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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