centre study, patients with first time detected antibodies and at least one transfusion in the past were

compared with controls with a negative antibody screening in the same centre. After adjustment for a limited number of confounders, this study confirmed known risk factors for antibody formation such as female sex (increased risk, since women are more susceptible to exposure of alloantigens during pregnancy, miscarriages, abortions and child birth[10]); lympho- proliferative disease and leukemia (lower risk attributed to lymphocyte dysfunction by concomitant chemotherapy and suppression of the immune response[11]). Also new and partly unexpected risk factors were found, such as diabetes and solid tumors (both increased risk). Although the latter patients do undergo chemotherapy as well, in this group antibodies might develop more easily because of their chronic inflammatory state[12]. The limitations of this case-control study[9], however, were i) the selection method for controls favored controls which had received more transfusions with also smaller transfusion time intervals as compared to the cases; ii) the relatively small number of patients reducing the detection of smaller relative risks; and iii) a relatively crude assessment of only a limited number of potential risk factors. Additionally the study design did not allow investigating the association with the actual factors at the time of the likely primary immunization / causal transfusion. We will not only try to confirm the observed potential risk factors in a larger cohort, but we aim to find other clinical, environmental factors as well as genetic factors. There is well documented evidence that certain HLA types are associated with enhanced response to red blood cell antigens like Kell, Duffy and Kidd[14-16]. HLA genes in this respect are particularly interesting because along with their polymorphisms, they have been shown to play an active role in autoimmune disorders and diseases which develop via T-cell mediated immunity.[17] Moreover, several of these genes have been identified in human studies to be associated with susceptibility and resistance to mycobacterial infection. Another strong correlation was shown between immunodeficient genotype (interferon gamma receptor 1 deficiency) and responsiveness to mycobacterium antigen.[18] Finally, specific SNP associations have been identified to play a role in viral immunity and variations in both humeral and cellular immunity following measles vaccination.[19,20] Although many genes are involved in the immune system, SNP's in genes (e.g. coding for HLA types) that modulate specific and innate immune responses will be of the first targets in our analyses. We hypothesize that this will yield genetic modulators on the patients' humoral response to particular erythrocyte expressed antigens but maybe even more broadly to other antigens as well.

By our questionnaire we will query environmental, life style factors and socio- economic status (SES) as those have been suggested to modulate the immune response. Environmental factors such as exposure to helminthic, fungal and parasitic infections do play a role in modulating the general set point of the immune response at young age[21]. The same is true for living in unsanitary conditions and for unhygienic occupations throughout life[22] Additional information on "immune modulating" conditions during childhood and youth will be collected from the vaccination status, completion of the vaccination programme, presence of pet animals, place of residence (urban/ rural) and visits to day care centers during childhood. The questionnaire will add to the knowledge to these possible confounders in cases and controls.

3. RESEARCH OBJECTIVE

The aim of the project is to examine the association between clinical, environmental and genetic characteristics of the recipient of erythrocyte transfusions and the risk of immunization against erythrocyte alloantigens that he/she was exposed to during that transfusion episode.

4. METHODOLOGY

4.1 Study Design and study population

We will perform a retrospective matched case- cohort study at hospitals nationwide from a period January 2005 to December 2011. Large red blood cell using hospitals will be selected as study bases. The study cohort will comprise of consecutive red blood cell transfused patients at the study center.

Cases are defined as first time ever irregular red blood cell antibody formers, with no prior history of red blood cell transfusions and alloimmunization before the study period.

Controls will be all consecutive transfused patients who had received their first and subsequent red blood transfusions at the study center with no prior history of red blood cell transfusions and alloimmunization.

Observational studies, if well conducted, are equipped to examine interesting transfusion research questions. With that in mind, we chose a case- cohort study design for our study. With the help of such a design, we can compare the cases occurring in a red blood cell transfused cohort with a randomly selected sample of the cohort. Using such an approach, for any one given case, we will select 2 controls that have had at least the same number or more transfusions than the case itself. This approach has following advantages:

This ensures that all the patients in the transfusion cohort with same or higher number of transfusions have an equal chance of being picked as controls. In essence, any member of the cohort who has been at a similar transfusion risk (of alloimmunization) at some point in their transfusion history can be selected as a control.

Cases also have an equal chance of getting selected as controls for other cases.

This study design minimizes the selection bias, if any. Such a study design allows us to include a number of patients which is sufficient to detect smaller effects and to adjust for other risk factors, as well as document potential risk factors extensively.

4.2 Matching

We will take in to account the number of transfusions a particular case received until the antibody forming episode, and match the 2 cases (selected per control) on the same number of transfusions.

To account for inter- hospital differences nationwide, we will also match the cases and controls on the site/ study center. (Figure 1)

4.3 Implicated Period

To examine the immune-modulating clinical risk factors surrounding the transfusions preceding the date of alloantibody formation, we will define a *clinical risk period* or an

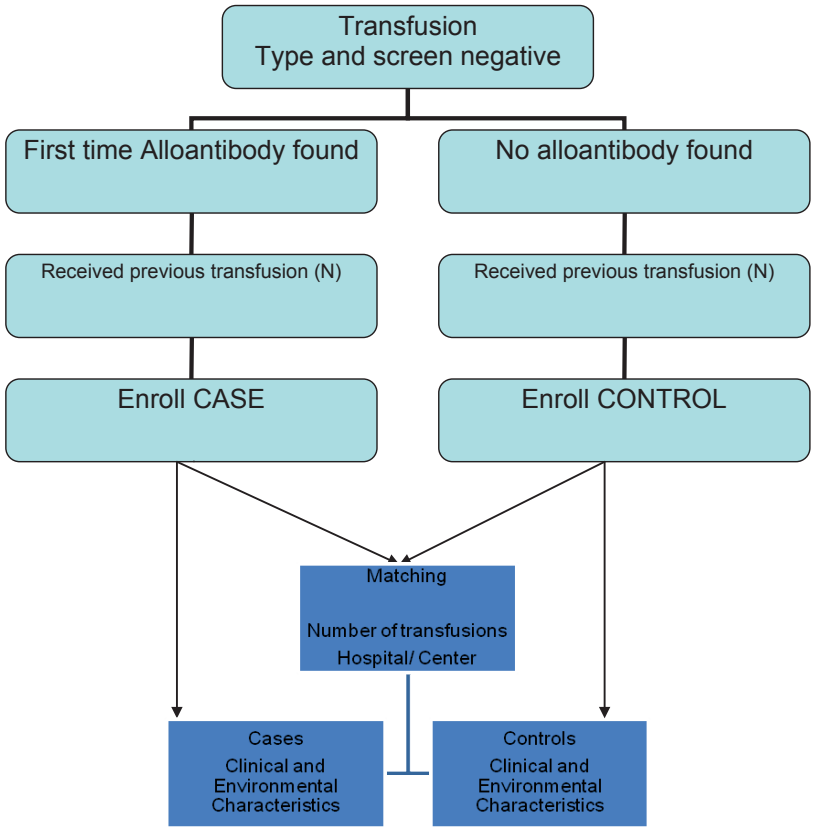

Figure 1. Flowchart of Study Design for the matched control group

implicated period of alloimmunization during which the case would have formed an irregular red blood cell antibody. This period would be the time (in days) between the date of a first ever positive screen for alloantibody to a calendar date 30 days before that positive screen. We will also introduce a lag period of minimum 7 days between that first ever positive screen and the last ever transfusion (*implicating transfusion*) before that positive screen. (Figure 2) This is to ensure that a patient's immune system has adequate time to respond to the transfusion exposure.

We will define a similar implicated period in the matched controls as well, retrospectively from the "*implicating transfusion*" to 30 calendar days back. (Figure 2)

4.4 First time formed alloantibody

Our endpoint for cases, or first time formed irregular red blood cell antibodies is defined as clinically significant antibodies as screened by a three cell serology panel at 37 degree Celsius. All patients were routinely screened for alloantibodies, which is repeated at least every 72 hours, if further transfusions as required. The antibodies are screened for by a three cell panel

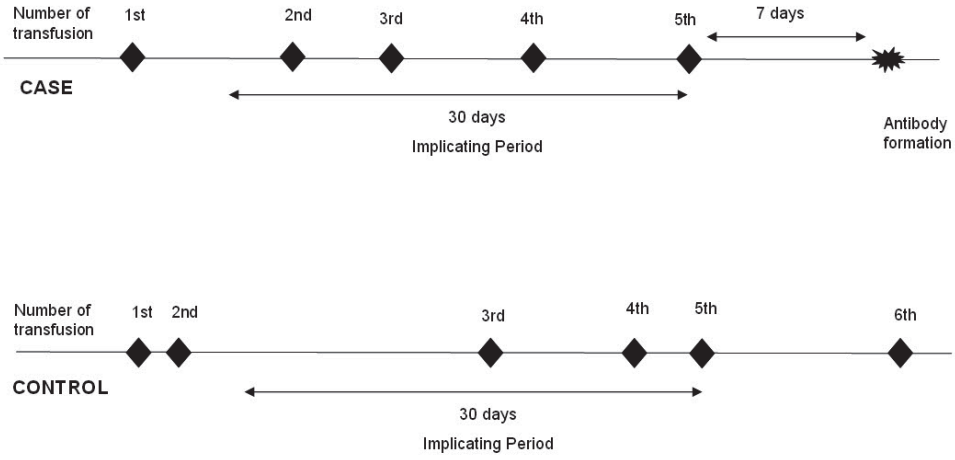

Figure 2. Implicating period of clinical data collection

including an indirect antiglobulin test (LISS Diamed ID gel system) and subsequently identifies by a standard 11 cell panels in the same gel system.

4.5 Data acquirement, measurements and handling

Transfusion cohort data will be acquired from the hospital blood transfusion services and on site patient records. Second we will use data from a patient questionnaire. Thirdly, we will determine the patients' racial background from blood of the included and consenting patients.

4.3.1 Patient Medical history and records

Potential clinical risk factors include hematological, oncological, surgical and medicinal data as well as auto- immune diseases and related conditions at the time of the implicated (likely causal) transfusion. Factors and conditions that will be actively scored are, infections (including the causal microorganisms) and active / chronic allergies (including the if known antigens), fever, cytopenia(s), systemic inflammatory response (a clinical response to a (non)-specific insult of either infectious or noninfectious origin), peripheral blood progenitor cells transplantation (autologous or allogenous), multi trauma, splenectomy, solid malignancies, autoimmune disorders (rheumatoid arthritis, diabetes mellitus type 1 etc.), chemotherapy, immunosuppressive drugs, cytostatics and antibiotics will be studied.

4.3.2 Questionnaire

Participants will be asked to fill out a printed questionnaire. The participants have also the option to fill in a web-based questionnaire, which will be accessible via a link provided in the information letter. After identification of control patients a similar mailing will be sent to these controls

Environmental and life style factors like vaccination status, previous pregnancies in case of females, level of education and current professions (as a proxy for socio- economic status)

will be obtained via the patient information questionnaire. The questionnaire will add to the knowledge to these possible confounders in cases and controls.

In general, many questions will involve "life-time" risk factors and information and are not particularly targeted at the time of implicated episode.

Racial confounder

Based on the knowledge that different ethnicities have varying frequencies of erythrocyte antigens, a so- called mismatch between a donor from one particular ethnicity and the recipient of another ethnicity does play a role in developing immune response to donor erythrocytes. Therefore, we will also attempt to document racial mismatch leading to red blood cell alloimmunization. This is attempted by one question in the questionnaire but will foremost rely on the blood group typing which usually determines the ethnicity.

Blood research and sampling

To investigate the effect of genetic factors on the risk of the development of alloantibodies, we will collect blood samples from all participants for extensively typing the blood to get an antigen profile and to look at genetic markers which influence immune system and vaccination efficiency. SNP's in candidate genes (e.g. coding for HLA types) modulating specific and innate immune responses will be assessed. Biomarkers typical for the activity of the immune response: cytokines and titers of antibodies against common (vaccinated) antigens can later be determined in the plasma and serum that are stored as well.

4.6 Statistical analysis

We expect to include a total of 500 case patients and 1000 controls.

Logistic regression models will be used to assess the association between the risk to develop antibodies and potential risk factors, adjusted for other risk factors and for the number of exposures to the antigen.

We will examine the association between the risk factor and alloimmunization using logistic regression.

We will also make a selection of all cases and controls on the most frequently found antibodies and if the relative impact of risk factors and immune modulators on the risk of all the antibody types(in separate analysis) is in the same direction, we will make a generalized observation.

With 1500 patients, and the conventional 80% power and a p-value of 0.05, we will be able to detect effects (odds ratio) of dichotomized risk factors of 1.35 or higher.

An additional analysis will be performed along the lines of a "case-crossover" design within the case patients. The "Hazard Period" (time period right before the detection of a positive antibody) will be compared to a "Control Component" (a specified time period other than the Hazard Period) in the case patient's medical history and the risk ratio for the transient effect risk factors will be calculated.

5. ETHICAL CONSIDERATIONS

5.1 Regulation statement

The study will have a multicenter design subjecting patients to a questionnaire and additional blood sampling. After approval by the central MEC of the LUMC, the study clearly requires a local Medical Ethical Committee approval for each site that detects an probable transfusion mediated alloimmunization. Help of local investigators, usually the local hematologist or clinical chemist in charge of the transfusion laboratory will be recruited to substantiate implementation of the study at the various sites. Each local investigator will in fact be responsible for ensuring that the study will be conducted in his centre in accordance with the protocol, the ethical principal of the Declaration of Helsinki, current ICH guidelines on Good Clinical Practice and applicable regulatory requirements.

5.2 Recruitment and Consent

Data will be collected at each hospital site, Sanquin and from medical records and files. All data will be coded for privacy reasons. As said, after identification of cases and controls, patients will be sent a short and concise letter and an information brochure explaining the purpose of the study. This letter will be combined with the questionnaire and foremost – an answer card expressing willingness or refusal to participate in the study to fill in and return to the study's contact address. Participants, moreover, will have an option of filling in the questionnaire via the study's website. The web link access will be explained in the patient information. After receiving a patient's positive response to our request to participate, a follow- up call will be made by the investigator to answer any additional queries and if applicable to make an appointment for the blood taking. The patients would be invited to LUMC or the participating centers for blood taking. Additionally to the signed answer card for blood taking, patients would be informed about the study once again at the blood taking appointment, and a final consent form in presence of the study coordinator and data manager will me signed before the blood taking commences. Proper tubing and transfer material will be provided to the non- LUMC sites.

5.3 The patient burden

The reading of the information and completing the questionnaire (estimated to take about 10 minutes) will be of minimal patient burden or stress and is absolutely voluntary. Apart from the questionnaire, the protocol involves a single blood sampling of 25 ml as main discomfort for cases and controls. However, the blood taking will preferably be combined with a regular control and if possible a blood sampling.

The blood taking will be organized centrally at the LUMC upon invitations. There are no further interventions within the study protocol. The study has absolute minimum invasive risk for the patients.

5.4 Medical information, data and sample handling and Reports

1. Per patient an electronic Case Record Form (CRF) with a unique study number (identifier) will be made. The CRFs will be subjected to independent data management. The principal investigators Anske van der Bom en J.J. Zwaginga, will be responsible for the CRF and data management.
2. Patient-identifying parameters such as name, the hospital patient number and the full birth date will not be entered and found in the electronic CRF. The key between these identifying data and the unique study number will be only available to the data management at the department of Epidemiology. These patient identifying parameters are only needed for sending the questionnaire and making an appointment for blood taking which will be done by the data management. The blood taking and further sampling will involve relabeling of the tubes to the specific study number.

There will be a provision to keep the patient personal details for the entire duration of storage of blood samples, with a possibility to track back and indentify the patients with their blood samples. Coding measure will ensure that this information is not available to a third party and is only accessible via an encoding key to the principal investigators of the R- FACT study. Individual medical and investigational information obtained during the study is considered confidential and disclosure to third parties is prohibited. The described strategy will guarantee effective study of data together with maintaining optimal patient privacy.

The blood samples will be stored in state-of-the-art storage facilities at the LUMC, with storage management software for 20 years.

The research, patient information, blood sampling and storage will be conducted in accordance with LUMC's Good Research Practice guidelines.

5.5 Withdrawal of individuals

Subjects can decide to have their samples removed from the serum, plasma, DNA and RNA bank and thus from further research in the future at any time and for any reason, i.e. meaning without consequences for their further clinical treatment.

5.6 Independent physician

Before consenting, patients can gather information or advice from the investigator but also from an independent physician: Tanja Netelenbos, MD. This name will be provided in the patient information.

5.7 Objection by minors or incapacitated subjects

Not applicable

5.8 Group related risk assessment and benefits

Not applicable

5.9 Incentives

Not applicable

6. ADMINISTRATIVE ASPECTS AND PUBLICATION

6.1 Handling and storage of data and documents

Data handling will comply with the Dutch Personal Data Protection Act.

A data-manager (employed on the project) and the PhD fellow will extract data from the study sites, and recode patients and locations to unique study codes under which non-patient identifying data are filed in a CRF per patient.

There will be no specific physical CRFs because of the massive patient / control numbers and electronic data sets can be often automatically extracted from the patient information systems present in most hospitals.

6.2 Amendments

All amendments will be notified to the MEC that gave a favorable opinion.

6.3 Annual Progress Report

The investigators will submit a progress summary of the study to Sanquin as sponsor of the study regularly. Information on inclusion of cases and controls, other problems and amendments will be provided as required by the regional and local MEC's.

6.4 End of the study report

The investigator will notify the accredited MECs of the end of the study within a period of 8 weeks. The end of the study is defined as the last data collected from medical records and case- control questionnaires.

6.5 Public disclosure and publication policy

The final publication of the study results will be written by the study coordinator(s) on the basis of the statistical analysis performed. A draft manuscript will be submitted to all co-authors for review. After revision the manuscript will be sent to a peer reviewed scientific journal.

Any publication, abstract or presentation based on patients included in the study must be approved by the study investigators and collaborators.

7. EXPECTED RESULTS

Our case-cohort study will quantify and characterize risks of patients and conditions for transfusion associated alloimmunization. Although a prospective serology study involving a first transfused cohort, would be most preferable to add to the insight in primary immunization (risk). However, 50% of first transfused patients never need new blood again, and escape follow up if not recalled. Moreover, the occurrence for the other 50% of the following

transfusion period is quite variable. Therefore, a prospective study is viewed as cumbersome. On the more practical side for a case control study, 50% of the transfused patients have been transfused before and these in principle are eligible as case or control patients. Indeed in accordance by the rules for inclusion these patients are already transfused at two different periods at least. Therefore, if we can define risk factors for alloimmunization then advanced matching of blood donors for this group should be regarded as valuable. Finally, strong synergy will be obtained between our study and the MATCH study by Schonewille et al. In the latter study, logistical / cost/ and benefit aspects of advanced matching after formation of a first antibody will be determined

Our study will contribute to classifying patients who could benefit from additional or extended typing and donor matching to prevent alloimmunization. We envision to contribute to a matching policy based on a prognostic risk score for immunization in general transfused patients.

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CHAPTER 3

RED BLOOD CELL ALLOIMMUNIZATION AND NUMBER OF RED BLOOD CELL TRANSFUSIONS

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Saurabh Zalpuri[1,2], Jaap Jan Zwaginga[2,3], Saskia le Cessie[1,4], José Elshuis[3], Henk Schonewille[2], Johanna G. van der Bom[1,2]

[1] Department of Clinical Epidemiology, Leiden University Medical Center; [2] Sanquin Blood Supply, Department Research, Leiden and Jon J. van Rood Center for Clinical Transfusion research, Leiden University Medical Center, Leiden; [3] Department of Immuno- hematology and Blood Transfusion, Leiden University Medical Center, Leiden; [4] Department of Medical Statistics, Leiden University Medical Center, Leiden;

Correspondence: Johanna van der Bom; Postbus 9600, 2300 RC, Leiden University Medical Centre, Leiden, The Netherlands; Email: j.g.van_der_bom@lumc.nl

Key words: Alloimmunization, general population, red blood cell transfusion, incidence, irregular antibodies

ABSTRACT

Background

Patients receiving red blood cells may form antibodies against the alloantigens expressed by red blood cells, with the risk of serious morbidity and the need for extensive phenotype-matching in subsequent transfusions. The incidence of alloimmunization is considered variable for specific patient groups and for first time antibody formation. We therefore studied the cumulative incidence of the first formed alloantibody as a function of red blood cells exposure.

Methods

We performed a new user cohort among all previously non-transfused non-alloimmunized patients that received non-extended matched (ABO and RhD) red blood cells transfusions from January 2005 to December 2009 in our university medical centre. Alloimmunization incidences were estimated by Kaplan-Meier survival-analysis.

Results

A total of 3002 previously non- transfused patients received 31,103 red blood cell units. A first time alloantibody forming event was experienced by 54 (1.8%) patients. The cumulative incidence of alloimmunization was 1.0% at 5 units, 2.4% at 10 units, 3.4% at 20 units, and 6.5% at 40 units of red blood cells transfused.

Conclusion

The risk to develop a first red blood cells alloantibody increases up to the 40th transfusion and is similar for men and women. More data is needed to examine the risk after 40th transfusion.

INTRODUCTION

Patients exposed to red blood cell alloantigens by transfusion, pregnancy, or transplantation may produce antibodies against alloantigens expressed by red blood cells. These can cause acute and delayed hemolytic transfusion reactions and potentially serious morbidity and even mortality. Additionally, alloimmunization makes more extensive matching of subsequent transfusions necessary.

Transfusion mediated alloimmunization could in theory be prevented by exact matching of all donor blood to the recipient's phenotype. Implementation of such a strategy, however, will be laborious and cost- consuming and its logistical burden may hamper the timely availability of matched blood for patients.

For some transfusion populations for e.g. sickle cell anemia and thalassemia, matching for Rh and K antigens to reduce the high immunization incidence associated with these diseases is often performed in most high income countries. We could maximally prevent alloimmunization by administrating matched blood to patients that are particularly at risk for alloimmunization, based on their clinical and transfusion risk factors. To estimate the feasibility of such an approach, an accurate antibody incidence measurement is an essential starting point. The reported frequency of red blood cell alloimmunization, however, varies considerably from 2 to 21%.[1-6] Besides, differences in study design and populations studied, the exact number of received transfusions before antibody formation is often unknown or poorly documented in many studies. Increased exposure to red blood cell antigens i.e. more red blood cell transfusions are likely to cause a higher risk for alloimmunization. Also, other studies included patients with already formed antibodies [7], or patients receiving large amounts of transfusions due to their predisposing indications [6, 8, 9].

Considering that red blood cell transfusions likely determine exposure to alloantigens and the risk for subsequent antibody formation and that preventative matching will alter the observed alloimmunization risk, we aimed to study the incidence of first alloantibody formation due to only ABO and D matched red blood transfusions in a general patient population from their first transfusion onwards. We also studied the incidence among females in the reproductive age for whom red blood cell transfusions are additionally matched for K antigen.

METHODS

Study Design and study population

We performed a retrospective incident new user cohort study among consecutive transfused patients at the Leiden University Medical Center (LUMC), the Netherlands from January 2005 to December 2009, to study adverse events (alloimmunization to red blood cell transfusions) among first time red blood cell transfused patients in an electronic patient follow up database. [10, 11] Eligible were all patients who received their first ever red blood cell transfusion within the study period in our centre. Transfusions were included only if they were preceded by a negative antibody screen and followed by a post transfusion antibody screen.

Patients who routinely received Rh phenotype (C, c, E and e) and K red blood cell transfusion, e.g. haemoglobinopathies and women who received intra uterine transfusions (IUT) were excluded. We also excluded infants under 6 months of age because they are presumed to have a reduced capacity to form red blood cell antibodies as their immune system is not fully developed [12-14]. According to Dutch transfusion guidelines (24), apart from ABO and RhD, K antigen blood group matching is mandatory since 2004 for female patients in the reproductive age (≤45 years). We did not exclude women who received ABO, RhD and K matched blood but analyzed them separately from the main cohort.

First time alloantibody

The endpoint of our study was the first time post-transfusion formation of clinically significant red blood cell alloantibodies as screened by a 3 cell serology panel at 37 degree Celsius, in patients with prior negative red blood cell antibody screens. All patients were routinely screened for alloantibodies before transfusion which is repeated at least every 72 hours, if further transfusions are required. The antibodies are screened for by a 3 cell panel including an indirect antiglobulin test (LISS Diamed ID gel system, Murten, Switzerland) and subsequently identified by standard 11 cell panels in the same gel test system. Additional techniques such as Poly-ethylene glycol, Bovine serum albumin and enzyme method were used when required. Non-red blood cell transfusion induced antibodies (5 cases of anti-D by passive acquisition), antibodies against low-frequency antigens that are not routinely present on antibody screenings panels (12 cases of anti Wr^a) and cold-reacting (12 cases) antibodies were not classified as endpoints.

Data acquisition

All data on antibody screening, antibody identification and transfusions were routinely recorded in the laboratory electronic data system General *Laboratory* Information *Management System* (GLIMS). We gathered transfusion dates for every transfused red blood cell unit, dates and results of antibody screens, antibody specificity, dates of birth and gender of all transfused patients.

Statistical analyses

The incidence of alloimmunization was estimated using Kaplan-Meier survival tables.

The cumulative transfusion exposure expressed as cumulative units of red blood cells transfused was used as the time axis.

We calculated incidences of new antibody development among all males and females older than 45 years. In addition, women under 45 years of age who had received K-matched red blood cells were analyzed separately. The association between sex and age of the patients and incidence of alloimmunization was also assessed and presented with log rank test p values.(significance level $p<0.05$)

RESULTS

After careful exclusion of patients that had received transfusions that had extended antigen matching, infants and patients without post-transfusion antibody follow-up (Table 1), the study cohort comprised of 3002 patients who had received a total of 31,103 red blood cell transfusions in the 5 year study period with a median of 6 units per patient (range 1-133). The main cohort comprised of almost twice more men than women, since the women with child bearing potential (under 45 years) were analyzed as a separate cohort on account of receiving transfusions that had limited antigen matching. After the first transfusion the median antibody follow-up period was 60 days and comparable for men (55 days) and women (69 days); (log rank p=0.12) (Table 2).

Table 1. The study population with the excluded number of patients

	Patients excluded	Patients in study
Transfused patients		8629
Haemoglobinopathies	100	8529
Intrauterine transfusions	49	8480
Infants <6 months of age	1089	7391
Patients with pre-transfusion positive screens	127	7264
Patients without antibody follow-up after a single transfusion event	3752	3512
Women ≤45 years of age (analyzed separately)	510	3002

Table 2. Transfused patient characteristics

Patient characteristics	Men	Women >45 yrs	Total	Women ≤45 yrs
Number of patients (%)	1929 (64)	1073 (36)	3002	510
Age in years (median, IQR)	60 (44, 70)	65 (56, 74)	62 (51, 71)	30 (15, 37)
Cumulative units	20,960	10,143	31,103	5093
Units per patient (median, IQR)	7 (4, 13)	6 (4, 11)	6 (4, 12)	6 (3, 12)
Follow-up period (median, IQR)[1]	55 (13, 256)	69 (14, 287)	60 (13, 266)	147 (22, 527)
Patients forming first antibodies (%)	36 (65)	18 (35)	54	7
Sensitization frequency (%)	1.9	1.7	1.8	1.4

[1] Follow-up period in days from first transfusion to last antibody screen in non-immunized patients and to first positive antibody screen in immunized patients.
IQR – Inter quartile range

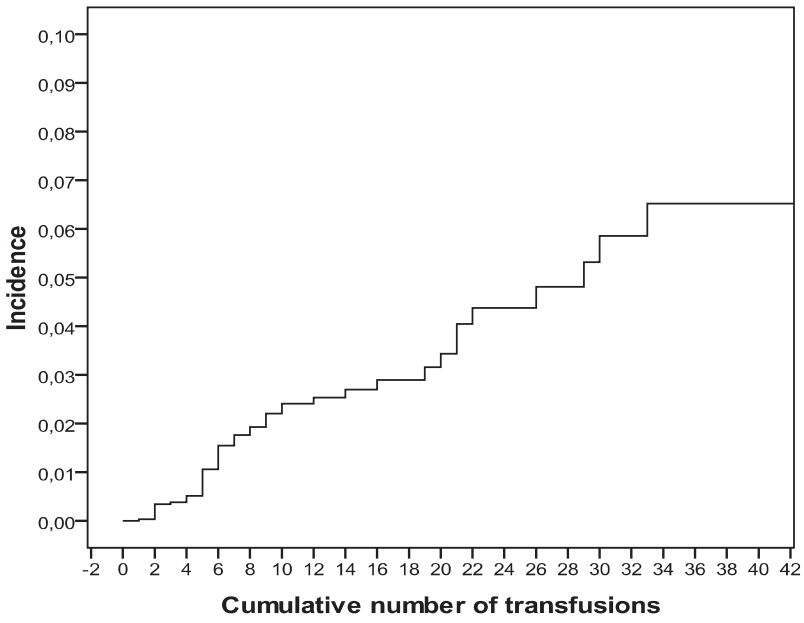

Figure 1. Red blood cell alloimmunization incidence in general red blood cell transfused population according to transfused units

Addendum to figure 1
Number of patients at risk at various cumulative transfusion units:

Number of units	Patients at risk
5	1830
10	953
20	370
40	94

In 54 patients (1.8%) 69 clinically significant antibodies were detected (Table 3). Single antibody specificities were found in 40 patients and multiple specificities in 14 patients. Multiple antibody combinations occurred with Anti E in 13 of 14 cases. Patients who developed antibodies had received a median of 6 red blood cell units (IQR 4, 10) and non-antibody formers had had a median of 6 red blood cell units (IQR 3, 11) units. Among women aged less than 45 years 7 women (1.4%) formed 9 alloantibodies (Table 3). We recorded 6 men and 2 women with a first time alloantibody formed in less than 2 weeks of their first transfusion at our center.(3x anti-Jk^a, 2x anti- E, 2x anti- Le^a and 1x anti-M). one male patient formed an anti-C^w after receiving 66 red blood cell units in 8 transfusion episodes.

The eventual cumulative incidence of alloimmunization as a function of exposure to the number of units, amongst 3002 red blood cell recipients was 1.0% at 5 units, 2.4% at

Table 3. Specificity and frequencies of antibodies among routine (ABO-D) and extended (ABO-D, K) matched patients between 2005-2009 at LUMC, Leiden

Alloantibody specificity	ABO, D matched			ABO, D, K-matched
	Men	Women	Total	Women ≤45
E	17	10	27	4
K	10	5	15	0
Jk^a	4	2	6	1
C^w	4	1	5	0
M	3	1	4	0
C	1	1	2	0
c	1	1	2	3
S	0	1	1	1
Fy^a	1	0	1	0
Le^a	2	0	2	0
e	2	0	2	0
Lu^a	1	0	1	0
Kp^a	1	0	1	0
Total	47	22	69	9

10 units, 3.4% at 20 units and 6.5% at 40 units of red blood cells transfused and was comparable for men and women (Fig. 2 and Table 4). In women aged less than 45 years (n=510), who had received K-matched transfusions, the incidence was 0.8% at 5 units and 2.5% after 10 units of red blood cells transfused (Fig. 2, Table 4). No association of age, categorized as young (<= 30 years), middle (> 31-60 years) and old (> 61 years) was found in the total study population with incidence of alloimmunization (log rank p= 0.18). Comparing men under 45 and over 45 years of age, again no association between age and alloimmunization was noticed. (Log rank p= 0.5)

Table 4. Antibody incidence according to number of transfused units in different transfusion recipient populations

Number of transfused units	Incidence of alloimmunization (95% CI)			
	Men (n=1929)	Women >45 years (n=1073)	All patients (n= 3002)	Women ≤45 years (n=510)
5	1.0 (0.9-5.6)	1.2 (1.1-8.4)	1.0 (0.9-3.8)	0.8 (0.7-20)
10	2.4 (2.1-5.7)	2.4 (2.3-9.7)	2.4 (1.8-4.2)	2.5 (2.4-16)
20	3.4 (3.1-8.0)	3.5 (3.3-15)	3.4 (2.6-5.9)	2.5 (2.4-16)
30	6.5 (4.9-11)	5.0 (4.9-21)	5.8 (4.4-9.1)	-
40	6.5 (4.9-11)	7.4 (7.1-24)	6.5 (4.9-9.9)	-

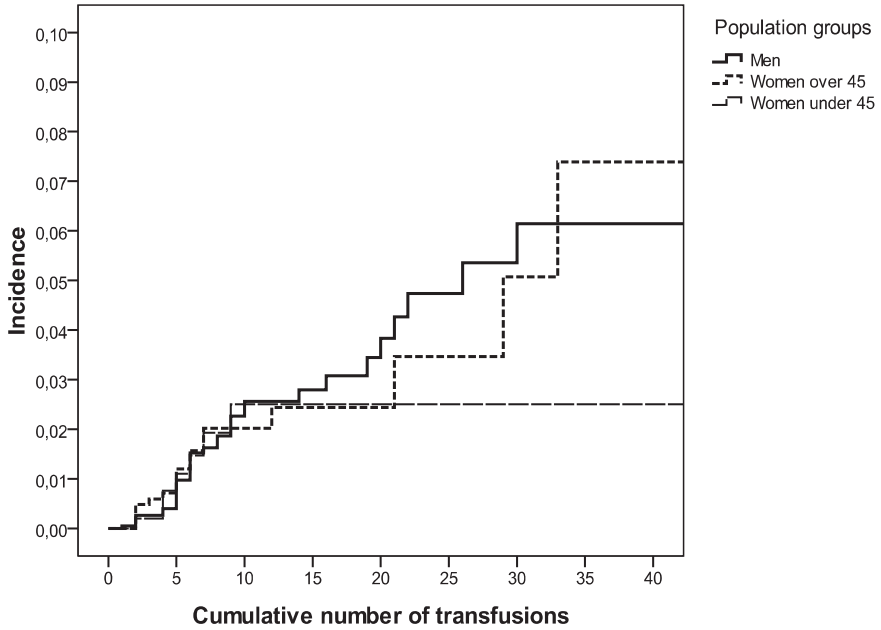

Figure 2. Red blood cell alloimmunization incidence in men, women >45 years (ABO, D matched) and women ≤45 years (ABO, D and K matched) according to transfused units

DISCUSSION

Our study, in general non-transfused and non-immunized transfusion recipients, demonstrated that the risk of first time alloimmunization to red blood cell antigens increases to 6.5 percent with the number of red blood cell transfusions up until 40th unit. This immunization rate was comparable for men and women over the age of 45 years as well as among different age groups. Young women who additionally received K- matched transfusions showed an immunization risk of 2.5% at the 10th transfusion, which was comparable to men and older women who received equal amounts of only ABO-D matched transfusions.

The alloimmunization risk to red blood cell antigens is debated and is suggested to be dependent on other factors such as the population studied, gender and genetic background. The number of transfusions however, seems most likely to have a major influence [7, 9, 15, 16]. Indeed, the incidences reported in several studies on transfused patients seem to indicate that the risk of alloantibody formation rises with an increasing number of transfusions. In sickle cell disease patients, Sarnaik et al. reported alloimmunization frequencies up to 11.5 percent, with increasing number of red blood cell transfusions [15], Reisner et al reported a 10 percent immunization rate in less than 50 units transfused [17] and in another study the rate of alloimmunization increased exponentially with higher numbers of transfused units [9]. In addition, Hoeltge et al showed, in a general transfused population, that the number of antibodies in a patient was positively correlated with the mean number

of red blood cell transfusions [16]. In these studies either the antibody specificities were not reported or clinically not- significant antibodies were included. Fluit et al, studying antibody specificities comparable to our study, reported an increase in first immunization frequencies from 4 percent before the 10th unit to 14 percent before the 40th unit.[7]. In lesser transfused populations (medical, obstetrics, trauma, elective surgery patients), as compared to the heavily transfused groups mentioned above, prospectively studied alloimmunization was described between 3 percent after a maximum of 24 units [18] and 8 percent after a maximum of 10 units [19]. In these two studies, however, more than 30 percent of patients had a pre-study transfusion history. The fact that our study comprised of a non-transfused population, likely explains our overall lower immunization rate. Seemingly in contrast with our findings, a recent study addressing this issue proposed that the number of transfusions was only a weak determinant of alloantibody formation [5]. In the latter study however, it is unclear how the patients with hemoglobinopathies and infants were dealt with. Such patients, along with patients that have previously formed antibodies – who were documented in the study- are likely to be receiving broader matched blood. Matching on more blood groups will decrease alloimmunization risk. Compared to other studies, we specifically excluded patients who had received extended matched blood for various chronic conditions. In addition, patients with previous alloimmunization were excluded, because of their enhanced immune response against donor red blood cell alloantigens compared to first antibody responders [20, 21].

In our data, we show that the association of the immunization rate and the number of transfusions is not weak, and increases up to 40th transfusion.

Finally, our study also showed a lower alloimmunization frequency among women aged less than 45 years of age, compared to the general female population (1.4 percent vs.1.7 percent), and an immunization rate of 2.5 percent after 10 units. The effectiveness of the current matching policies can be seen in these women where no K antibodies were observed.

A strength of our study was using the incident new user cohort study design, that reduces the investigator error as well as avoids selection bias, without compromising on study validity. [10, 11] Our data collection approach allowed a prospective follow up of previously non-transfused and non-immunized patients during subsequent transfusions up to the appearance of a first alloantibody. We thus feel that this cohort represents the general transfused population and is appropriate to study the incidence of first time alloimmunization. Another strength of the study is that the incidence of alloimmunization increased with the increased number of units despite the median and range of red blood cell units being relatively similar among men, women > 45 years and women < 45 years of age.

Although we did exclude all patients who had received transfusions before 2005, there could be a possibility that the patients entering the cohort have had transfusions prior to the start of study period in other hospitals.

In addition, the incidence of alloimmunization, in the antibody forming patients as well as in the patients without observed alloimmunization in our study, may be under-estimated, because antibodies can stay undetected due to variable follow up intervals after transfusion, e.g. between 1 and 1825 days in our study. Moreover, the time that is needed before

antibodies can be detected differs and once formed they can disappear again, depending on antigen types [22]. This inherently will lead to a number of undetected alloimmunizations and thus, our presented results could be an underestimate of actual incidence.

Our data being retrospectively acquired unfortunately did not allow for a comprehensive check on previous pregnancies. But we did exclude women receiving Intra Uterine transfusions and pre-transfusion antibodies. Additionally, we also analyzed women under 45 cohort separately and it did not show different results. Pregnancy induced antibodies can lead to an overestimation of transfusion induced first time alloantibody respondents. We, however, feel that this potential problem plays a minor part in our study.

Alloimmunization to red blood cell transfusions is the single largest transfusion adverse reaction category reported in the Netherlands every year, since 2002 (23). Accurate incidence figures of alloimmunization provide a good platform to further study clinical implications and environmental risk factors of all immunization.

We conclude that the risk to develop alloantibodies increases with the cumulative number of red blood cell transfused units. This risk increases at least up to 6.5% at 40 transfusions and does not differ for men and women. We were not able to find a plateau of sensitization and even beyond 40 units, there maybe be more alloimmunization taking place. More data is required to examine the risk of alloimmunization after more than 40th transfused unit. (addendum to figure 1)

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CHAPTER 4

EFFECT OF STORAGE OF RED BLOOD CELLS ON ALLOIMMUNIZATION

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Saurabh Zalpuri[1,3] MD, MSc, Henk Schonewille[1] PhD, Rutger Middelburg[1,3] PhD, Leo van de Watering[1] MD, PhD, Karen de Vooght[4], James Zimring MD, PhD[5], Johanna G. van der Bom[1,2,3] MD, PhD, Jaap Jan Zwaginga[1,3,6] MD, PhD

[1] Center for Clinical Transfusion Research, Sanquin Blood supply, Leiden, Netherlands; [2] Department of Clinical Epidemiology, Leiden University Medical Center, Leiden, Netherlands; [3] Jon J van Rood center for Clinical Transfusion research, Leiden University Medical Center, Leiden, Netherlands; [4] Blood transfusion, pediatric and point of care, Clinical chemistry and hematology laboratory, University Medical Center Utrecht, Utrecht, Netherlands; [5] Puget Sound Blood Center Research Institute, Seattle, WA, USA; [6] Department of Immuno- hematology and blood transfusion, Leiden University Medical Center, Leiden, Netherlands

Correspondence: Dr. J.J. Zwaginga; Leiden University Medical Center, Department of Immunohematology and Blood transfusion; Albinusdreef 2, Building 1, E3-Q; P.O. Box 9600, 2300 RC Leiden, The Netherlands; t +31.71.5265129, f +31.71.5265267; j.j.zwaginga@lumc.nl

Key words: Alloimmunization, storage time, red blood cell transfusion, risk- factor

ABSTRACT

Background

Red blood cells (RBCs) undergo changes during storage. Various studies have suggested a higher risk of adverse and often multi-factorial clinical outcomes associated with older stored RBCs. Our aim therefore was to examine if storage of transfused RBCs is also associated with the risk of RBC specific alloantibody formation.

Methods

A two-center retrospective case- referent study was performed where case patients and control subjects were sampled from all consecutive patients who had received their first and subsequent red blood transfusions in one of the two centers only. Cases were defined as patients who developed a first RBC alloantibody. Control subjects were patients without detectable RBC alloantibodies, who were matched to the case patients regarding number of RBC transfusions. Binary logistic regression analysis was used to examine the association between storage time of RBC and the occurrence of alloimmunization.

Results

A total of 144 cases and 286 controls were selected for our study, who had received a total 5478 RBC units. Comparing patients receiving units stored shorter than a certain number of days versus older units (with various storage periods up to 4 weeks) did not reveal an association or a trend between alloimmunization risk and storage time categories.

Interpretation

Our findings suggest that storage times of transfused RBCs between one and four weeks do not affect the risk of alloimmunization.

INTRODUCTION

Patients undergoing red blood cell (RBC) transfusions are exposed to non-self, donor red cell antigens, and as a result may develop antibodies to such foreign antigens. The alloimmunization risk is influenced by clinical[1] and genetic predisposition[2], as well as by the number of transfusions[3].

Red blood cells undergo various biochemical and biomechanical changes during storage, known collectively as the storage lesions. Such changes include membrane changes, changes in the vaso- dilatory capacity of micro-vessels due to NO release by free hemoglobin, 2, 3-DPG depletion and release of potassium[4,5]. Residual leucocytes and platelets are present, and the accumulation of released lipids, cytokines and histamine have been reported in suspension solution[6,7]. These lipids and micro-vesicles are biologically-active, with pro-inflammatory and pro-coagulant activity[8]. Currently, the clinical importance of these changes in stored RBCs is not clear, and there is much debate on the topic[8-17].The definition of aged red cells varies in the available literature. Most often a storage time period of 14 days[9-12] is used as a cut-off point to define older versus younger blood but this is arbitrarily based on the depletion of 2,3 DPG in 2 weeks stored red cells[4,13]. Other storage lesion markers might well result in another differentiation of young vs. old blood.

It was demonstrated that storage of RBC transfused in mice results in stronger immunization to one very immunogenic model antigen[14]. A similar phenomenon, however, was not observed for anti-D formation following Rhesus D incompatible transfusions in humans[15]. To clarify this issue for current transfusion practice storage times, we studied if the storage time of transfused RBCs was associated with the risk of alloantibody formation in a previously non-alloimmunized transfusion population.

METHODS

Study design and study population

A case referent study at the Leiden University Medical Center, Leiden and the University Medical Center Utrecht, Utrecht, in the Netherlands was performed. This study is part of an ongoing study on Risk Factor for Alloimmunization after red blood Cell Transfusions (R-FACT). The full protocol of the study has been published previously[16]. The study was approved by the ethics committees of both participating hospitals.

The source population consisted of all not previously transfused, non-alloimmunized patients who had received their first RBC transfusion, including at least one pre- and post-transfusion antibody screening, at the same study center. The study period was January 2005 to December 2010 at Leiden University Medical Center and January 2006 to December 2011 at University Medical Center Utrecht, Utrecht. Cases were all patients in the cohort with a first time ever RBC alloantibody. For each case two controls were sampled and matched to case patients based on the total number of red cell transfusions received. This was done following the "risk-set" sampling strategy from the total transfusion cohort, who were at

risk (i.e., had at least received the same number of RBC units) of becoming a case at the time the case was diagnosed[22].

Children were excluded, because in the participating hospitals children routinely received RBC stored for ≤7 days, as a matter of transfusion policy.

Storage time was categorized in weekly periods up to 4 weeks for the analysis.

Implicated Period

An "implicated period" or a "clinical risk period for alloimmunization" was defined for case and control patients as the period in which a transfusion most likely caused the observed primary alloimmunization. This implicated period was the time (in days) between the last transfusion before a first ever positive alloantibody screen and 30 days earlier[16]. To optimize the likelihood that our cases were primary immunizations, and not secondary booster immunizations, we considered an alloimmune response to need a minimum "lag period" of 7 days between the first finding of the antibody and the last preceding transfusion. As part of a study on clinical risk factors, a similar implicating period was selected for the controls[16]. (details presented in the published R-FACT study protocol[16])

Red Blood Cells and First time formed RBC alloantibody

Routinely transfused red blood cells in the Netherlands are in SAGM, pre-storage leukoreduced, not irradiated. RBC alloantibodies were defined as warm reacting clinically significant antibodies (C, E, c, e, C^w, K, Fy^a, Fy^b, Jk^a, Jk^b, Le^a, Le^b, Lu^a, Lu^b, M, N, S and s), screened for using a three cell panel including an indirect antiglobulin test (LISS Diamed ID gel system) and subsequently identified by a standard 11 cell panel using the same technique. All patients were routinely screened for alloantibodies before transfusions. If transfusions were given at subsequent days, the maximum interval between screens was 72 hours.

Data acquisition

Transfusion dates and dates of donation of every RBC unit received, dates and results of the antibody investigations, patients' dates of birth, gender and ward of hospitalization were gathered from the hospitals' electronic laboratory information management systems.

Data Analysis

Binary logistic regression analysis was used to examine the association between storage time of RBC and the occurrence of alloimmunization. Analysis was conducted based on various cut-off days for younger and older stored RBC units. Patients receiving on average fresher RBC unites were compared to patients receiving on average older units. In addition, patients who had received only younger (fresher) stored RBC units were compared to patients who received only older RBC units, to exclude any effect of the mixed stored (a combination of young and old blood units) RBC units[17].

All odds ratios (ORs, 95% confidence interval, CI) were corrected for the number of transfusions for which the cases and controls were matched. We presented adjusted ORs,

adjusted for possible determinants that could have confounded the association, independent of their statistical significance in the univariate analysis.

Confounders

We corrected for the number of transfusions in the implicating period (or, clinical risk period) since RBC units stored for a longer period have a tendency to be given out to patients requiring low numbers of units. This is due to the often practiced 'first in, first out' inventory management policies. Patients with indications for massive transfusions usually receive shorter stored RBC units, as a result of "first in, first out" inventory management policies. Additional categorical variables were patient age, sex and clinical ward of transfusion requirement (with acute requirements like surgical wards vs non-acute requirements like hematology wards), hospital and year (2005-2011) of donation of the transfused products. The latter two can be regarded as possible indicators of changes in blood collection, manipulation and different local transfusion strategies, if any.

RESULTS

Population Characteristics

During the study period, 17767 patients received first ever RBC transfusion at our study centers and 156 formed clinically relevant RBC alloantibodies.

The study population initially comprised of 468 patients (156 cases, 312 controls). After excluding 38 children, the remaining 144 cases and 286 controls had received a median of 9 RBC units in the study period and median 4 units in the implicated period. The distribution of age, sex, mean storage time per RBC unit and ward of hospitalization in cases and controls are presented in table 1. The median storage time of transfused RBC units was 16 days.

Table 1. Characteristics of RBC alloimmunization case and control patients

Characteristics	Case patients	Control patients
Number of patients	144	286
Males (n) Male to Female ratio	82 (1.3)	161 (1.3)
Age (mean, standard deviation)	57.5 (18.3)	57.5 (18.2)
Total number of RBC units – Median (Inter quartile range)	1834 9 (11)	3644 9 (10)
Total number of RBC units in implicated period – Median (Inter quartile range)	815 3 (4)	1953 4 (6)
Median storage time in implicated period (in days) – (Inter quartile range)	16 (8)	15 (7)
Patients in wards with acute RBC requirements, n (%)	67 (47)	147 (51)

Storage time and risk of alloimmunization

The majority of case patients (50%) were present in the average storage time category of day 15-21 days, (week 3) and was chosen as the reference group, in order to establish a robust comparison group. Patients receiving on average stored blood less than 2 weeks old; and patients receiving on average stored blood more than 3 weeks old compared to week 3 had adjusted ORs for alloimmunization of 1.05 (95% CI 0.6-1.8) and 0.9 (95% CI 0.7-1.3) respectively. (Table 2)

Table 2. Association between storage times considered as the average storage time per patient, categorized into weeks, and the occurrence of alloimmunization

Reference (3rd week)	Cases (n) 72	Controls (n) 144	Crude OR * (95% CI) 1 (ref)	Adjusted OR ** (95% CI) 1 (ref)
≤2 weeks	43	82	1.08 (0.6-1.8)	1.05 (0.6-1.8)
>3 weeks	29	60	1.003 (0.8-1.3)	0.9 (0.7-1.3)

* Odds ratio adjusted for number of matched transfusions
** Odds ratio adjusted for number of matched transfusions, sex, ward of hospitalization, hospital, patient age and transfusions received in implicated period

Table 3. Association between average storage time of RBC unit per patient and risk of alloimmunization: Comparison of average blood older than 14 days to average blood younger than 14 days and the occurrence of alloimmunization

"X" days vs. <= 14 days	Cases (n) 43	Controls (n) 82	Crude OR *	Adjusted OR **
> **14**	101	204	0.9 (0.6-1.4)	0.9 (0.6-1.5)
> **15**	89	175	1.01 (0.6-1.6)	1.02 (0.6-1.7)
> **16**	80	155	1.04 (0.6-1.7)	1.1 (0.6-1.7)
> **17**	61	136	0.8 (0.5-1.4)	0.8 (0.5-1.4)
> **18**	52	120	0.7 (0.4-1.2)	0.7 (0.4-1.2)
> **19**	46	98	0.8 (0.4-1.4)	0.7 (0.4-1.3)
> **20**	36	80	0.7 (0.4-1.4)	0.7 (0.4-1.4)
> **21**	29	60	0.8 (0.4-1.4)	0.7 (0.4-1.4)

* Odds ratio adjusted for number of matched transfusions
** Odds ratio adjusted for number of matched transfusions, sex, ward of hospitalization, hospital, patient age and transfusions received in implicated period

Next, patients transfused with units with average storage times more than 14 days up to more than 21 days were compared to patients with transfused units with average storage times less than 14 days and observed no differences, with adjusted ORs in the ranges of 0.7-0.9 (95% CI ranges 0.4-1.7) (Table 3)

Lastly, to try and disentangle the effect of mixed blood (fresher and older stored transfused units) and compare only old versus only fresh transfused blood, patients receiving only RBC units stored for less than 14 days were compared with patients receiving RBC units stored only longer than 14 days up to only longer than 21 days and observed no clear association. Only in patients receiving exclusively units stored for more than 21 days as compared to less than 14 days, an adjusted OR of 0.4 (95% CI 0.1-1.01) was found.(Table 4)

Table 4. Association between storage time of RBC units per patient and risk of alloimmunization: Comparison of patients receiving only more than 14 days stored blood to patients receiving only less than 14 days stored blood

"X" days vs. <= 14 days	Cases (n) 25	Controls (n) 51	Crude OR *	Adjusted OR **
> **14**	75	138	0.9 (0.5-1.7)	0.9 (0.5-1.8)
> **15**	62	116	0.9 (0.9-1.8)	0.9 (0.5-1.8)
> **16**	54	100	0.9 (0.5-1.9)	1.01 (0.5-1.9)
> **17**	42	90	0.8 (0.4-1.5)	0.7 (0.4-1.5)
> **18**	37	80	0.7 (0.3-1.4)	0.6 (0.3-1.3)
> **19**	34	69	0.7 (0.3-1.5)	0.6 (0.3-1.4)
> **20**	26	51	0.8 (0.4-1.7)	0.7 (0.3-1.6)
> **21**	21	43	0.6 (0.3-1.4)	0.4 (0.1-1.01)

* Odds ratio adjusted for number of matched transfusions
** Odds ratio adjusted for number of matched transfusions, sex, ward of hospitalization, hospital, patient age and transfusions received in implicated period

DISCUSSION

In our case-referent study in a previously non-transfused, non-immunized population, storage times of RBC units were not associated with the post-transfusion risk of alloimmunization against clinically significant RBC antigens. We did observe an association between storage time and alloimmunization at the >21 days versus ≤14 days comparison cut-off; however, given our study size, this could be a chance finding.

To our knowledge, this is the first study examining the effect of clinically relevant storage times of red blood cells and clinically relevant alloimmunization against non-AB and non rhesus D RBC antigens that patients normally are not matched for.

The association between the receipt of older stored red cells and other adverse clinical outcomes has been studied before[10,11,20,21]. Most of the available literature has suggested adverse effects of the older stored red cells on clinical endpoints such as post-operative infections and complications, multi-organ failure and mortality. There is no agreement, however, on whether these associations are causal[4].

By excluding children from our study, a source of confounding by indication was avoided, since children were routinely transfused (at both the study centers) with blood stored for less than 7 days. In addition, we adjusted for numerous variables in our study that could have had an influence on the storage time of RBC as well as the risk of alloimmunization.

Failure to detect case patients in our study cohort (measurement bias) might be due to the variable follow-up in post transfusion antibody screening. The study design with "risk- set" sampling of our controls from the source cohort, we feel prevents this possible measurement bias from affecting our exposure (storage time of red cells) distribution in the different comparison groups[20]. Furthermore, we cannot unequivocally rule out secondary immunizations. However, by including only pre-transfusion non-alloimmunized patients as well as introducing the 7 day lag period between first antibody detection and the date of the last preceding transfusion to define the implicated transfusion period, we feel to have efficiently minimized booster reactions. A relative limitation of the study was that there were very few patients who received only red cells stored for less than 7 days or only red cells stored for more than 28 days. The effect estimates of these extreme ends of storage time on alloimmunization were therefore not reliable (wide 95% CIs, data not shown). The inability to obtain such extreme end data – although of conceptual value – seems to indicate their small relevance for day to day practice.

Our 95% confidence intervals clearly justify our conclusions for case and control patients who received RBCs within the storage time ranges of 7-28 days. This storage time range is a reflection of the average RBC storage time range with which 95% of patients in our study centers are transfused.

A hypothesis which could be considered for our observed lack of association with the older stored RBC units (as well as fresher RBC units) could be that two different and opposing "heightened" periods of danger may exist – 1) increasing immunogenicity by leukocyte activity in fresher units that decreases with storage, and 2) immunogenicity by accumulation of cytokines, lipids, histamines and micro-vesicles that increases with storage; where these might largely cancel out each other's effect.

So far murine studies have shown that exclusive transfusion of older units of red cells (expressing a model antigen consisting of hen egg lysozyme fused to ovalbumin fused to human Duffy[b] (HOD antigen) resulted in a *stronger* antibody response than fresh units. Moreover, the co-incident transfusion of fresh RBCs dampened the immunogenicity of the stored RBCs[17]. It is worth noting that these findings involved the height of antibody titers

against a very immunogenic antigen causing universal immunization. The non-AB, non-D RBC antigens in humans that are not matched for in transfusion practice, are usually much less immunogenic. RBC alloimmunization for non AB, non rhesus D antigens in humans, as a function of RBC storage, therefore can only be studied in very large observational datasets. With this, we have to accept that the routine assays around these immunizations do not include validated antibody titer determinations. The immunization frequencies in humans, as in our study, therefore cannot be directly compared with results on antibody titers in universally immunized mice. Additionally, alloimmunogenicity in the murine studies did not substantially increase until a point in storage that can be considered to be equivalent to 42 days of human RBC storage.

A potential important modulator for immunization that studies of this type neither are able to study involve donor RBC and patient factors that determine RBC survival in the patient's circulation. This post transfusion survival[23] as 'biological age' of a given unit might likely be more important for alloimmunization than the "chronological (storage) age". Currently, as RBC survival studies are not routinely done in clinical studies, this issue cannot be assessed with normal laboratory practice.

In conclusion, our patient population, study design and storage time ranges showed that so far, there is no evidence that pre-transfusion storage time (within the ranges of 7-28 days) of red blood cells modulates the risk of alloimmunization. Some indication of a protective effect of older RBC (older than >21 days) was seen but given the study size, this may well be a chance association.

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CHAPTER 5

INTENSIVE RED CELL TRANSFUSIONS AND RISK OF ALLOIMMUNIZATION

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Saurabh Zalpuri[1,2] MD, MSc, Rutger Middelburg[1,2] PhD, Henk Schonewille[1,3] PhD, Karen M.K. de Vooght[5] PharmD, PhD, Saskia le Cessie[2,6] PhD, Johanna G. van der Bom[1,2,3] MD, PhD, Jaap Jan Zwaginga[1,3,4] MD, PhD

[1] Center for Clinical Transfusion Research, Sanquin Research, Leiden, Netherlands; [2] Department of Clinical Epidemiology, Leiden University Medical Center, Leiden, Netherlands; [3] Jon J van Rood center for Clinical Transfusion research, Leiden University Medical Center, Leiden, Netherlands; [4] Department of Immuno- hematology and blood transfusion, Leiden University Medical Center, Leiden, Netherlands; [5] Department of Clinical Chemistry and Hematology, University Medical Center Utrecht, Utrecht, Netherlands; [6] Department of Medical Statistics, Leiden University Medical Center, Leiden

Correspondence: Dr. J.J. Zwaginga; Leiden University Medical Center, Department of Immunohematology and Blood transfusion; Albinusdreef 2, Building 1, E3-Q, P.O. Box 9600, 2300 RC Leiden, The Netherlands; t +31.71.5265129, f +31.71.5265267; j.j.zwaginga@lumc.nl

Key words: transfusion intensity, alloimmunization, risk, red cells, massive transfusion

ABSTRACT

Introduction

Exposure to allogenic red blood cells may lead to formation of antibodies against non-self antigens in transfused patients. While alloimmunization rates are known to increase with the number of transfusions, the transfusion course in patients can vary from receiving multiple units during a single transfusion event or getting them dispersed over a long(er) period. In the present study we compared the immunization risk between different transfusion intensities.

Methods

An incident new-user cohort study was conducted among consecutive transfused patients at two university medical centers. Eligible were all patients who received their first red blood cell transfusion within the study period from January 2005 to December 2011. Intensive transfusions were defined as ≥5, ≥10 and ≥20 RBC units within 48 hours. Alloimmunization hazard rates (HR), comparing patients receiving intensive transfusions to patients never receiving intensive transfusions were estimated.

Results

The study cohort comprised of 5812 patients who had received a median of 7 (Inter Quartile Range=4, 12) units. RBC alloantibodies were formed by 156 patients. The adjusted Cox-regression hazard rates for alloimmunization, with number of units as the time covariate and adjusted for patient age, sex and the follow up time after first transfusion, ranged from 0.8 to 1.2 (95% ranges CI- 0.4 to 2.6).

Conclusion

The occurrence of RBC alloimmunization in patients receiving intensive transfusions did not differ significantly from patients receiving non-intensive transfusions.

INTRODUCTION

Exposure to allogenic red blood cells (RBC) may lead to formation of antibodies against non-self antigens in transfused patients. Besides genetic predisposition[1] and clinical immune modulating (e.g. medications or infection) factors[2], the risk of alloantibody formation is also influenced by the number of transfused RBC units[3,4].

Intensive (or massive) transfusions refer to a large amount of transfusions in a short period of time, and there is a lack of general consensus[5,6] in defining massive or intensive transfusions in the literature. Massive transfusions have been demonstrated to be associated with systemic inflammatory response syndrome[7,8]; higher rate of post-operative infections[9] and prolonged ICU stays[9].

Most transfusion recipients fail to react to donor alloantigens after multiple transfusions which may depend on antigen dosage but also on immune modulation factors of tolerance induction or otherwise. In healthy Rh D negative donors, a high (80-95%) anti-D rate after deliberate immunization against very low doses of D antigen has been demonstrated.[10,11] On the other hand, much lower (15%) rates of D immunization have been found in acutely transfused patients undergoing liver and heart[12] transplants and even an inverse correlation between number of D-incompatible transfusions and anti D formation was reported[13]. These examples underline that the minimum doses of alloantigens (or transfusions), the number of doses and the interval between doses may be important determinants of immune response.

Noting that the impact of intensive transfusions on alloimmunization is surprisingly unknown, we examined the association of transfusion intensity and the risk of clinically relevant RBC alloantibody formation in a previously non-transfused, non-alloimmunized cohort.

METHODS

Study design and study population

An incident new user cohort study among consecutive transfused patients at the Leiden University Medical Center (LUMC) and the University Medical Center Utrecht (UMCU), in the Netherlands was conducted. The study period was January 2005 to December 2010 at Leiden University Medical Center and January 2006 to December 2011 at University Medical Center Utrecht, Utrecht. Eligible patients were all patients who received their first red blood cell transfusion within the study period in our study centers. We could not exclude patients with previous transfusion history in other hospitals, due to absence of such information in the transfusion records of the study centers. To minimize inclusion of previous transfusion recipients, transfusions were included when preceded by a negative antibody screen and followed by a post transfusion antibody screen.

With this cohort design, the transfused population was followed up until the event (alloantibody formation), or the last negative antibody screen.

Red blood cells and first time formed RBC alloantibody

Routinely transfused RBC in the Netherlands are in SAGM, pre-storage leukoreduced. RBC alloantibodies were defined as warm reacting clinically significant antibodies (C, E, c, e, C^w, K, Fy^a, Fy^b, Jk^a, Jk^b, Le^a, Le^b, Lu^a, Lu^b, M, S and s), first screened for using a three cell panel using an indirect antiglobulin test (LISS Diamed ID gel system) and subsequently identified by a standard 11 cell panel using the same technique. Alloantibodies of other specificities other than those mentioned, as well as cold reacting alloantibodies were ignored. The reason was that the antibodies which were included were measured routinely at the study centers before every transfusion, by a standard 3 cell panel. Other antibodies might be present, but they were not measured as a routine clinical practice, hence go undetected. Since it would be invalid to present data on such antibodies that were not measured in all patients, we decided to only study alloantibodies present on a standard 3 cell panel used for antibody detection.

All patients were routinely screened for alloantibodies before transfusions. If transfusions were given at subsequent days, the maximum interval between screens was 72 hours.

Same antibody screening methods were employed during the total study period.

Transfusion intensity [or Intensive transfusions]

Transfusion intensity was defined as the number of units transfused per transfusion event within 48 hours. This 48 hour period was chosen to cover the overnight (past-midnight) transfusions from one calendar date to the next. To study the effect on alloimmunization rate in patients receiving multiple units at the same time versus multiple units over a longer period (non-intensive transfusions), calculations were performed with increasing cut-off values for intensive transfusion, i.e. ≥5, ≥10 and ≥20 RBC units within 48 hours, respectively. The study population comprised of three types of patients based on their transfusion patterns:

1. Patients who received intensive transfusions during their first transfusion event.
2. Patients who received intensive transfusions during a later time point in their transfusion history.
3. Patients who never received intensive transfusions.

To analyze the influence of intensive transfusions during a single transfusion event on alloimmunization rate, and exclude any influence of previous transfusion events on a later intensive transfusion event, patients who received the defined RBC units (≥5, ≥10 and ≥20) during their first event (type 1) were compared to those without intensive transfusions. For reasons of clarity we decided to concentrate on the effect of intensive transfusions only during the first transfusion, independent of what happens afterwards. Patients who received intensive transfusions during a later transfusion time point (type 2) were followed until that intensive transfusion event (excluding the event itself) and their transfusion information up until that time point was combined with patients who never had intensive transfusions (type 3).

Follow Up time

A transfusion at our two study centers is routinely preceded with an RBC antibody screen, as a matter of transfusion practice. Therefore, follow up time for antibody formers was calculated

as the time between their first transfusion and first ever alloantibody positive screen and for non alloantibody formers, the time between their first and the last transfusion, preceded by a negative antibody screen. From the total cohort, only those patients were selected who had a minimum follow up time of 7 days. We hypothesized that a minimum period of 7 days after a transfusion is required to mount an antibody response while earlier detection of antibodies more likely indicates a booster (previous) immunization.

Data Analyses

Baseline characteristics were presented as medians with inter-quartile range (IQR).

Kaplan Meier survival analysis with number of transfusions as the time axis was used to describe alloimmunization incidences.

Cox regression analysis was performed to estimate the hazard ratios (HR) of alloimmunization with number of transfusions as the time covariate. Crude and adjusted HR, adjusted for patient age (<= 25, 26 to 50, 51 to 75 and > 75 years), sex and the follow up time after first transfusion (adjusted as a continuous and a quadratic expression), were presented with 95% confidence intervals.

We conducted a sensitivity observed/ expected analysis. The observed number of antibodies in the intensive transfused group was known. To calculate an expected number of antibodies in this intensive group, were they hypothetically to be non-intensively transfused, we first calculated a cumulative incidence of antibody formation in the non-intensively transfused group (with standard errors). This cumulative incidence (from the non-intensive group) was used to calculate an expected cumulative incidence of antibody formation in the intensively transfused group. Thus, an expected number of antibodies was obtained in the intensively transfused group, if they were to be transfused with the same number of RBC units, but in a non-intensive manner. With the observed (actual) and the expected number of antibodies in the intensive group, we calculated a risk ratio and using bootstrapping methods, a 95% confidence interval.

RESULTS

Characteristics of the study population

The study population comprised of 5812 patients of which 156 (2.8%) formed clinically relevant red cell alloantibodies. The median age of the study population was 55 years (IQR = 25, 68), comprising of 56.4% males receiving a median of 7 (IQR = 4, 12) transfusions. The median follow-up period was 66 days (IQR = 22, 245), and almost twice as long in antibody formers as compared to non-antibody formers (Table 1).

Twelve patients in the intensively transfused group formed 15 antibodies, compared to 142 patients in the non-intensively transfused group forming 184 antibodies. The antibodies formed per patient in intensively and non-intensively transfused patients were 1.25 and 1.30 respectively.

Table 1. Characteristics of antibody formers and non-antibody formers in the cohort*

	Antibody formers	Non-antibody formers
Number of patients	156	5656
Male/ Female ratio	1.3	1.3
Age (years)	59 (38, 70)	55 (24, 68)
Follow- up in days	123 (24, 314)	65 (22, 244)
Transfused units	6 (2, 10)	7 (4, 13)

*Data are presented as medians and Inter Quartile Range

Characteristics of intensively transfused (≥ 10 units) and non-intensively transfused population

After excluding patients with less than 7 days of follow up, 5746 patients remained in the cohort out of which 183 (3.2%) were intensively transfused and 12 (6.5%) of them formed alloantibodies as compared to 142 (2.6%) of non-intensively transfused patients.

This cohort of 5746 patients (with 67.2% males) received 7 median units (IQR = 4, 12). The intensively transfused among this cohort received almost 3 times as many median units

Table 2. Characteristics of Intensively transfused (≥ 10) and non-Intensively transfused patients*

	Intensively transfused	Non-Intensively transfused
Number of patients	183	5563
Antibody formers (%)	12 (6.5%)	142 (2.6%)
Male/ Female ratio	1.9	1.3
Age (years)	60 (44, 71)	55 (23, 68)
Follow-up in days	23 (13, 90)	68 (22, 248)
Transfused units	20 (15, 32)	7 (4, 11)

*Data are presented as medians and Inter Quartile Range

Table 3. Intensively transfused patients and their indications for intensive (≥ 10) transfusions

	Intensively transfused, n (%) Total= 183	Immunized, n (%) Total= 12
Cardio- thoracic surgery	66 (36.1)	6 (9.1)
Transplants	16 (8.7)	1 (6.3)
Surgery, other*	69 (37.2)	1 (1.4)
Trauma	31 (16.9)	4 (12.9)

* emergency, neuro, spinal, vascular and abdominal surgeries; fluxus- post partum

than the non-intensive group (Table 2) and the median follow-up period was 23 days (IQR = 13, 90), almost 3 times shorter than in the non-intensive group, 7 (IQR = 4, 11). Table 3 presents the indications for transfusion among the intensively transfused patients (indication for intensive transfusion was missing for one patient).

Intensive transfusion and risk of alloimmunization

The Kaplan-Meier analysis showed no difference in the risk of alloimmunization between the intensively transfused group (≥ 10 units) as compared to the non-intensive group (Figure 1). Similar Kaplan-Meier analyses were done for the other cut-off values of intensive transfusions (i.e. ≥5 and ≥20 units within 48 hours) and no difference in alloimmunization risk was observed (Figures 1 and 2 respectively, shown in appendix).

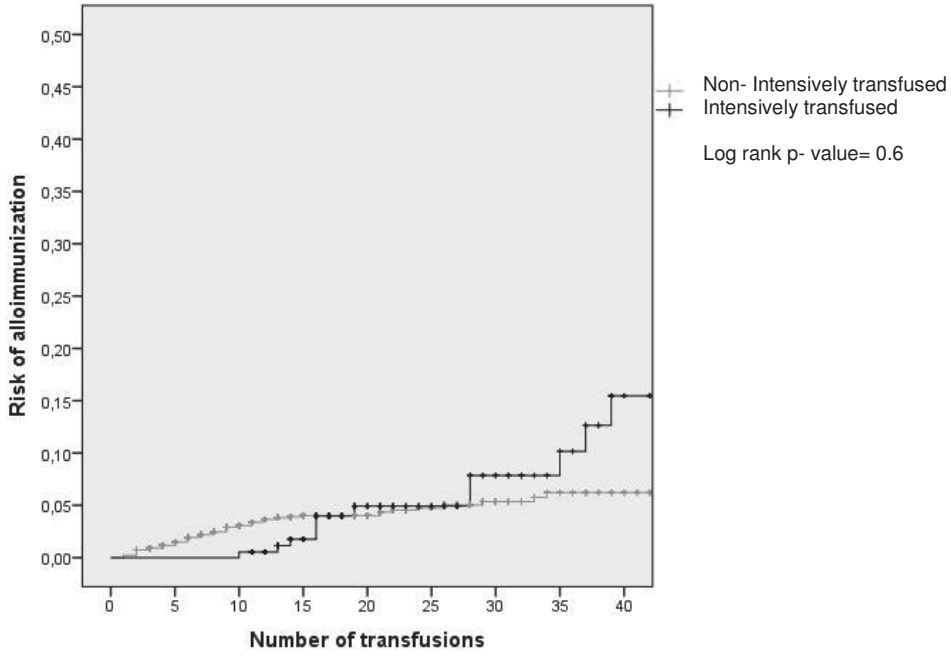

Figure 1. Kaplan Meier estimate of alloimmunization in intensively transfused (≥10 units) and non-intensively transfused patients

Number of patients remaining at transfusion number:

Transfusions →	*10*	*20*	*40*
Intensive	182	94	24
Non-Intensive	1817	602	151

The crude and adjusted Cox-regression hazard ratios, with number of transfusions as the time covariate revealed no differences in alloimmunization risk in the intensively transfused group as compared to the non-intensively transfused group regardless of cut-off values for intensive transfusions (Table 4).

Table 4. Intensive and non-Intensive transfusions and the risk of alloimmunization (HR) using the number of units as the time covariate**

Intensive transfusion	Hazard Ratio (95% CI) ≥5 units		≥10 units		≥20 units	
	Crude	Adjusted*	Crude	Adjusted*	Crude	Adjusted*
Intensive vs. non-Intensive	1.2 (0.8-1.8)	1.1 (0.7-1.6)	1.2 (0.7-2.1)	1.1 (0.6-1.9)	0.9 (0.3-2.6)	0.9 (0.3-2.4)

* Adjusted for sex, age and follow up time after first transfusion.
** The intensive group receiving ≥5 units comprised of 550 patients with 5.5% antibody formers, the numbers for the ≥20 group were 45 with 8.8% antibody formers.

Sensitivity analysis

The observed number of antibodies in the intensive group (≥ 10 units) was 12. An expected 9.2 antibodies were calculated in this group, giving a risk ratio of 1.3 (95% CI 0.7-2.1). The confidence intervals were estimated after 10,000 bootstrapping runs. The risk ratios were similar in other intensive transfusion categories (≥5 and ≥20 units).

DISCUSSION

In our study, no statistically significant difference in the risk of alloimmunization in intensively transfused patients and non-intensively transfused patients was found (stratified on the number of transfusions and using three cut-off values for intensive transfusions).

Strength of our study was the incident new user cohort study design, which avoids inclusion of prevalent users in the study cohort[14]. This study design and its data collection approach allowed a prospective follow up of previously non-transfused and non-immunized patients during subsequent transfusions up to the appearance of a first alloantibody. Although all the patients with a previous transfusion history in our study centers prior to 2005 were excluded, the possibility of previous transfusions in a different hospital cannot be ruled out entirely. Secondary immune responses may have occurred. We, however, do not expect this to affect our study findings. There is no reason to believe that patients with unknown previous transfusions and with unknown previous antibodies are more likely to get a massive transfusion.

The difference in follow up times between intensively transfused (median 23 days) and no intensively transfused (median 68 days) can be attributed the fact that the intensively transfused patients were censored right after their intensive transfusion events, (hence

the shorter follow up time) as compared to non- intensively transfused patients who were followed up until the end of the study period.

This cohort, in our opinion, is a good representation of a general transfused population. In the absence of a clear consensus on the definition of a massive transfusion event, three definitions were used to study the effect of intensity of transfused units on the risk of alloimmunization.

In addition to the Cox regression analysis, we performed an observed/ expected analysis to verify the results from Cox models. The Cox model calculates "instantaneous" antibody incidence, censoring a patient as soon as an antibody is formed. Intensive transfused patients receive many transfusions without a gap in between transfusions to measure antibodies after every transfusion, which makes it difficult to pin-point the exact transfusion after which an antibody was formed. This may bias the Cox estimate. An observed/ expected analysis is based on cumulative incidence, hence less sensitive to the potential mis-estimation by the Cox model.

In our approach, subsequent non-intensive transfusions after the intensive transfusion event in the intensively transfused group were included. This might affect the immunization risk estimate of the intensive transfusion event. However, there is no *a priori* reason to believe that for example, the 20th transfusion after the intensive episode carries a different risk of alloimmunization than the same 20th transfusion in the non-intensively transfused group. We furthermore acknowledge the possible presence of immunologic non-responders to alloantigens in our study cohort, as postulated in other studies[15,16]. This is not regarded as a source of confounding in our study because it cannot be envisioned that being a (non-) responder is related to receiving intensive or non-intensive transfusions. Their distribution in the intensive and non-intensive transfused group, thus, is independent of being a responder or a non-responder.

So far, the effect of intensity of blood transfusions on alloimmunization against clinically relevant (non Rh D) red cell antibodies has not been reported. Intensive or massive transfusion, and more specifically, the circumstances in which intensive transfusions are usually administered namely, major thoracic surgeries, surgeries post- trauma etc. combined with medications and the administration of a massive load of non-self transfused antigens, are likely to modulate the immune system and with it possibly responses to non-self antigens. In this respect, the immunological "danger model" theory on one hand proposes that an immune response may be exaggerated by not only the non-self antigen but also by the extent of trauma/ tissue damage[17,18]. The alloantigens in the transfused blood together with the danger signals from the damaged tissue collaborate to trigger an immunologic alarm and mount an immune response. In line with this, a chronic inflammatory state which for instance accompanies sickle cell disease appears to enhance alloimmunization[19]. Moreover, previous febrile reaction/ inflammatory response to platelets was associated with higher risk of RBC alloimmunization[20]. Finally, murine studies confirm that some pro- inflammatory stimuli[21] and increased inflammatory state[22] enhances humoral immunization to transfused alloantigens.

On the other hand, it has been suggested that the conditions surrounding acute transfusions lead to suppression of the immune system. Trauma to the tissue induced by surgery is known to be immuno-suppressive[23]. Trauma associated transfusions can lead to micro-chimerism, and these individuals undergo global immuno-suppression[24] that predisposes these post trauma transfused patients to donor leucocytes tolerance as well as diminished alloantigenic responses[24,25]. Medications, with corticosteroids being an evident example, administered in the setting of massive transfusion can also suppress patient's immune response[12]. Donor leucocytes may both activate or attenuate the recipient's immune system with beneficial tolerance as shown in renal grafts[26] as well as deleterious effects like increase in infections[27].

The dose (intensity) of red blood cells in our study was categorized in to three categories but it is worth noting that the dose of exposure to a particular antigen itself could not be quantified in our study. The amount of antigen being taken up by the immune system itself was another variable which was unquantifiable in our observational study. An intensively transfused patient might haemorrhage a lot of blood while being transfused, and may end up with the same amount of antigen to the immune system as a non-intensively transfused patient. This antigen dose related questions might be relevant and should be addressed in future research on this theme.

Given our study population, study design and intensive transfusion definitions, we did not find an association between intensively and non-intensively transfused patients, and the risk of alloimmunization. Future research is warranted to study the clinical risk factors of alloimmunization.

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APPENDIX

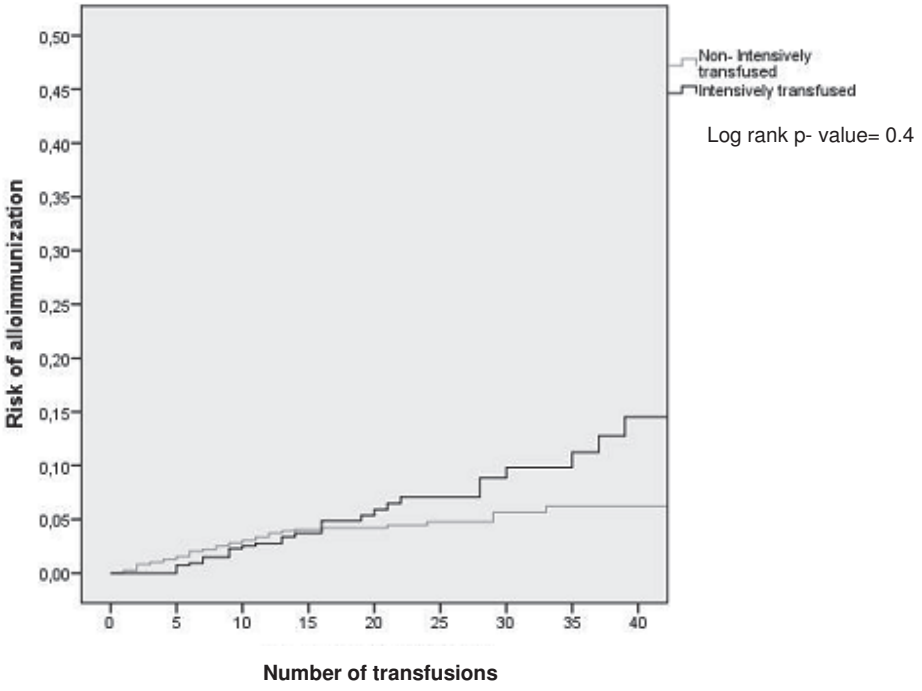

Figure 1. Kaplan Meier estimate of alloimmunization in intensively transfused (≥5 units) and non-intensively transfused patients

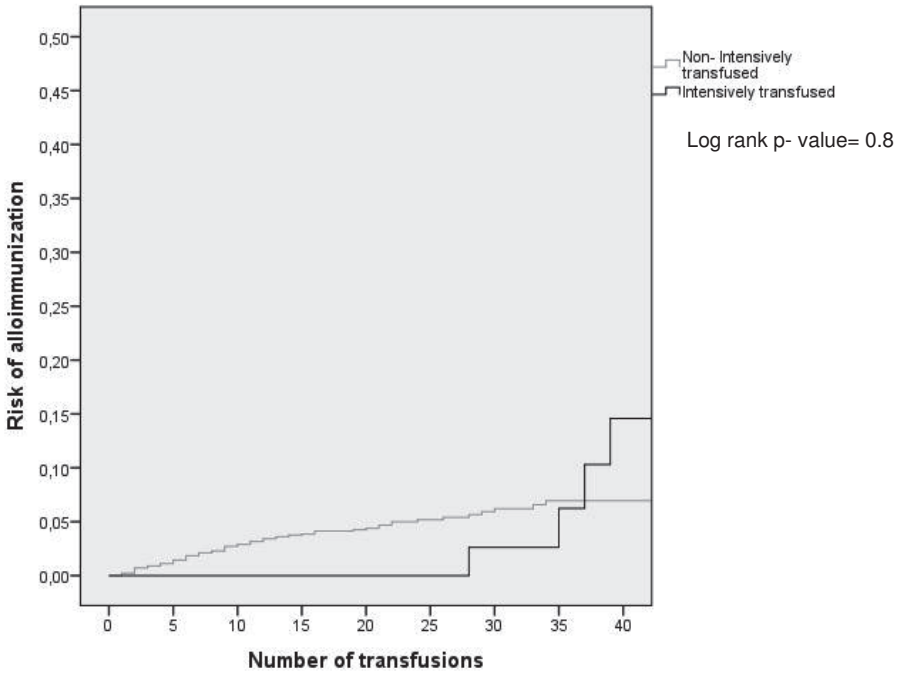

Figure 2. Kaplan Meier estimate of alloimmunization in intensively transfused (≥20 units) and non-intensively transfused patients

Table 1. Intensive* and non-Intensive transfusions and the risk of alloimmunization (HR) using the number of units as the time covariate; using =>14, =>21 and => 28 days minimum follow up cut-offs

Minimum follow up time in days		
<= 14 (HR**, 95% CI)	<=21 (HR**, 95% CI)	<=28 (HR**, 95% CI)
1.2 (0.6-2.4)	1.3 (0.6-2.7)	(0.5-2.6)

* Intensive transfusion: ≥10 units
** Adjusted for sex, age and follow up time after first transfusion.

CHAPTER 6

IMMUNOSUPPRESSANT USE AND ALLOIMMUNIZATION AGAINST RED BLOOD CELL TRANSFUSIONS

Manuscript in preparation

Saurabh Zalpuri[1] MD, MSc, Jaap Jan Zwaginga[1,3] MD, PhD, Henk Schonewille[1], PhD, Karen M.K. de Vooght[5] PharmD, PhD, Saskia le Cessie[4] PhD, Johanna G. van der Bom[1,2] MD, PhD

[1] Center for Clinical Transfusion Research, Sanquin Research, Leiden; [2] Department of Clinical Epidemiology, Leiden University Medical Center, Leiden; [3] Department of Immuno- hematology and blood transfusion, Leiden University Medical Center, Leiden; [4] Department of Medical Statistics, Leiden University Medical Center, Leiden; [5] Department of Clinical Chemistry and Hematology, University Medical Center Utrecht, Utrecht, Netherlands

Correspondence: Dr. Johanna G. van der Bom; Centre for Clinical Transfusion Research, Sanquin Research; Plesmanlaan 1a, 2333 BZ Leiden, The Netherlands; Tel1: +31 71 568 5053 (assistant), Tel2: +31 71 526 8871, Fax: 071-5685191; Email: j.g.vanderbom@lumc.nl

Key words: Alloimmunization, corticosteroids, immunosuppressants, red cell antibodies, transfusions

ABSTRACT

Introduction

Patients receiving red blood cell transfusions are at risk of developing alloantibodies against donor red cell antigens. The risk of alloimmunization is dependent on the number of units administered and patient's genetic predisposition, but has also been suggested to be modulated by a patient's clinical profile. Our aim was to examine whether immunosuppressants suppress the development of clinically relevant RBC antibodies.

Methods

A two-center retrospective case- referent study was performed where case patients and control patients were sampled from all consecutive patients (17,750 patients) who had received their first and subsequent red cell transfusions in a five year period in the study centers. Cases were all patients with a first detected RBC alloantibody preceded by negative antibody screens. Control patients were two-to-one matched to the case patients on the number of RBC transfusions. Logistic regression analysis was used to examine the association between immunosuppressant exposure and the subsequent occurrence of RBC alloimmunization.

Results

Among the total study population, 98 patients received immunosuppressive therapy, with 46 patients receiving only corticosteroids, 16 receiving only other immunosuppressants and 36 receiving both. A total of 156 case patients and 312 control patients in the study received a median of 6 transfusions (interquartile ranges 3, 11). The incidence of alloimmunization among patients using immunosuppressants was lower than among other patients receiving red blood cells, adjusted relative rate (RR) 0.46 (confidence interval, CI 0.23-0.89).

Interpretation

Our findings support a considerably lower risk of alloimmunization with the use of immunosuppressive medications.

INTRODUCTION

Patients receiving red blood cell transfusions are at risk of developing alloantibodies against donor red blood cell antigens[1]. Alloimmunization against clinically relevant red cell antigens can cause serious complications like acute and delayed hemolytic transfusion reactions. In light of this, it becomes important to study the risk factors associated with alloimmunization in detail, in order to predict which patients are most vulnerable to alloimmunization; and thus they may be considered for more extended matched red blood cell transfusions to prevent alloimmunization. On the other hand identifying clinical factors protecting patients against alloimmunization would be equally important.

The risk of alloimmunization is dependent on the number of red cell units administered[1]. The extent of alloimmunization has been studied in various populations with the incidence of alloimmunization increasing with the number of units, ranging from 7%[1] (after 40 transfused units) to 13%[2] (estimated) in a general transfused population. The risk of alloimmunization is also determined by a patient's genetic predisposition to form an immune response to these non-self antigens[3]. In addition, it has been suggested that a patient's clinical condition[4] is associated with modulation of the alloimmunization risk. Immunosuppressive therapy could be of particular importance in this respect, because red blood cell transfusions and immunosuppressive therapy often coincide in intensive care, trauma, active autoimmune disorder, cancer and organ transplant patients.

The use of immunosuppressants among a general transfused population and its effect on the risk of clinically relevant RBC alloimmunization, however, has not been reported and was the purpose of this study.

METHODS

Design and study population

A matched case-referent study was performed at two study centers- Leiden University Medical Center, Leiden and University Medical Center Utrecht, Utrecht, in the Netherlands. Details of our case- referent study design have been previously described[5]. In short, the source population comprised of all previously non-transfused, non-alloimmunized patients who received their first RBC transfusion at one of the study centers. The study period was January 2005 to December 2010 at Leiden University Medical Center and January 2006 to December 2011 at University Medical Center Utrecht, Utrecht.

Case patients were patients with first time detected clinically relevant red cell antibodies and control patients were patients who did not have clinical red cell antibodies after the same number of transfusions as the matched case. The control sampling was conducted on the principles of a risk-set sampling strategy[6], e.g. for any given case (with *N* units up until alloantibody formation), two control patients with at least the same number of units were randomly selected from the source population (figure 1). Control patients were then matched to case patients on *N* number of units (figure 1). Case and control patients were also matched on the study center[5].

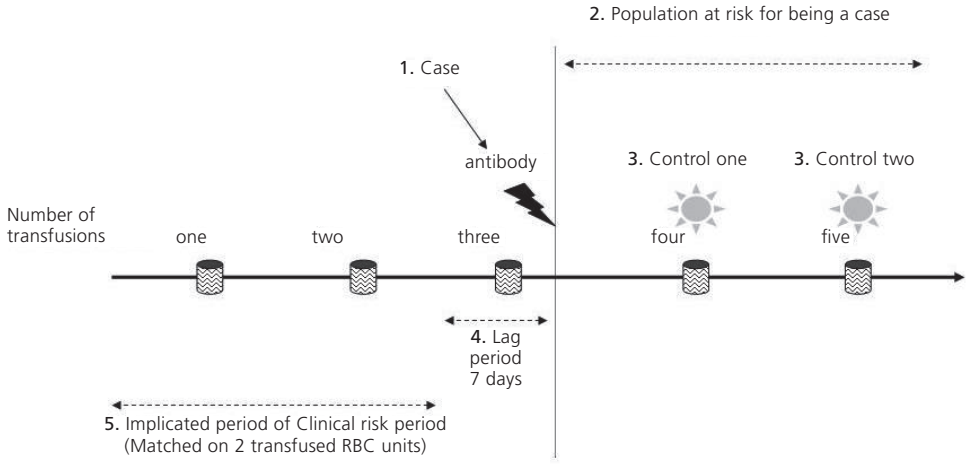

Figure 1. Control patient selection and Clinical risk period*

** The chronological order from case patient identification to clinical risk period definition is marked from number 1 to 5.*

The transfusion policy in the study centers was as follows: 1) routinely transfused RBC concentrates were in SAGM and pre-storage leukoreduced and 2) all patients were routinely screened for alloantibodies before transfusion which was repeated at least every 72 hours, if further transfusions were required.

Clinical risk period (Implicated Period) of alloimmunization

We first set out to define an 'immunization risk' period preceding the antibody finding in order to identify the concurrent clinical conditions that in combination with an antigen mismatched transfused unit (implicated unit) could have led to alloimmunization. We measured all the study variables within this clinical risk period.

For the case patients, this risk period[5,7] was defined as a 30 day period preceding the date of the transfusion immediately before) the first positive alloantibody screen[5]. We chose the risk period not to include the week just before the positive screen to "give" antibodies at least one week to develop. The risk period definition is illustrated in Figure 1. A similar clinical risk period was defined for the control patients, as the period of 30 days preceding the transfusion, at which case and control patients had been matched (figure 1).

Using the above defined method to pick a clinical risk period (the so called implicated period) of alloimmunization, we found in the majority (88%) of our case patients at least one transfusion with the mismatched antigen in the risk period immediately preceding the antibody identification. For the remainder of case patients, we looked further back into their transfusion history to identify the mismatched antigen transfusion unit and re-defined the implicated period as per the above mentioned[5] definition of implicated period, around that particular mismatched transfusion.

There were 20 cases with antibodies like Fya, Jka, M for whom an antigen mismatched red blood cell unit could not be confirmed. This is because the donor red cell units are usually not typed for antigens except for ABO, D, other rhesus (C, E, c, e) and K antigens.

First time formed clinically relevant red cell alloantibodies

Red cell alloantibodies were defined as warm reacting clinically significant antibodies (C, E, c, e, C^w, K, Fy^a, Fy^b, Jk^a, Jk^b, Le^a, Le^b, Lu^a, Lu^b, M, S and s), and were screened for using a three cell panel including an indirect antiglobulin test (LISS Diamed ID gel system) throughout the study period. Positive screening in the three cell panel led to subsequent identification of the antibody or antibodies by a standard 11 cell panel using the same technique.

Alloantibodies of other specificities than those mentioned, as well as cold reacting alloantibodies are not routinely detected by the three cell panel screening method and thus were not considered to be included as cases of clinical alloimmunization.

Medication classification

To classify the immunosuppressive therapy into corticosteroids and other immunosuppressants categories (table 1), the World Health Organization's ATC (Anatomical Therapeutic Chemical) classification index was used (source: http://www.whocc.no/atc_ddd_index/). Medications classified under category H, sub-category H02 were included as corticosteroids; medications classified under category L, sub-category L04 were included as (other) immunosuppressants (table 1).

Table 1. Immunosuppressive medication use among total study population of 468 patients

Immunosuppressive medications (98 patients)	
Number of patients (%) Using corticosteroids	
Prednisolone	50 (51)
Prednisone	15 (15)
Dexamethasone	12 (12)
Triamcinolone	5 (5)
Hydrocortisone	4 (4)
Methylprednisolone	1 (1)
Betamethasone	1 (1)
Using other immunosuppressants	
Cyclosporine	34 (34)
Mycofenolaat mofetil	22 (23)
Azathioprine	3 (3)
Lenalidomide	2 (2)
Everolimus	1 (1)
Methotrexat	1 (1)
Thalidomide	1 (1)

Data Collection and Definitions

Transfusion dates, results of the antibody investigations, patients' dates of birth, gender, chronic obstructive pulmonary disease (COPD), infections (bacterial, viral, fungal- diagnosed by laboratory serological techniques including blood and tissue cultures), fever (temperature above 38 degree Celsius), transplants (organ and stem cell), allergies (food, dust, animal and chemical), autoimmune diseases (including rheumatoid arthritis), leukemia (acute lymphoblastic, chronic lymphocytic, acute myeloid, juvenile myelomonocytic, myelodysplastic syndrome and myeloma), lymphoma, chemotherapy (yes or no), surgeries (thoracic, abdominal, cranial and facial, upper and lower limbs and excluding coronary bypass and transluminal angiography), traumas (high impact traumas including cars, motorbikes and bicycles; falls) and diabetes (type 1 and type 2) were collected from clinical files within the defined clinical risk period (implicated period) of alloimmunization. Immunosuppressive medications- corticosteroids and other immunosuppressants used within this risk period were gathered from the hospitals' electronic patient dossiers and information management systems.

Data Analyses

Specific corticosteroids and other immunosuppressants types and their usage (in numbers and percentages) were presented.

The association between the use of immunosuppressive medications and alloimmunization was modeled using a logistic regression model. Odds ratios were interpreted as relative rates throughout the manuscript. All relative rates (RR) were corrected for the matching factors- total number of transfusions and study center and presented with a 95% confidence interval (CI).

We compared patients receiving 1) any immunosuppressives, 2) exclusively corticosteroids, 3) exclusively other immunosuppressants and 4) exclusively both of these in combination, to patients not exposed to any of these medications, within the implicated period.

The distribution of potential confounders in controls with and without corticosteroids and other immunosuppressants as well as among case patients and total study population (presented in the appendix) were presented in numbers and percentages, or median with interquartile range (IQR).

The adjusted relative rates were adjusted for – sex and age (categorical with <=25, 26-50, 50-75 and > 75 year categories), chronic obstructive pulmonary disease (COPD, infection, fever, transplant, allergies, auto-immune diseases, rheumatoid arthritis (RA), leukemia, lymphoma, chemotherapy, surgery, trauma and diabetes type 1 and type 2 during the implicated period.

RESULTS

Characteristics of the study population

Out of a total of 17,750 transfused patients, 468 patients were studied (156 case patients, 312 control patients). 56% (261) patients were from Utrecht and 44% (207) patients were from the Leiden study center. The study population had a median age of 59 years, (IQR 38,

70) and comprised of 56% males. Cases had received a median of 6 units of red cells (IQR 3, 11; range 1-66) units before antibody formation. Antibodies were detected for the first time after a median of 123 days (IQR 25, 333) following the first transfusion.

Use of immunosuppressive therapy in the clinical risk period (implicated period):

A total of 98 patients used any immunosuppressant medications. Prednisone (50%), prednisolone (15%) and dexamethasone (12%) were the most used corticosteroids and cyclosporine (34%) and mycofenolaat mofetil (22%) the most used other immunosuppressants (table 1).

Control patients using immunosuppressive medications (in the month before the matched transfusion) were more often females, 51% to 43% and younger (48 vs. 62 years) as compared to the patients not exposed to immunosuppressive medications. Patients exposed to immunosuppressive medications more often had infections (51% to 24%), fever (33% to 23%), transplants (36% to 3%), allergies (12% to 4%), leukemia (28% to 7%), lymphoma (7% to 4%), chemotherapy (17% to 14%); and a lower percentage of auto-immune diseases (1.3% to 3%), surgeries (37% to 58%) and traumas (none to 9%) compared to patients not using immunosuppressive medications (table 2). The distribution of diabetes type 1 (2% to 1%) and type 2 (8% to 9%) was similar in both patient populations.

Similar group distributions for case patients and the total study population were presented as well (Appendix table 1 and table 2).

Immunosuppressives and risk of alloimmunization

Eight patients were left out of the adjusted multivariable analysis due to missing data on at least one confounder. Patients receiving immunosuppressive medications had a lower alloimmunization rate than those not receiving these medications. The crude relative rate (RR) was 0.50 (95% CI, 0.29-0.88) and the adjusted RR 0.46 (95% CI 0.23-0.89). With these results, we analyzed specifically patients using only corticosteroids, only other immunosuppressants or both (table 3).

Compared with patients not using any immunosuppressive medications, patients using only corticosteroids, only other immunosuppressants and patients using both all had a lower alloimmunization rate, an adjusted RR 0.52 (95% CI 0.23-1.16); 0.24 (95% CI 0.05-1.20) and 0.52 (95% CI 0.19-1.40) respectively (table 3).

INTERPRETATION AND DISCUSSION

In our case referent study among previously non transfused, non alloimmunized patients, exposure to immunosuppressives was associated with a lower incidence of clinically relevant red cell alloantibodies against donor red blood cells.

To appreciate these findings, several aspects need to be discussed. Strength of our study is the control sampling strategy. By using a risk-set sampling strategy, our control patients formed a representative sample of the source population. In this study we

Table 2. Patient characteristics as observed during the clinical risk period, according to their exposure to immunosuppressive medications; Among *control patients* (n= 312)

Patient Characteristics***, n (%)	None n= 237	Corticosteroids or other Immunosuppressant n= 75	Only Corticosteroids n= 35	Only other Immunosuppressants n= 14	Both n= 26
Sex, males	136 (57.4)	37 (49.4)	15 (42.9)	7 (50)	15 (57.7)
Age* (years)	62 (43, 73)	48 (29, 59)	51 (32, 64)	36 (14, 49)	51 (29, 63)
COPD	7 (3.0)	4 (5.5)	2 (5.9)	0 (0.0)	2 (7.7)
Infection**	57 (24.1)	38 (50.7)	17 (48.6)	6 (42.9)	15 (57.7)
Fever	55 (23.2)	25 (33.3)	11 (31.4)	7 (50.0)	7 (26.9)
Transplants (organ and stem cell)	7 (3.0)	27 (36.0)	9 (25.7)	6 (42.9)	12 (46.2)
Allergies	10 (4.2)	9 (12.0)	4 (11.4)	1 (7.1)	4 (15.4)
Auto-immune diseases (including Rheumatoid Arthritis)	5 (2.1)	1 (1.3)	1 (2.9)	0 (0.0)	0 (0.0)
Leukemia	17 (7.2)	21 (28.0)	11 (31.4)	5 (35.7)	5 (19.2)
Lymphoma	9 (3.8)	5 (6.7)	2 (5.7)	2 (14.3)	1 (3.8)
Chemotherapy	32 (13.5)	13 (17.3)	10 (28.6)	2 (14.3)	1 (3.8)
Surgeries	138 (58.2)	28 (37.3)	14 (40.0)	3 (21.4)	11 (42.3)
Trauma	21 (8.9)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Diabetes Type 1	5 (2.1)	1 (1.3)	0 (0.0)	1 (7.1)	0 (0.0)
Diabetes Type 2	19 (8.0)	7 (9.3)	3 (8.6)	2 (14.3)	2 (7.7)

Values are number (%) or *median (interquartile range)
** includes bacterial, viral and fungal infections
*** all characteristics were measured during the implicated period

Table 3. Relative rate of alloimmunization in patients using only corticosteroids, only other immunosuppressants and both as compared to using none of these

	Case patients	Control patients	Crude RR* (95% CI)	Adjusted RR** (95% CI)
None	133	237	1 (ref)	1 (ref)
Only Corticosteroids	11	35	0.52 (0.24-1.12)	0.52 (0.23-1.16)
Only Immunosuppressant	2	14	0.23 (0.05-1.08)	0.24 (0.05-1.20)
Both	10	26	0.64 (0.28-1.43)	0.52 (0.19-1.40)

* adjusted for number of matched transfusions and hospital
** adjusted for number of matched transfusions and hospital; sex, age, COPD, infection, fever, transplants, allergies auto-immune diseases, leukemia, lymphoma, chemotherapy, surgeries, trauma diabetes type 1 and diabetes type 2.

RR- Relative Rate; 95% CI- 95% Confidence Interval

examined the combined immune modulating effects of transfusion exposure and that of immunosuppressives administered in the defined implicated period. For this purpose, we carefully chose an implicated period. The aim of defining this clinical risk period in which the transfusion mediated exposure to mismatch antigens occurred, was to enable us to study clinical concurrent events with possible immune modulating effects. While the observed protective association between immunosuppressive therapy and alloimmunization may in part be the result of other risk factors for alloimmunization that are also associated with the use of immunosuppressants i.e. confounding factors, we carefully measured all other risk factors and adjusted for them in our analyses.

Although the possibility of unknown transfusions at a different hospital cannot be entirely ruled out by our strategy, all selected patients needed to have a negative antibody screen preceding the first transfusion and at least followed by one post transfusion antibody screen. This strategy is not totally excluding secondary ("boostered") immune responses. We, however, do not expect this to affect our study findings. There is no reason to believe that patients with unknown previous transfusions and with unknown previous antibodies are more likely to be exposed (or unexposed) to any of the potential confounding variables. The same reasoning is true for the fact that we could not exclude patients with possible previous transfusion history in other hospitals, due to absence of such information in the transfusion records of the study centers.

To our knowledge this is the first study in humans that shows the presence and extent of the protective effect of immune suppressive medications on alloimmunization against clinically relevant red cell antigens. A causal nature of the observed association with use of immunosuppressants is biologically plausible. Their role in suppressing the transplant rejection in the patients undergoing organ transplants[8] has been documented. In addition, immunosuppressive therapy has been shown to impair humoral immune responses to vaccines[9] and antigens[10]. With respect to corticosteroids, hydrocortisone has been shown to diminish in vitro responses to streptokinase- streptodornase and tetanus toxoid[11] vaccinations

as indication of a suppressed immune response. This diminished immune response in presence of corticosteroids has been attributed to transient lymphocytopenia, by the redistribution of circulating T-cells to other body compartments[12]. It has been also demonstrated that proliferation of T-cells can be inhibited by corticosteroids[13-18]. For example, glucocorticoids inhibit production of T-cell growth factor and block the clonal expansion necessary to amplify a primary response[16,19,20].

Other immunosuppressive drugs also suppress T-cell responses[21]. Proliferation of B and T lymphocytes is inhibited by immunosuppressants like mycophenolate[22] and rituximab[10]; while drugs like cyclosporine and tacrolimus inhibit the activation and differentiation of T-cells by inhibiting calcineurin. In addition, a lower influenza vaccine antibody response and diminished T-cell proliferation responses have been shown in with these drugs immunosuppressed liver transplant patients[23].

Considering the mechanisms of the studied alloimmunization against red cell antigens, they are both B- and T helper cell dependent. Although the short lived formation of non-naturally occurring IgM antibodies by B-cell derived plasma cells is mainly T-cell independent, the subsequent memory B-cell response and the formation of more high affinity IgG is T-cell helper dependent. It is therefore likely that in presence of corticosteroids and the other immunosuppressive drugs, the T-cell mediated responses to donor red cell antigens are impaired. Of course, the observed immunosuppression therapy mediated risk reduction of alloimmunization need not be entirely caused by this therapy but a direct attributive effect is strongly plausible.

Therefore when aiming for an eventual alloimmunization risk prediction on the basis of clinical factors, immunosuppressives might be added to such a prediction risk score. This may enable to distinguish high risk patients for alloimmunization that might benefit from cost effective extended donor blood phenotype matching strategies.

In summary, corticosteroids and other immunosuppressant medications appear to have a considerable protective effect on alloimmunization in patients transfused with donor red blood cells. While immune activating conditions are often the reason to start these drugs and coincide with their use, the inhibiting effect that was observed in our studies might be even an underestimation of the true effectiveness of these drugs to block the alloimmunization response.

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APPENDIX

Appendix table 1. Patient characteristics according to use of immunosuppressive medications; Among *total study population* (n= 468)

Patient Characteristics***, n (%)	None n=370	Corticosteroids or other Immunosuppressant n= 98	Only Corticosteroids n= 46	Only other Immunosuppressants n= 16	Both n= 36
Sex, males	211 (57)	50 (51)	20 (44)	9 (56)	21 (58)
Age* (years)	62 (43-73)	49 (29-60)	51 (34, 63)	28 (9, 48)	52 (23, 63)
COPD	14 (3.8)	4 (4.2)	2 (4.4)	0 (0)	2 (5.7)
Infection**	95 (25.7)	51 (52)	21 (45.7)	8 (50)	22 (61.1)
Fever	84 (22.7)	34 (34.7)	13 (28.3)	8 (50)	13 (36.1)
Transplants (organ and stem cell)	10 (2.7)	36 (36.7)	10 (21.7)	6 (37.5)	20 (55.6)
Allergies	18 (4.9)	12 (12.2)	5 (10.9)	1 (6.2)	6 (16.7)
Auto-immune diseases (including Rheumatoid Arthritis)	7 (1.9)	2 (2.0)	1 (2.2)	0 (0.0)	1 (2.8)
Leukemia	26 (7)	26 (26.5)	13 (28.3)	6 (37.5)	7 (19.4)
Lymphoma	10 (2.7)	5 (5.1)	2 (4.3)	2 (12.5)	1 (2.8)
Chemotherapy	43 (11.6)	19 (19.4)	15 (32.6)	2 (12.5)	2 (5.6)
Surgeries	223 (60)	37 (37.8)	19 (41)	3 (19)	15 (42)
Trauma	33 (8.9)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Diabetes Type 1	5 (1.4)	1 (1.0)	0 (0.0)	1 (6.2)	0 (0.0)
Diabetes Type 2	38 (10.3)	10 (10.2)	4 (8.7)	2 (12.5)	4 (11.1)

Values are number (%) or *median (interquartile range)
** includes bacterial, viral and fungal infections
*** all characteristics were measured during the implicated period

Appendix table 2. Patient characteristics according to use of immunosuppressive medications; Among all *case patients* (n= 156)

Patient Characteristics***, n (%)	None n= 133	Corticosteroids or other Immunosuppressant n= 23	Only Corticosteroids n= 11	Only other Immunosuppressants n= 2	Both n= 10
Sex, males	75 (56.4)	13 (56.5)	5 (45.5)	2 (100.0)	6 (60.0)
Age* (years)	61 (39-72)	51 (26-62)	51 (39-62)	10 (7-10)	53 (21-64)
COPD	7 (5.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Infection**	38 (28.6)	13 (56.5)	4 (36.4)	2 (100.0)	7 (70.0)
Fever	29 (21.8)	9 (39.1)	2 (18.2)	1 (50.0)	6 (60.0)
Transplants (organ and stem cell)	3 (2.3)	9 (39.1)	1 (9.1)	0 (0.0)	8 (80.0)
Allergies	8 (6.0)	3 (13.0)	1 (9.1)	0 (0.0)	2 (20.0)
Auto-immune diseases (including Rheumatoid Arthritis)	2 (1.5)	1 (4.3)	0 (0.0)	0 (0.0)	1 (10.0)
Leukemia	9 (6.8)	5 (21.7)	2 (18.2)	1 (50.0)	2 (20.0)
Lymphoma	1 (0.8)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Chemotherapy	11 (8.3)	6 (26.1)	5 (45.5)	0 (0.0)	1 (10.0)
Surgeries	85 (63.9)	9 (39.1)	5 (45.5)	0 (0.0)	4 (40.0)
Trauma	12 (9.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Diabetes Type 1	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Diabetes Type 2	19 (14.3)	3 (13.0)	1 (9.1)	0 (0.0)	2 (20.0)

Values are number (%) or *median (interquartile range)
** includes bacterial, viral and fungal infections
*** all characteristics were measured during the implicated period

CHAPTER 7

CAUSAL INFERENCE FROM PREDICTION RESEARCH: ERRONEOUS ASSUMPTIONS IN TRANSFUSION MEDICINE RESEARCH

CORRELATION VERSUS CAUSALITY IN TRANSFUSION MEDICINE: UNDERSTANDING MULTIVARIABLE ANALYSIS IN PREDICTIVE VERSUS ETIOLOGY RESEARCH

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Saurabh Zalpuri[1,2] MD, MSc, Rutger A. Middelburg[1,2] PhD, Leo van de Watering[1] MD, PhD, Eleftherios Vamvakas, MD, PhD, MPH[4], Jaap Jan Zwaginga[1,3] MD, PhD, Johanna G. van der Bom[1,2] MD, PhD

[1] Center for Clinical Transfusion research, Sanquin Research, Leiden; [2] Department of Clinical Epidemiology, Leiden University Medical Center, Leiden; [3] Department of Immuno- hematology and blood transfusion, Leiden University Medical Center, Leiden; [4] Cedars-Sinai Medical Center, Los Angeles

Correspondence: Dr. Johanna G. van der Bom; Centre for Clinical Transfusion Research; Sanquin Research; Plesmanlaan 1a, 2333 BZ Leiden, The Netherlands; Tel1: +31 71 568 5053 (assistant), Tel2: +31 71 526 8871, Fax: 071-5685191; Email: j.g.vanderbom@lumc.nl

Key words: Transfusion, etiology, prediction, multivariable models, confounding.

ABSTRACT

Background and Aim

In the current medical literature, etiologic and prediction research aims are frequently confused. Investigators tend to use principles from prediction research for their etiologic research questions, which results in misleading interpretation of risk-factor findings at hand. We used a questionnaire-based survey to quantify the proportion of ISBT 2012, Cancun visitors who felt confident with a causal interpretation of a stepwise logistic regression model.

Methods

We designed and distributed a short online questionnaire survey addressing questions about a constructed abstract entitled "Association of transfusion and clinical outcomes in a large cohort" among the participants of ISBT 2012, Cancun. In addition to asking questions about the demographics (age, sex, country of employment, and highest education level) of the participants, we designed seven statements representing possible interpretations of the findings presented in the abstract and asked the participants to mark Agree, Disagree or Do Not Know for each statement.

Results

Based on the responses to these statements, we quantified the proportion of participants who inferred causality from stepwise multivariable models built to examine a question of association (or prediction).Thirty to 40% of the respondents agreed that a stepwise model was a valid method to adjust for confounding, and 60% of them agreed to a causal interpretation of a model built for prediction purposes.

Conclusion

These findings suggest that a large proportion of ISBT visitors confuse etiology with prediction in the published transfusion medicine research.

Significance

Using the results as a platform, we aim to delineate the distinction between etiologic and prediction research, issues of confounding accompanying these research aims and how a multivariable model deals with confounding.

INTRODUCTION

There is considerable controversy regarding the beneficial and adverse effects of blood transfusion[1]. In published observational studies, blood transfusions more often than not are associated with worse clinical outcomes[2;3]. Some authors may present these associations using terms that suggest causality. There is a question, however, as to whether proper methods have been used to investigate cause-and-effect relationships (as opposed to merely describing apparent associations) and to arrive at the stated conclusions. Statements on etiologic relations (that is, cause-and-effect relationships) and statements on predictive associations are frequently confused in the medical literature. This is most often illustrated by the fact that investigators use methods of prediction research to investigate etiologic research questions[4;5].

The aim of prediction research is to measure the likelihood of occurrence of a disease based on risk factors associated with the disease; the aim of etiologic research is to examine the causal relationship between a risk factor and the occurrence of a particular disease or outcome.

In transfusion-related studies, this misuse of prediction research methods intended for etiologic (causal) questions leads to misinterpretation of the "independent risk factors" identified in multivariable regression models as causal factors. To investigate the extent of this misinterpretation (or the extent to which an inference of causality was drawn from multivariable models), we conducted a survey of transfusion-medicine specialists, clinicians and researchers who attended the International Society for Blood Transfusion conference in Cancun in July 2012. In this report, we use the results of the survey as a basis for discussion of the methodological differences between etiologic research and prediction research, including the role of confounding and how multivariable models should be used differently to study questions from prediction research versus etiologic research.

QUESTIONNAIRE BASED SURVEY METHODS

We designed a short online questionnaire survey addressing questions about a constructed abstract entitled "Association of transfusion and clinical outcomes in a large cohort" (Figure 1). This abstract was based on an actual abstract presented in one of the previously-held major hematology conferences. The questionnaire was sent out to the International Society of Blood Transfusion (ISBT) members who attended the ISBT conference at Cancun, Mexico in July 2012. In addition to asking questions about the demographics (age, sex, country of employment, and highest education level) of the participants, we designed seven statements representing possible interpretations of the findings presented in the abstract (or of the inferences that could be made from the methods used in the hypothetical study), and we asked the participants to mark Agree, Disagree or Do Not Know for each statement. Based on the responses to these statements, we quantified the proportion of participants who inferred causality from stepwise multivariable models built to examine a question of association (or prediction). An Apple iPad 2 was awarded to a lucky winner out of all the participants who completed the questionnaire.

RESULTS FROM THE QUESTIONNAIRE

Of 435 participants, 398 provided complete demographic information. The mean (Standard Deviation) age of participants was 52.6 (11) years and did not differ between male and female attendees. Nearly 40% of participants had a master's degree/ professional degree or field of specialization; 35% of participants had a doctorate (Appendix- Figure A). Almost a quarter of all responders were employed in Northern or Western Europe, 18% in North America and 12% in South East Asia or the Indian subcontinent (Appendix- Figure B). Not all participants responded to all 7 statements of the questionnaire pertaining to the interpretation of the results of the abstract, with fewer and fewer participants committing themselves to each successive statement. In this way, 351 participants answered the first statement, while 298 participants responded to all 7 statements.

Figure 2 presents the seven abstract-related statements and the frequency of responses to these statements. Statements 2, 4, 5 and 6 deal with how methods of prediction research are misused in studies aiming to answer cause-and-effect research questions. Between 35 and 50% of responders agreed (wrongly) with these statements (Figure 2). Statements 1 and 7 straightforwardly refer to a causal interpretation of the research aim of the abstract, despite the fact that prediction research methods were used in the study. Between 45-60% responders agreed (wrongly) with these statements.

The answers to statement 3 were difficult to interpret since the phrasing "cannot" might have been interpreted as "were not" by survey participants.

The correct combination of answers was disagree, disagree, agree, disagree, disagree, disagree and disagree. This combination was "achieved" by 17 participants.

CONCLUSIONS BASED ON SURVEY RESULTS

Our findings show that almost 60% of our sample of attendees at the 2012 ISBT conference at Cancun inferred causality from models built to serve mere prediction purposes (Figure 2, statement 7).

DISCUSSION

How do questions addressing etiology differ from those addressing prediction?

"Predicting outcomes is not synonymous with their cause. Every causal factor is a predictor—albeit sometimes a weak one—but not every predictor is a cause"[6].

An Etiologic question by definition aims to *establish causality*. In other words, the aim is to asses if an outcome can be attributed to a particular risk factor, following adjustment for other potentially causal factors or confounders. The focus usually is on a single potentially causal factor at a time and confounding is the cardinal issue that must be dealt with in etiologic research. This is discussed in detail in the following sections.

A Prediction question by definition is *merely descriptive*. It aims to estimate the probability of occurrence of an outcome given a combination of a patient's clinical or non-clinical attributes. Predictive research does not asses the cause of the outcome; predictive factors may or may not be causally related to the outcome but confounding is a non-issue in predictive research[6]. The development of the widely used Sokal Score for the prognosis of chronic myeloid leukemia (CML) is a good example of predictive research. In this instrument[7], age, cytogenetic evaluation, circulating blast cells and spleen size are used as predictive factors to estimate the probability of a good versus bad prognosis for CML patients. The factors included in the instrument need not be causal (for example, spleen size may be predictive of the outcome in CML, but is surely not the cause of CML), nor do they need to be associated with mortality from CML; all they need to do is predict the course or prognosis of CML.

The importance of confounding (especially in etiologic research as opposed to prediction research) makes it essential to clearly define the study aim and the risk factors in question; to understand which multivariable methods have to be used for etiologic (as opposed to predictive) questions; and also to understand what multivariable modeling can (and cannot) do. The bottom line is that a different model needs to be built if the question addressed by the study is etiologic versus predictive.

Which myths on multivariable modeling lead to its misuse?

What does a multivariable model do? A multivariable model attempts to *describe* the data using multiple independent variables. Depending on the method to select these variables, a multivariable model attempts to create the best model fit. Given the information (from independent variables) at hand, a multivariable model attempts to determine a formula (or a regression line) which best describes the data.

Myths of multivariable models in etiologic research

A common assumption is that a multivariable model, resulting from entering multiple variables available from the dataset into a logistic (or linear) model, will ensure that all confounding in the study has been taken care of. This is an incorrect assumption. Three key issues need to be addressed regarding confounders and a multivariable regression model- a) selection of confounders, b) possible introduction of selection bias by adjusting for a non- confounder and c) treatment of intermediate variables as true confounders.

a) Confounder selection with stepwise regression

"The data analyst knows more than the computer and failure to use that knowledge produces inadequate data analysis"[14].

Automated procedures like stepwise regression are used to select variables (covariates- a covariate is a variable in a multivariable model) for a multivariable model, based on the p values. A multivariable stepwise regression uses every strong (statistically significant) association (between each of the variables and the outcome) to describe the data. Although statistically strong predictive associations between covariates and the outcome need not be causal, they are mistaken as causal when the results are interpreted.

In essence, a researcher lets the software decide on the covariates based on the entry criteria of the stepwise regression, which is a low p value or some equivalent of the p value. However, by this approach, important confounders can be removed from the model whereas "background-noise" variables may be retained[15]. This problem has been recognized and eloquently summarized before[16]. Subject matter knowledge coupled with good statistical oversight offer better guidance and they are essential to ensure that only relevant covariates are selected and entered into a model[8].

b) Introduction of selection bias by adjusting for a non- confounder

Consider an example below where one intends to examine the association between presence of HLA antibodies in patients and mortality. We will proceed with an example using directed acyclic graphs[9] (DAGs, Appendix: Rules for drawing a Directed Acyclic Graph) as a visual representation aid. Few things that we know *a priori* are (see figure 3 below):

1. Patient's sex is associated with the presence of HLA antibodies. Women tend to have a higher rate of HLA antibodies due to pregnancy.
2. Patient's age is associated with mortality. Older patients are at a higher risk of dying.

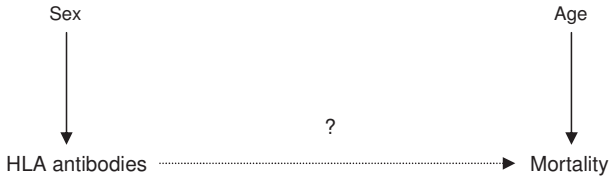

Figure 3

Next, one decides to adjust for number of red cell transfusions as a confounder. A couple of things we know *a priori* are (see figure 4 below):

1. Number of transfusions is associated with patient sex. Male patients on average receive more transfusions than female patients because of a higher rate of traumas and cardio-vascular surgeries.
2. Number of transfusions is associated with patient age. Older patients tend to receive more transfusions than younger patients due to the severity of the underlying diseases.

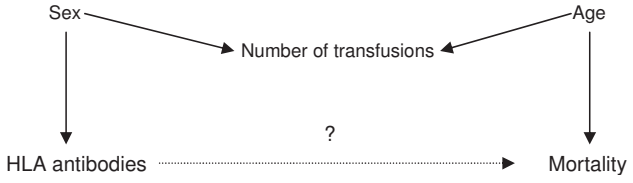

Figure 4

Now, controlling for number of transfusions would create a (false) association between patient sex and age (see figure 5 below). This implies that if patients with higher number of transfusions are female, they must be old; while younger patients must be male. Otherwise they would not have received these many transfusions. Thus, the probability of dying is higher in women, due to the average older age in this group, which is a result of the false association created by controlling for the number of transfusions. HLA antibodies occur predominantly in female patients and therefore also become associated, through higher age of female patients, with mortality.

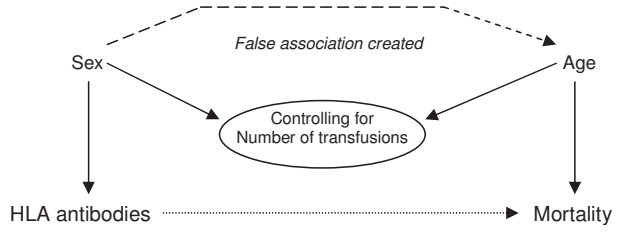

Figure 5

This is a classic example of "M- Bias"[9] (referring to the shape of the DAG) in epidemiological research methods which induces a spurious statistical association between two variables[9;10].

c) Adjusting for an intermediate variable in the causal pathway

As an illustration, one can think of a study aiming to examine a *causal* relationship between a patient's hemoglobin level and mortality. It is often presumed that entering the number of red-blood-cell (RBC) transfusions received by the patient into the model will control for confounding by transfusion. This is an unfounded assumption because such a variable is not a true confounder but an intermediate variable; consequently, entering it into a multivariable model will not ensure that it has been adjusted for. This is illustrated in more detail in the next section.

The myths of multivariable models discussed above do not pertain to prediction models, but etiologic research models only.

CONFOUNDING

A variable is a confounder if it is a common cause of the single etiologic determinant of interest (the "exposure") and the outcome under study. For example, in a study (Figure 6 below) to determine the association between number of RBC transfusions and mortality, the severity of a patient's illness would be a confounder.

In an etiologic study to determine if the number of transfusions causes patient mortality, the severity of a patient's illness would be a confounder which distorts any true

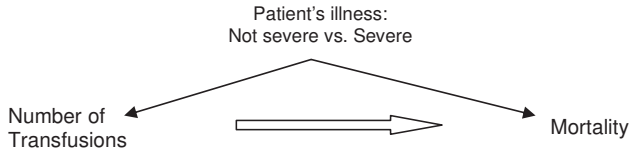

Figure 6

causal relationship between the number of transfusions and the mortality. Directed Acyclic Graphs (DAGs)[9] are an elegant way to visualize and thus facilitate identification of potential confounders. It is important to adequately plan the measurement of potential confounders in the design phase of the study. In this way, they can be documented during the study and then controlled (adjusted) for in a multivariable model. (see below)

Next, a variable is *not* a confounder if it merely lies in the causal pathway between the exposure of interest and the outcome. The exposure of interest should not determine the presence or absence of this "confounder". For example in figure 7, we show the association between receiving plasma or platelet transfusions and mortality, among patients who experienced massive blood loss after the transfusion event. Decreased blood loss after platelets/ plasma transfusion is not a true confounder; instead, the decreased blood loss after plasma/ platelet transfusions is a causal intermediate variable in the pathway between plasma/ platelet transfusions and mortality (Figure 7 below).

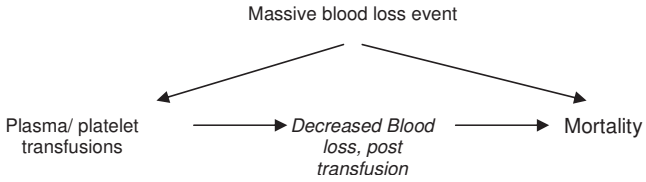

Figure 7

Confounding by Indication

Patients receive blood transfusions based on their need for such intervention, in other words, based on their indication for transfusion. Transfusion medicine research with causal claims is rife with this type of confounding. This is particularly the case if researchers attempt to find causality between transfusions and adverse outcomes.

Sicker patients tend to get more transfusions, and also tend to have worse outcomes. This causes distorted associations between amount of transfusions and worse outcomes, and consequently the danger that these associations are over-interpreted. This type of confounding usually goes unmeasured[11], but if measured well, it could be dealt with[12]:

a. at the stage of study design by 1) clearly defining the outcomes of interest and the exposure; 2) careful considering other determinants which may be related to the risk

factors for the outcomes (for example, by careful consideration of the clinical setting in which blood is given) and 3) only studying patients with similar risk factors and measuring such risk factors well to allow for correction at the analysis stage;

b. at the stage of statistical analysis by using stratification of patients in risk groups. This approach helps to characterize the interdependence between the potential confounding factors and transfusion (which is the single exposure of interest).

In summary, identification of all possible and likely confounders (and illustrating them for example, by using causal diagrams/ directed acyclic graphs) and adequate measurement and correction for the identified confounders are all necessary in etiologic/causal research. Computerized stepwise multivariable modeling on the basis of p values is the wrong method for doing this, because it may very well omit true confounders. Why this is so will be further illustrated in the next three sections.

How regression modeling deals with (or adjusts for) confounding in etiologic research?

Step 1: Unadjusted Regression model

Regression is a technique used to assess the association between two variables. For example, looking at the association between number of RBC transfusions and patient mortality (Fig. 8) the linear regression equation would be $y = \beta_0 + \beta_1 x$, where

y = outcome: mortality

x = determinant: number of transfusions

β_0 = intercept: estimated value of y when x = 0, (Base value)

β_1 = coefficient (slope of regression line): change in mortality risk per unit increase in the number of transfusions

The regression equation is Mortality = Base value + (co-efficient*number of transfusions)

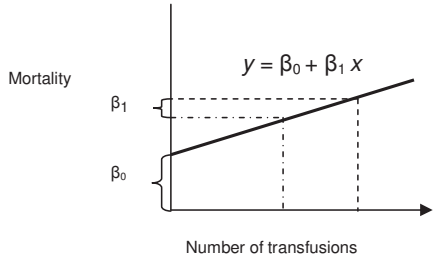

Figure 8. Unadjusted model

Figure 8 above represents that for each additional RBC transfusion, the risk of patient mortality increases by a factor of β_1.

Confounder

As described previously (Figure 8 above); severity of illness will be a confounder if it is a common cause of the outcome of interest (mortality) as well as the exposure (number of transfusions). We present it graphically in two steps.

Step 2: Confounder associated with the outcome

Severity of illness is associated with patient mortality. More severely ill patients will have a higher risk of dying (see below, figure 9).

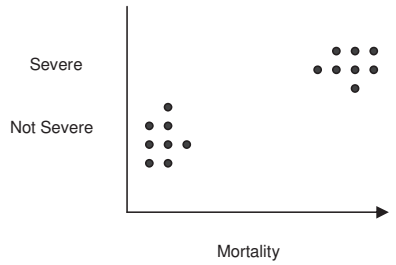

Figure 9. Severity of illness as the cause of mortality

Step 3: Confounder associated with the exposure

Severity of illness is also associated with the number of transfusions. More severe patients will receive a higher number of transfusions (see below, figure 10).

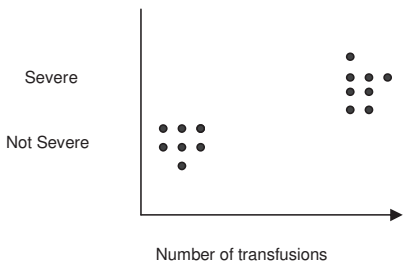

Figure 10. Severity of illness as the cause of number of transfusions

Step 4: Adjustment for confounding

We can visualize the confounding by stratifying the data by severity of illness or by correcting for the confounder as in Fig 11 below. By doing this, we indeed observe that the patient mortality is not dependent on RBC transfusions (it is a horizontal line) if we examine this association separately within the group of severely-ill and within the group of not-severely-ill patients. Yet, the unadjusted (dotted) regression line in Step 1, Fig 8) shows an association

between increasing number of transfusions and risk of dying, because the model is merely trying to best fit a line through the data to account for the increased mortality in the severely-ill patients. However, the resulting fitted line does not depict a true relationship between the number of RBC transfusions and mortality; instead, it demonstrates illness severity as a cause for both the number of RBC transfusions received and mortality (shown in Step 2 and Step 3). By stratifying the data into potential confounder categories, it becomes apparent why the unadjusted line shows the association of mortality with a patient's increasing number of transfusions (Fig 11).

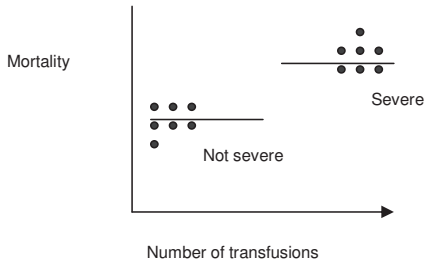

Figure 11

Building a multivariable model in predictive research

Predictor selection

Conduct univariate analysis for each (previously identified or newly suspected) predictor. This provides an idea on potentially important predictors based on their statistical significance, and it also serves as a guide to cluster/combine determinants/predictors.

Predictor inclusion

Based on the p values from the univariate analysis and pre-existing knowledge, costs, invasiveness of the tests or procedures pertaining to each predictor and other practicalities, run a multivariable analysis including or excluding predictors based on a pre-determined inclusion/ exclusion criterion based usually on p values. The next step is calibration and discrimination of a prediction model, which is beyond the aim and scope of this article.

Building a multivariable model in etiologic research

Confounder inclusion

One should include confounding variables based on a plausible biological hypothesis or an *a priori* reasoning, irrespective of their statistical significance, that is, regardless of the p values.

Confounder selection

One should first tabulate risk factors according to presence or absence of the exposure of interest to obtain a summary of risk-factor distribution in the study population. If a risk factor

differs between those with and without the causal factor of interest, it is a confounder of the association in the study.

When interpreting the results, one should discuss incomplete adjustment from residual confounding. This may occur if important confounders are not known or are not measured during the study; if an improper surrogate variable (for a confounder under investigation) has been used; or if the categories of a confounding variable have not been defined correctly, especially when recoding a continuous confounder in to a categories.

CONCLUSION

Our findings from a blood transfusion survey conducted among the participants of ISBT 2012, Cancun suggest that a large proportion of ISBT visitors confused etiology with prediction in the published transfusion medicine research. We feel that in the conduct of clinical transfusion medicine research, as well as at a major international transfusion and hematology conference, there is insufficient emphasis on research methodologies, strategies to handle confounding, and the fundamentals of data analysis. International and regional seminars, congresses, focus groups and working party meetings can all be opportunities to organize special education sessions on these matters. Furthermore, additional modules or sessions on research methodology must be added to the formal training of transfusion medicine researchers and specialists to better equip these professionals to bridge the gap between the evidence-base for transfusion medicine and clinical practice.

Focus Points

1. Association arising from a prediction research methodology does not imply causation.
2. Confounding is the cardinal issue that must be dealt with in etiologic research.
3. Causality is often implied from the output of a multivariable model. Careful review of study aims and research methods is required to interpret causation from association.
4. Multivariable model by itself does not take care of confounding.

No Conflict of Interest

The authors certify that they have no affiliation with or financial involvement in any organization or entity with a direct financial interest in the subject matter or materials discussed in this manuscript (e.g., employment, consultancies, board membership, stock ownership, honoraria), except as disclosed in an attachment. Any research or project support is identified on the title page of the manuscript.

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DETAILED READING

Causal diagrams

Greenland S, Pearl J, Robins JM. Causal diagrams for epidemiologic research. Epidemiology 1999;10:37-48.

Causal inferences

Robins JM. Data, design, and background knowledge in etiologic inference. Epidemiology 2001;12:313-320.

Confounding

Book by Szklo and Nieto: Epidemiology: Beyond the basics

Confounding evaluation

Hernan MA, Hernandez-Diaz S, Robins JM. A structural approach to selection bias. Epidemiology 2004;15:615-625.

Confounding by indication

Middelburg RA, van de Watering LM, van der Bom JG. Blood transfusions: good or bad? Confounding by indication, an underestimated problem in clinical transfusion research. Transfusion 2010;50:1181-1183.

Etiological and Prediction research

Book by Grobbee and Hoes: Clinical Epidemiology

CHAPTER 8

GENERAL DISCUSSION

The aim of the research presented in this thesis was to quantify the problem of alloimmunization among a general transfusions receiving, previously non-alloimmunized, and non-transfused population; and to examine potential transfusion-related and clinical risk factors associated with alloimmunization.

Every red blood cell transfusion (obligatory) introduces a myriad of foreign antigens, yet the majority of transfusion recipients do not alloimmunize against non-self red blood cells[1]. Alloimmunization is a multi-factorial immune event involving a genetic and a non-genetic component in which several risk factors need to be simultaneously present. In essence, every transfusion recipient has a unique or specific clinical profile, has been exposed to certain environmental immune modulating conditions and possesses a unique set of genes governing the immune response.

The primary aim of the R-FACT study at the time of its inception was to be able to classify transfusions receiving patients as a high risk or a low risk group for alloimmunization. If successful, a risk specific preventive matching strategy could be applied to especially such high risk patients thus avoiding alloimmunization in the first place. To put it in perspective, low at risk patients against alloimmunization would not require preventive matching strategies thus avoiding the logistical burden of obtaining timely and proper matched blood for their phenotype, as well as saving costs.

To study risk factors associated with a first time alloimmunization event in a general transfused patient population in an observational setting, it is essential to have a robust study design; and a considerable amount of time and effort is required. To appreciate the findings presented in this thesis, it is necessary to first discuss the nuances of the study designs chosen (chapter 2).

Our source population was based on an incident new user cohort. All the case patients were incident case patients with no prior history of transfusions and alloimmunization, to the best of our knowledge. Such a new user cohort avoids selection of prevalent transfusion recipients as well as existing (prevalent) case patients in the source population. Our data collection approach allowed a prospective follow up of previously non-transfused and non-immunized patients during subsequent transfusions up to the appearance of a first alloantibody. We thus feel that this cohort ideally represents the general transfused population and is appropriate to study the incidence of first time alloimmunization.

A matched case- referent study was designed where cases were defined as first time ever alloantibody formers against clinically relevant red cell antigens with no previous transfusion history. Next, selection of control patients is important, as it should be a representative sample of the source cohort. Potential controls were all consecutive first time transfused patients at our two study centers with no previous history of alloimmunization. For every case patient, we selected two control patients from the new user cohort, who had at least the same number or more transfusions than the case patient. This ensured that all the patients in the transfusion cohort with the same or higher number of transfusions had an equal chance of being picked as control patients. In essence, any member of the cohort (including case patients) who had been at a similar transfusion risk (of alloimmunization) at some point in

their transfusion history could be selected as a control patient. In short, we used a risk-set sampling strategy (*Book: Modern Epidemiology- 3rd edition by Kenneth J. Rothman; chapter 8- Case- Control studies- Variants of case- control design*), and then matched control patients to the case patients on the number of transfusions received up until antibody formation, and the hospital. Straightforward comparison of alloimmunizers and non- alloimmunizers from a hospital database leaves room for selection bias where control patients do not represent the source population well in terms of transfusion exposure and well as other risk factors[2].

Further, since the exposure itself is the foremost risk factor for an adverse event, we matched the case patients and control patients on the number of transfusions to study the clinical risk factors in a matched case- referent study design. In the studies where we used a follow up new user cohort design (chapters 3 and 5), we stratified patients on the number of red cell units and presented their alloimmunization risk. This was especially relevant since a transfusion and every subsequent transfusion might present a different risk of alloimmunization. Thus, by using a Kaplan- Meier survival analysis and using the number of cumulative transfusions as the time axis, we were able to quantify alloimmunization risk with a relative simple but elegant approach.

To study immune modulating clinical risk factors and their effects of alloimmunization, another obstacle needs to be addressed. It is important to identify and define a clinical risk period (or a so called implicated period) in which the antigen mismatch (exposure) coincided with pre-defined risk factors leading to modulation of the immune.

A "clinical risk period for alloimmunization" or an "*implicated period*" was thus defined for cases as the period in which transfusion most likely caused the observed primary alloimmunization. This implicated period was the time (in days) between the last transfusion (N^{th}) before the first positive alloantibody screen and 30 days earlier[3]. A similar implicated period was selected for the controls, which was a period of 30 days preceding the N^{th} transfusion. Potential transfusion related risk factors and clinical risk factors present in this implicated period were studied.

Confounding is a major concern in etiological studies. Combining a priori knowledge together with subject matter knowledge, we defined and measured the confounders and then adjusted for them in our analyses.

TRANSFUSION EXPOSURE

What was known?

Red blood cell transfusions likely determine exposure to alloantigens and the risk for subsequent antibody formation. Existing evidence quantifying the problem of alloimmunization has been overwhelmingly documented in either specific patient groups[4,5] or in patients with a pre-study transfusion history[6,7]. Evidence in the literature also pointed out to an increased risk of alloantibody formation with higher number of transfusions, although some of these studies again included patients with pre-existing antibodies[8] or included patients receiving extensively matched transfusions due to their predisposing conditions[9,10]. Alloimmunization

risk for first time ever formed antibodies as a function of the number of transfusions was not reported before.

Besides the number of transfusions as a risk factor against alloimmunization, the dose or intensity of these RBC transfusions could potentially also have an impact on alloimmunization risk. The impact of intensive (or massive) transfusions on adverse outcomes has been reported with massively transfused patients at a higher risk of developing systemic inflammatory response syndrome (SIRS)[11,12] and mortality[12] as adverse patient outcomes[11-13], yet their impact on alloimmunization was surprisingly unknown.

What did we add?

In **chapter 3**, we designed a new incident user cohort[14] study in a general transfused patient population with no pre-study transfusion and alloimmunization history documenting the cumulative incidence of a first time ever red cell alloimmunization. Stratified on the number of transfusions received, we found that the risk of alloimmunization increases up to 7% at the 40th transfused unit, and that the risk was comparable between men and women.

In **chapter 5**, using a new user cohort study design, we examined the association between transfusion intensity and the risk of clinically relevant RBC alloantibody formation in a previously non-transfused, non-alloimmunized cohort. Special emphasis was put on different amounts of intensive transfusions, since there is no consensus on a uniform definition in the literature[15,16]. However, we did not find a difference between the intensively transfused (with intensive transfusions studied separately as ≥5, ≥10 and ≥20 units transfused in 48 hours) and non-intensively transfused patients, and their risk of alloimmunization.

Interpretation

Patients receive mismatched blood during their transfusion histories, but most of these do not for alloantibodies against the mismatched blood. Patients who do not alloimmunize after few initial transfusions tend not to make antibodies against subsequent transfusions. We observed this in our study estimating the incidence of alloimmunization in a general transfused population and found no more than 7% alloimmunizers even with very high exposure of transfusions. The "responder" hypothesis[1,17] in this respect signifies that only a small part of the population is able to mount a red blood cell alloimmunization. Some additional patients however do form alloantibodies even up until 40th transfusion and within the studied patient cohort we could not yet observe a leveling of the frequency of alloimmunization as a function of exposure. The latter could indicate that there really exists a limited population of *responders*, who will eventually respond to an alloantigen. What we do can conclude is that within clinically relevant transfused amounts there is a very large population of patients that form no antibodies. Off course this large population includes patients that are heterozygotic for many antigens and that therefore despite receiving large number of transfusion may not encounter a rare antigen (for example- K, 9% in Caucasians, 2% in blacks and 25% in Arabs) mismatch and that thus are not triggered to form antibodies even in a large number of transfusions.

Secondly, within the responders we have to acknowledge that alloimmunization is a multi-factorial determined event, clinical and other non-genetic determined risk factors for alloimmunization that can only be detected in patients that detect non-self antigens. Finally, it cannot be said if responders are perhaps genetically programmed to *respond* or that non responders are genetically protected.

What next?

With the evidence from our study on the incidence of alloimmunization and the number of red cell transfusions, a hypothesis is generated that many patients with many exposures (more than 40 transfusions) may be required to assess if there is a fixed number of responders in the general transfused population. Using similar principles of incidence new user cohort but with a larger study population as well as longer follow up period in this respect would be useful in following patients who received greater than 40 red cell transfusions. Although there are very few patients receiving such large number of transfusions, and hence may not seem to be of clinical relevance, nonetheless this would enable to also identify a certain group of never-responding transfusion recipients. Such patients might harbor a potential "protective" genotype or phenotype that prevents alloimmunization.

STORAGE TIME OF TRANSFUSED RED CELLS

What was known?

Red blood cells undergo various biochemical and biomechanical changes during storage. Besides, there are residual leucocytes and platelets present in stored blood, and the accumulation of released lipids, cytokines and histamine have been reported in suspension solution[18-22]. The clinical importance of these changes in stored red cells is much debated[23-26]. Currently there is no evidence in humans if and how the present transfusion storage times modulate the risk of alloimmunization.

What did we add?

Using the case referent study design, we next studied if the storage time of transfused red blood cells was associated with the risk of alloimmunization, (**chapter 4**). Given our study design and population, we found that the storage time of red blood cells is not associated with post-transfusion risk of alloimmunization within the clinically relevant storage time ranges of 7-28 days.

Interpretation

An interpretation and explanation of the fact that we did not observe an association between older (or younger stored red cells) in our study could in theory be attributed to two "heightened" periods of danger that change differently with storage time – 1) increasing immunogenicity by leukocyte activity in fresher units and decreasing with storage, and 2) immunogenicity by accumulation of cytokines, lipids, histamines and micro-vesicles that increases with storage. The similar alloimmunization risk that we observed for 'old and

young' blood might thus be due to (e.g. the two mentioned) risk factors which sum remains constant throughout the investigated storage period.

What next?

Of conceptual value would be to study the effect of less than 7 days (very young) stored blood and blood stored for more than 28 days (very old) on the risk of alloantibody formation. The debate in literature is how long blood can be stored before it induces detrimental clinical effects. Studying such questions and comparing various contrasts from 7 days younger to 28 days and older would give an indication on "when does the stored blood start displaying immunologic activity"; the study on the biological mechanisms could follow.

PATIENT RELATED RISK FACTORS

Sex

What was known?

The alloimmunization risk to red blood cell antigens is suggested to be higher among women as studies have pointed out female sex to be an independent risk factor for alloimmunization[2,5,27,28]. It is important to note that this higher risk in the above mentioned studies is found in the presence of various selection biases- a) higher number of transfusions[29], b) more women with diseases with an intrinsic higher allo-response like auto immune hemolytic anemia[30] or sickle cell disease and c) longevity of women with such diseases (sickle cell diseases[31]) and d) previous pregnancies as well known trigger/ primer for alloimmunization. The review[31] suggested that women not be considered as a high risk group for alloimmunization.

What we added?

In our new incident user cohort (**chapter 2**), we showed that the alloimmunization rate was comparable for men and women over the age of 45 years. Additionally, young women in potentially reproductive age who additionally received K- matched transfusions (as per transfusion policy in the Netherlands) showed an immunization rate comparable to men and older women who received equal amounts of only ABO-D matched transfusions.

Furthermore, in our case- referent study design, we examined the association of sex with the risk of alloimmunization and found again (results not shown in this thesis) to be similar between men and women.

Interpretation

It has to be noted that information on previous pregnancies in women was not available in our studies, due to the limited or no availability of this information in the hospital patient management systems. Yet, adjusting for other potential confounders including number of transfusions, age, co-morbidities etc., we feel that the female sex is not a risk factor for alloimmunization.

Immunosuppressive therapy

What was known?

Antigen mismatched transfusions are the most obvious requirement for an allo-response. However, inflammation state of a transfusion recipient as dictated by his or her clinical morbidity are also likely to influence immune responses[17]. In this respect not only existing morbidities but also the use of concomitant medication could play a role in adaptive immune response towards alloantigens[2]. Diabetes, solid malignancies and progenitor cell transplants were associated with a higher risk of clinical alloimmunization[2]; and lympho-proliferative disorders and atherosclerosis with a lower risk of alloimmunization[2].

To study potential patient related risk factors against alloimmunization, we identified immunosuppressive therapy as one of the most interesting starting points. We found this especially interesting because many patients – such as trauma patients, intensive care patients, patients with active autoimmune disorders, patients with cancer and patients undergoing organ transplants- receive both red cell transfusions as well as immune suppressing drugs-. Use of corticosteroids and other immunosuppressive therapy among a general transfused population and its implicated inhibiting effect on the risk of alloimmunization against clinically relevant red cell antigens, however, has not been studied.

What did we add?

Using the case-referent study design of the ongoing R-FACT study with matched cases and controls (**chapter 6)**, we found that exposure to immunosuppressives was associated with a lower incidence of clinically relevant red cell alloantibodies against donor red blood cells.

Interpretation

A causal nature of the observed association with use of immunosuppressants is biologically plausible. Immunosuppressive drugs, including corticosteroids have been shown to impair humoral responses to vaccines[32,33]; T- cells have been shown to lose their proliferative ability under corticosteroids and other immunosuppressive drugs impair T-cell responses[34-39]. With the notion that antibody responses against red cell antigens are T- cell help dependent, it is therefore likely that corticosteroids and the other immunosuppressive drugs might in part inhibit red cell alloimmunization via T-cell modulation. Of course, while only associations were tested, and while the observed immunosuppression therapy mediated risk reduction of alloimmunization need not be the entirely caused by this therapy, but a direct attributive effect is strongly plausible.

What next?

For many reasons, immunosuppressives cannot be standard administered to transfusion recipients in order to lower their alloimmunization risk. But importantly, this knowledge should be applied to a clinical risk score (in combination with other clinical risk factors) to discern a high (or low) risk patient group and give them pre-emptive extended matched blood.

Other clinical factors to consider would be presence or absence of chronic diseases like diabetes, auto-immune diseases, allergies; acute stresses like surgeries, infection, stem cell transplants and in addition- leukemia, carcinoma; thus a list of factors representing a generally activated immune system. Apart from that, certain medication types (anti-neoplastic medications, systemic hormones, antibiotics, chemotherapy) are likely to influence (or alter) the immune system's responses towards foreign antigens and thus the process of alloimmunization. Assessing them each in detail would shed light on a possible high risk group of patients who are susceptible to alloimmunize; which are the aims of the ongoing R-FACT study.

ADDITIONAL RISK FACTORS FOR FUTURE CONSIDERATION

Environment

What was known?

The "antigenic" environment or the nurture where one was born or raised with might also be associated with an altered "education" of the immune system and with it, another set point of the response to non-self antigens. Environmental factors such as exposure to helminthic, fungal and parasitic agents do play a role in modulating the general set point of the immune response at young age[40]. The same is true for living in unsanitary conditions and for unhygienic occupations throughout life[41].

The hygiene "hypothesis" in this respect is supported by epidemiologic studies and proposes that insufficient stimulation of T helper 1 cells (by bacteria and viruses) leads to an over active T helper 2 cell response skewing towards antibody mediated immune response[42]. It moreover, suggests that a lack of exposure to antigens, micro-organisms and parasites during early life could leave a person susceptible to immune system impairment in later life[43]. Certain autoimmune and allergic diseases have been linked to such skewed hygiene conditions[43,44].

What next?

Information on the antigenic environments during formative years- country, rural or urban places of residence, regular contact with farm animals and pets, stay at day care centers during childhood and socio-economic status information; could add to the knowledge in predicting a patient's risk against alloimmunization. This information on transfusion recipient's environment related immune modulation conditions is currently being collected via questionnaires in the R-FACT study. Such information on immune modulating environmental condition should be added as well to a prediction risk score discerning high and low at risk for alloimmunization patients. In addition, this information would also stimulate further research on the mechanism of immunization in general- on how T helper 1 and T helper 2 cell imbalances influence the immune responses.

Genetics

What was known?

A patient's inherent genetic predisposition to mount a response against alloantigens could be an additional important risk factor. HLA genes in this respect are particularly interesting because along with their polymorphisms, they have been related to autoimmune disorders and diseases which develop via T-cell mediated immunity[45]. Certain HLA (human leukocyte antigen) gene types indeed are similarly also associated with an enhanced response to red cell antigens like Fy^a (Duffy group), Jk^a (Kidd group) and K (Kell)[46-48]. Such evidence thus points to a set of genetic factors that predispose for being a responder[17] (or a non-responder). Such "nature" related factors might be especially important for lending credibility to the "responder theory" discussed previously. In this respect the risk of alloimmunization varying according to clinical and environmental factors should be especially studied in patients with the most favorable genetic make up to mount humoral immunity against red blood cell antigens. The evidence for this however needs to be expanded.

What next?

An interesting way to study these genetic factors could be to look at genetic markers which influence immune system and vaccination efficiency. SNP's in candidate genes (e.g. coding for HLA types) modulating specific and innate immune responses should be assessed. HLA types already implicated with some antigen groups should be extended to study for all the clinically relevant antigen types mentioned in the R-FACT study protocol. Admittedly, R-FACT study numbers so far are low to find any small effect. Merging the datasets and bio-banks (with stored patient tissue) with other ongoing initiative nationwide (or continent-wide) could yield potentially useful results.

Transfusing patients based on these genetic types would be an elegant yet currently an expensive solution. Perhaps, identification of a high at- risk sub-population would make transfusions based on extensive phenotype matching more viable and cost effective.

Given the evidence that we have been able to produce in our study population, with our study designs and the studied transfusion and patient risk factors; they could be tabulated as follows:

Transfusion and Patient risk factors	Risk of alloimmunization
Number of transfusions	Risk *increases* with the number of transfusions
Intensity of transfusions	*Similar risk* in intensively and non- intensively transfused
Storage time of red cells	Does *not* affect the risk of alloimmunization
Patient Sex	Does *not* affect the risk of alloimmunization
Patient Age	Does *not* affect the risk of alloimmunization
Immune suppressant therapy	*Decreases* the risk of alloimmunization

Next, assessing the scientific evidence on clinical transfusion medicine research, we observed that the investigators tend to use principles from prediction research to answer etiologic research questions. This often results in misleading interpretation of risk factor findings at hand[49-52]. Therefore it seems warranted to question in studies on transfusion associated risk factors- if and how multivariate models are being used and interpreted; and if the important issue of confounding is properly dealt with. To first investigate the public acknowledgement of these issues, we used a questionnaire-based survey to quantify the proportion of 32nd meeting of the International Society of Blood Transfusion ISBT 2012, Cancun, Mexico visitors who felt confident with a causal interpretation of a stepwise logistic regression model. Thirty to 40% of the respondents agreed that a stepwise model was a valid method to adjust for confounding, and 60% of them agreed to a causal interpretation of a model built for prediction purposes. These findings suggest that a large proportion of ISBT visitors (transfusion medicine experts) often confuse etiology with prediction in the published transfusion medicine research. Conclusions in present literature based on flawed study designs, methods and analysis are thus not often questioned. Using these results as a platform, we aimed to delineate the distinction between etiologic and prediction research, issues of confounding accompanying these research aims and how a multivariate model deals with confounding. To this effect, our **chapter 7** aims to provide an education based point of reference dealing with these issues.

Future research following our studies should pragmatically aim at identifying and studying other potential clinical risk or protective factors for alloimmunization. The research should be based on robust study designs and extensive data sets, inspired and aided by subject matter knowledge. Our ongoing R-FACT study (of which the first results are reported in this thesis) is in our mind an example of a setting wherein patient diagnosis, medication and therapy profiles, potentially immune modulating environmental factors in early life and importantly, certain HLA types, single nucleotide polymorphisms (SNPs) and other such indicators of humoral response can be studied extensively. The next step will be to combine the information from this thesis with the future results of the R-FACT study into, a clinical risk score to identify high (or low) risk groups for alloimmunization. Based on such a clinical prediction risk score – the eventual aim of the on-going R-FACT study – future patients might be selectively matched to their blood group phenotype.

In conclusion, the results from this thesis point to an increase in the risk of alloimmunization with an increased number of transfusions. Intensity of red cell transfusions and the storage time of red blood cells do not influence the risk of alloimmunization. For recipient related factors, the results differ. Surprisingly, risk of alloimmunization does not differ between men and women. However, use of concomitant immunosuppressives in patients receiving red cell transfusions decreases the risk of alloimmunization. The conduct of observational studies like ours, that make use of existing datasets, presents greater demands than is often realized, and needs considerable amounts of thought about the study design and analysis. In the research literature about transfusion medicine the pitfalls of confounding by indication are often neglected, and associations are confused with causality. Therefore, caution is often needed to

interpret the results from the existing literature in our field. Apart from the findings reported in this thesis, we hope that the studies that are presented will engender a robust debate about how to conduct clinical observational research on the hazard of alloimmunization by transfusions.

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ADDENDUM

SHORT SUMMARY

KORTE SAMENVATTING (DUTCH SUMMARY)

ACKNOWLEDGMENTS (DANKWOORD)

CURRICULUM VITAE

SHORT SUMMARY

A matched case- referent study was designed (presented in **chapter 2**) where a "clinical risk period for alloimmunization" was defined for cases as the period in which transfusion most likely caused the observed primary alloimmunization. Potential transfusion related risk factors and clinical risk factors were studied in this implicating period. We designed a new incident user cohort study in a general transfused patient population in **chapter 3**, and observed that the risk of alloimmunization increases up to 7% up until 40th transfused unit, and the risk was comparable between men and women. We then focused on the transfusion related risk factors for alloimmunization. Next, we studied if the storage time of transfused RBCs was a risk factor of alloimmunization in **chapter 4**; and found that given our study design and population, the storage time of RBCs is not associated with post-transfusion risk of alloimmunization within the clinically relevant storage time ranges of 7-28 days. In **chapter 5**, we examined the association between the transfusion intensity and the risk of clinically relevant RBC alloantibody formation in a previously non-transfused, non-alloimmunized cohort using another incident new user cohort study design; and did not find a difference between the intensively transfused (with intensive transfusions studies separately as ≥5, ≥10 and ≥20 units transfused in 48 hours) and non-intensively transfused patients, and their risk of alloimmunization. In **chapter 6**, we showed that the use of corticosteroids and other immunosuppressants was associated with a lower risk of clinically relevant red cell alloantibodies against donor red blood cells. Finally, using results from ISBT 2012 conference at Cancun, we aimed to delineate the distinction between etiologic and prediction research, issues of confounding accompanying these research aims and how a multivariate model deals with confounding. To this effect we provided educational messages that might serve as a point of reference to deal with these methodological issues in **chapter 7**.

SAMENVATTING (DUTCH SUMMARY)

In **hoofdstuk 2** hebben we een cohort gevolgd van patiënten die voor het eerst een transfusie ontvingen. Het risico op allo-immunisatie liep op tot 7% bij de 40st transfusie en was vergelijkbaar tussen mannen en vrouwen. Om transfusie-gerelateerde risicofactoren voor allo-immunisatie verder te onderzoeken hebben we vervolgens een gematchte case-referent studie opgezet (zoals beschreven in **hoofdstuk 3**). Hierbij werd een "klinische risico periode voor allo-immunisatie" voor cases gedefinieerd als de periode waarin gegeven transfusies het de hoogste waarschijnlijkheid voor het veroorzaken van een primaire allo-immunisatie hadden. Mogelijke transfusie-gerelateerd en klinische risicofactoren werden voor deze risico-periode geanalyseerd. Vervolgens onderzochten we in **hoofdstuk 4** of de bewaarduur van RBC een risicofactor voor allo-immunisatie was. Met onze studie-opzet en binnen deze populatie vonden we geen associatie van klinische relevante bewaarduren van 7-28 dagen met het risico op allo-immunisatie na transfusie. In **hoofdstuk 5** onderzochten we of transfusie intensiteit geassocieerd was met het risico op klinisch relevante RBC allo-antilichamen in niet eerder getransfundeerde en niet eerder ge-allo-immuniseerde patiënten en vonden geen verschil in het allo-immunisatie-risico tussen intensief getransfundeerde (gedefinieerd als ≥5, ≥10 en ≥20 eenheden binnen 48 uur) en niet-intensief getransfundeerde patiënten. In **hoofdstuk 6** laten we zien dat het gebruik van corticosteroïden en andere immuunsuppressiva geassocieerd is met een lager risico op klinisch relevante allo-antilichamen tegen rode bloed cellen van de donor. Tot slot hebben we resultaten van een survey onder bezoekers van de ISBT 2012 in Cancun gebruikt om het onderscheid tussen etiologisch en predictie onderzoek duidelijk te maken en de verschillende rollen van confounding en het gebruik multivariate modellen in beide typen onderzoek te benadrukken. Hiertoe geven we in **hoofdstuk 7** een educatieve uitleg over deze onderwerpen.

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CURRICULUM VITAE

Saurabh Zalpuri was born on the 19th of February, 1982 in Chandigarh, India to Susheel Zalpuri and Padma Zalpuri. In April 2000, at the age of 18 years, he graduated from Bharatiya Vidya Bhavan High School, Chandigarh with biology, chemistry, physics, English and sports education as core subjects. He then enrolled in Moscow Medical Academy I.M. Sechenov in September 2000 and graduated as a medical doctor in June 2006. Following his graduation, he was admitted to Utrecht University, Bio-Medical Sciences Faculty on a Utrecht Excellence Scholarship for a two year prestige master's programme in Clinical Epidemiology. During the master's programme, he completed his practical internship at the Julius Center, Utrecht under the supervision of Dr. Cuno Uiterwaal; an optional internship at Charité, Berlin at the Department of Social Medicine, and finished his master's thesis at the AIDS foundation East-West, Amsterdam. After graduating as a Clinical Epidemiologist in August 2008, he began his PhD project at the department of Clinical Epidemiology, Leiden University Medical Center and the department of Clinical Transfusion, Sanquin Research in Leiden. The results from this PhD project are described and discussed in this thesis. He is currently employed at MSD/ Merck, the Netherlands, as an associate principal epidemiologist.