Protocol Title: **Risk factors for alloimmunization after erythrocyte transfusion: a case control study.**

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R- FACT Study- Risk Factors for Alloimmunization against red blood Cell Transfusion
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Abbreviation List:

1. RBC: Red Blood Cell
2. TRIP: Transfusion Reaction in Patients registry
3. SNP: Single Nucleotide Polymorphism
4. HLA: Human Leukocyte Antigen
5. SES: Socio- Economic Status
6. EIN: Unit Identification Number
7. MEC: Medical Ethical Committee
8. CRF: Case Report Form
9. 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, 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 or resistance to immunization against erythrocyte alloantigens exposed to during that transfusion episode.

Study design: A nationwide multicenter case-control study.

Study population: Patients/cases will be formed by a first time alloimmunization episode during the study period. Controls are defined as transfused individuals matched to cases and that are not immunized. A third group of “non-responders” is defined as individuals that have received a multitude of transfusions without antibody formation at all.
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), 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 alloimmunisation 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 alloimmunisation risk would be a more feasible step forward. This strategy would be especially valuable because as soon as immunisation for one antigen develops, additional immunisations tend to develop more frequently.
Characterization of patients and clinical conditions with high immunisation risk can be derived from studying the possible correlations between the actual immunisation 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. An additional patient group that irrespective of many transfusions and thus, exposure to alloantigens still fails to generate antibodies could serve to define indeed protective factors explaining this group’s resistance to immunisation.
We expect a two-fold impact from our study: a) to identify a set of transfusion recipients who need to be extensively matched, and maybe also a set of recipients who are being particularly resistant to immunisation and need not be extensively matched, 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. In this respect, it is generally recognized that immunocompromised patients have a lower risk to develop such antibodies. Relatively little is known, however, about other patient-related risk factors.

A recent study examined such patient-related risk factors in a case-control study among 101 cases developing erythrocyte alloantibodies and 87 controls. 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 childbirth); lympho-proliferative disease and leukaemia (lower risk attributed to lymphocyte dysfunction by concomitant chemotherapy and suppression of the immune response). Also new and partly unexpected risk factors were found, such as diabetes and solid tumours (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. Unexpectedly in the previous study, the number of red blood cells received was not associated with a risk of alloimmunisation. The limitations of this case-control study, however, were i) the selection method for controls favoured 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 immunisation / 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. Moreover, we will characterize genetic variants in patients who never develop an alloantibody even after undergoing multiple transfusions and thus may express genotypes that protect against immunization to erythrocytic antigens. There is well documented evidence that certain HLA types are associated with enhanced
response to red blood cell antigens like Kell, Duffy and Kidd\textsuperscript{13-15}. 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.\textsuperscript{16} 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.\textsuperscript{17} 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.\textsuperscript{18,19} 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\textsuperscript{20}. The same is true for living in unsanitary conditions and for unhygienic occupations throughout life\textsuperscript{21} 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 centres 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 or resistance to immunization against erythrocyte alloantigens that he/she was exposed to during that transfusion episode.
4. METHODOLOGY

Study Design
A matched case-control study will be performed.
With an “ideal” set of cases and controls in mind, the study design should enable inclusion of cases matched to controls on the number of previous transfusions, the transfusion free interval, the number of donors, hospital and calendar date and importantly, on the same “antigen mismatch” i.e. controls should be equally mismatched on antigen exposure as the cases. The following example will help with better understanding.

With Kell (K) antigen as an example; the frequency of K antigen in the population is 9% and the frequency of K antibody in patients that are immunized after transfusion is 25%. Although 90% of patients are K negative and thus can be immunized against Kell, only 1 in 10 donors transfers this antigen. The high frequency of immunization against Kell seems thus to indicate a high antigenicity of Kell as compared to other antigens. An ideal set of a single matched case-control pair would mean that they are both Kell negative and both exposed to K antigen during transfusion while the case is immunized and the control is not. So from a list of potential controls, the ideal one would have the same K exposure status as the case: the same number of previous transfusions and transfusion free period (diagram 1). This will require a large sampling of the control population to ensure such ideally matched control. However, since we plan on sampling 2000 controls, we believe there will be enough proper matched controls at least for the most frequent antibodies. For less frequently found alloantibodies, we will follow the best possible approach by matching on estimations of antigenicity and frequencies of antigens.
Figure 1. Flowchart of Study Design for the matched Environmental control group
Diagram 1. The “ideal” Case-Control Matching

CASE

Same hospital and date for case and control

“X” Days

Transfusions

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CONTROL

“X” Days

Transfusions

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4.1 Design and Study Population

We propose to perform a case control study in which i) the cases are defined as patients with a first and most likely transfusion related immunisation against a RBC antigen. (This group of case patients will be compared with 2 separate groups of control patients, to study 2 different questions) ii) the selection of one group of controls- “environmental control group” that is matched on the number of previous exposure episodes to erythrocytes of the cases, iii) another separate group of patients- “genetic control group”, who have been proven to be extremely resistant (non-responders) to multiple erythrocyte antigenic exposures will be included iv) the number of included patients is sufficient to detect smaller effects and to adjust for other risk factors, and v) documentation of potential risk factors is performed more extensively. In detail, we will characterize differences in possible genetic and acquired determinants of immunisation in the different groups. Moreover, we will look for possible immune modulating external conditions (like medication or disease) in the immunisation implicated transfusion period.

The research objective will be approached as follows. We will perform a nationwide multicenter case-control study in the general transfusion population with cases as mentioned reported with first alloantibodies against transfusion received red blood cells. Controls will be derived from two source populations.

1. The first control group (environmental control group) will be matched to the cases on the number of previous erythrocyte transfusions, and the transfusion free/immunization period. These controls serve to assess the differences in exposure distribution to environmental risk factors as compared to the cases.

2. The second control group (genetic control group) will be derived from multi-transfused patients who have not developed any antibodies to red blood cells despite numerous transfusions. These controls are sampled to better identify genetic variants that protect against immunization.

We envision to contribute to a matching policy based on a prognostic risk score for immunisation in general transfused patients. That, however, will be part of future studies.

Case patients

Eligible cases of alloimmunization will be selected from all patients that are transfused and that have formed a new and first alloantibody against a RBC antigen.
The patient and the antibody type will be obtained from two sources: a) national registry of Transfusion Reactions in Patients (TRIP) b) large red blood cell products using hospitals nationwide. Partly because the reporting of alloantibodies to TRIP is optional, under reporting is estimated for this source. Therefore, we will also approach the largest hospitals directly. The case data collected from the hospital files will include a) information on potential risk factors of the patient, b) the (probable) causal transfusion and amount of transfusions preceding the antibody formation and c) identification of the RBC typing of the donors that are most probably implicated in the alloantibody formation via the EIN nos. In this manner we can calculate the exposure/ amount of the immunizing antigen.

As cases for our study will qualify:
- Patients for the first time diagnosed with transfusion induced red blood cell alloantibodies (= patients who have received transfusions before detection of alloantibodies).
- Patients that are not a priori receiving more extensively typed RBC transfusions.

**Matched control patients (Environmental Control Group)**

Selection of controls is done using matching, a sampling procedure that makes controls and cases comparable with respect to the study base. We will sample at least 2 controls from the case site for one case.

Control-patients are matched to a) the case-patients according to the number of donors (units of blood), b) number of previous red blood cell transfusion episodes c) the length of the transfusion free period, d) the hospital (of the implicated episodes in cases) and calendar date and e) on the same antigen mismatch as the cases. The same antigen mismatch (similar to the one experience by the cases) is significant as it makes the matched case- control pair comparable at baseline risk. Transfusion free period for cases is effectively defined as the time of positive antibody screening to the last negative screening, and the same period for controls is defined as the negative antibody screening on the same day (as case’s positive screening) to the last negative antibody screening. (Figure 1)

We will allow for flexibility in the matching criteria and select for the closest match, (on the defined matching factors) to the case patients.
From controls thus qualified, we will similarly obtain the donor blood type via the EIN numbers of the transfused donor products.

**Unmatched control patients (Genetic Control Group)**

Based on our hypothesis that some genetic variants have a protective effect against the immunization to erythrocyte antigens, we will sample another control group who have undergone > 30 transfusions and >3 transfusion events, and have not developed an immune response to erythrocyte antigens. The association between these genetic variants and the inability to develop erythrocyte alloantibodies will be thus assessed.

### 4.3 Data acquirement, measurements and handling

Data will be acquired first from TRIP, 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 haematological, 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.

For the case patients, these conditions will be measured during the time leading up to the implicated transfusion episode or the immunization period. For controls, the measurements will be recorded for the same interval i.e. between the 2 negative screenings. This information is well documented in patients’ medical case records and files and will be obtained from their respective hospital databases.
Donor blood type information, which will be critical in documenting the donor-patient blood type mismatch, will be obtained from the electronic transfusion records from the hospitals as well.

4.3.2 Questionnaire
Participants will be asked to fill out a printed or a web-based questionnaire. Normally, after detection of an antibody/alloimmunization episode, a Blood Group Card is sent by the site/hospital to the cases informing them about the condition, we propose to use or follow up on this action with an additional explanatory note about the study and a questionnaire along with it. 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.

4.3.3 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 titres of
antibodies against common (vaccinated) antigens can later be determined in the plasma and serum that are stored as well.
4.5 Statistical analyses and power

Based on the current TRIP incidence figures in the Netherlands (involving patients with clinical relevant transfusion induced alloantibodies), we expect to include a total of 1000 case patients (500 per year).

For the first matched Environmental control group, we plan to take 2 controls for every case, into the study.

For the second unmatched Genetic control group, based on average numbers of patients (at Leiden University Medical Centre) with > 30 transfusions/ year, we hope to include 500 patients for identifying genetic variants that might be protective against immunization.

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 perform separate analysis for each antibody type. We will select all antigen negative subjects (for e.g. Kell negative) and stratify them according to the number of previous exposures to that particular antigen (for e.g. Kell antigen) and examine the association between the risk factor and alloimmunization using logistic regression. We will make thus 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 1000 cases and their controls, 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 alloimmunisation. Help of local investigators, usually the local haematologist 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 and at 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 explaining the purpose of the study. This letter will be combined with the questionnaire and foremost the informed consent declaration 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. There are different options that the patients can choose from for the blood taking: at their own hospital during the first coming admittance or outpatient visit, at the family practitioner and at the LUMC itself. 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 as well as locally, at the attending hospitals of participants, which ever is the more convenient option for them. There are no further interventions within the study protocol.

### 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.

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: Professor Rene de Vries. 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 favourable 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-control study will quantify and characterize risks of patients and conditions for transfusion associated alloimmunisation. 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 alloimmunisation 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 alloimmunisation.
Reference List


12. Hendrickson JE, Desmarets M, Deshpande SS, Chadwick TE, Hillyer CD, Roback JD, Zimring JC. Recipient inflammation affects the frequency and magnitude of immunization to transfused red blood cells Transfusion 2006 Sep;46(9):1526-36.


