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Clinical determinants of red cell alloimmunizatiom, implications for preventative antigen matching strategies

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7

SUMMARY AND FUTURE PERSPECTIVES

Summary and future perspectives

In this thesis, we report on various determinants which we found associated with red cell alloimmunization in humans, with the eventual aim to reduce red cell alloimmunization and its potentially detrimental consequences by risk factor based matching strategies. Here, we first highlight the identified risk factors against the background of former evidence. Finally, we discuss future research perspectives.

Optimizing red cell antigen matching: critical antigens

Antibody formation to red cell alloantigens requires exposure to alloantigens together with a certain activation of the recipient's humoral immune system. The reported alloimmunization prevalences are surprisingly variable ranging from only 3% to as high as 58%.[1-9] These wide ranges likely reflect the differences in study designs and selected patients, e.g. inclusion of previously transfused and thus more exposed patients, inclusion of previously alloimmunized patients, and the length of serological follow-up. Our strategy of use of an incident new-user cohort enables estimation of the incidences of alloimmunization as a function of exposure within a cohort of transfusion-naive patients. With this approach, our group previously managed to confirm the intuitive assumption that the risk to develop alloantibodies increases with the transfusion burden.[10]

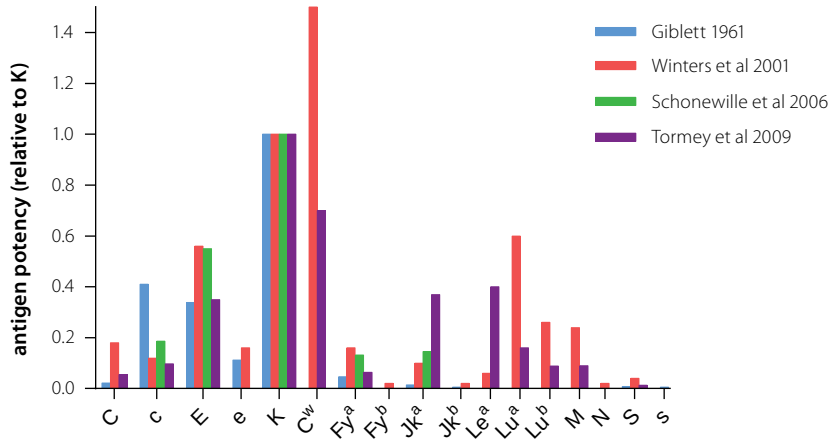
Expansion of our cohort from two to six participating hospitals allowed us to assess antigen-specific alloimmunization incidences and with it the exposure corrected immunogenicity of these antigens. In **chapter 2**, we illustrate that anti-E, anti-K, anti-Jk^a, and anti-c are the most prevalent formed alloantibodies among the 7.7% of transfused patients who formed alloantibodies after having received at least 40 units of red cells. With a policy of serological matching against their cognate antigens, the population would have benefited from a 74% reduction of red cell alloimmunization.

Considering prevention by matching for certain antigens, it is important to realize that antigens are not equally distributed nor are they equally immunogenic. Several studies reported on antigen immunogenicity estimates over the past decades.[11-14] Likely related to the often used 'Giblett-equation', these studies yielded conflicting conclusions regarding the potency of all antigens, except for K (Figure 1). Giblett-based calculations deduce immunogenicity estimates from prevalence figures by comparing the observed numbers of antigen-specific antibodies with the calculated probability of non-self antigen exposure.

However, several factors potentially influencing the results obtained by this equation need to be taken in consideration:

First, the Giblett equation is based on average antigen frequencies in a donor and recipient population of Caucasian origin and assumes that the chance of alloimmunization

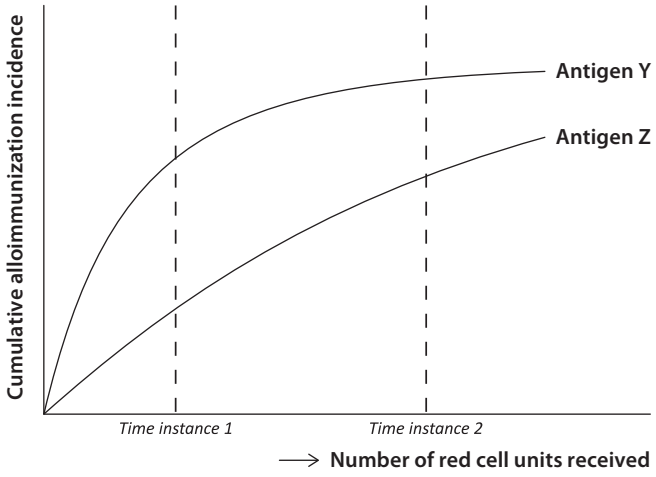
Figure 1 Summary of previously estimates of antigen immunogenicity with K as reference.



is linearly increasing with antigen exposure. However, it seems more likely that when one has not formed an alloantibody after multiply mismatched units, the likelihood to do so after the next mismatched unit will be even smaller because of the recipient being a so called 'non-responder' patient. Accordingly, most hemophilia patients receiving prophylactic clotting factor infusions form inhibitors to these products early after initiation of regular suppletion.[15] Consequently, for an antigen with moderate frequency (e.g. Jk^a), the cumulative incidence curve rises relatively early during accumulation of red cell transfusions and then flattens after *N* red cell transfusions, because patients lacking expression of this antigen will reasonably be fastly exposed and immunized. Contrary, for an antigen with low frequency (e.g. K) the initial increase of the incidence curve will be slower because, although most red cell recipients do not express the antigen themselves, this also applies to the donor population. Thus, the odds of encountering non-self K as compared to non-self Jk^a per transfused red cell unit are far lower. Figure 2 illustrates this exposure-related flattening for a fictitious antigen Y with moderate frequency and an antigen Z with low frequency. At time point 1, the number of patients who have formed anti-Y far exceeds those who have formed anti-Z, while at time point 2 these numbers approximate one another. Ultimately, prevalence-based immunogenicity estimates derived from the Giblett equation will induce an overestimation of the relative immunogenicity of low-frequent antigens (e.g. K), especially in a multiply transfused population.

A second important caveat in assessing antigen immunogenicity concerns current RhD matching strategies. In fact, the likelihood to be exposed to the C, c, E, and e antigens is determined by the influence of *RHD* and *RHCE* gene-linkage. That is, as only 6% of the

Figure 2 The estimated relative immunogenicity of antigens is dependent on exposure.



As fictitious antigen Z represents a low-frequent antigen as compared to the fictitious moderate frequent antigen Y, most responsive patients will have been exposed and thus have formed anti-Y at time point 1, while primary exposure still occurs after time point 1 for antigen Z. Consequently, the four-fold higher estimated potency of antigen Y as compared to antigen Z at time point 1 decreases to a factor 2 at time point 2.

Caucasian RhD-negative population express the E antigen,[16] RhD-negative patients receiving only RhD negative red cell products are rarely exposed to E. Conversely, as 67% of RhD-positive individuals lack E,[16] 23% of RhD-positive patients are at risk of E alloimmunization with routine RhD matching. None of the reported prevalence-based calculations accounted for this gene-linkage. Consequently, when matching for one antigen indirectly also involves matching for another (gene-linked) antigen, inaccurate estimations of antigen exposure, and thus of the antigens' immune system stimulating potencies, will be made.

Third, prevalence-based calculations estimate the antigen's potency relative to another (e.g. relative to K), however, do not inform about absolute risks according to alloantigen exposure. The latter, however, will be specifically decisive when debating extended matching for the individual patient.

Finally, previously reported studies did not consider higher immunization risks due to intrinsic antigen differences between e.g. Caucasian donors and recipients from non-Caucasian origin (e.g. hemoglobinopathy patients), neither considered the lower risk of some patients due to receiving extended matched products (e.g. auto-immunized or

previously alloimmunized patients or women in the reproductive age). As the odds for antigen exposure in all these patient populations differs from that in the general, non-extended matched Caucasian patient population, earlier presented immunogenicity calculations represent a mixture of these risks.

Considering the above, we limited our incident new-user cohort to primarily Caucasian red blood cell recipients, while we plotted the incidence of antigen-specific alloimmunization as a function of exposure to all units received and second to only all antigen-positive units received by all antigen-negative patients. The latter enabled us to deduce unbiased relative antigen immunogenicities from these incidences. With this approach, we confirm in **chapter 2** that K indeed is the most immunogenic antigen, followed by E, C^w, e, Jk^a, and c. Based on these data, prophylactic extended matching for the K, E, e, Jk^a, and c antigens would prevent 74% of primary alloimmunization events. While K, E, e, and c matching is often attempted for in high risk patients, prevention of anti-Jk^a is currently not aimed for in developed countries.[17, 18] Yet, our results underline the importance of Jk^a matching as this antigen is shown to be highly potent in eliciting an antibody response with the antibody known to easily induce complement mediated hemolysis. Although the observed high immunogenicity of C^w might come as a surprise, we do not recommend to implement extended matching for this antigen. The chance of subsequent exposure after primary immunization is only 2% per transfused unit[16] and, more importantly, severe hemolysis by anti-C^w is rare.[19-21]

Finally, we have to realize that even with complete (molecular) typing of red cell recipients, it is unlikely to find a completely matched red cell unit for all patients due to limited donor resources.[12] Furthermore, matching logistics will be even more compromised in countries with less organized blood collecting services as compared to the Netherlands, as well as in case of red cell recipients with a different ethnic background from the donor population, and in situations of acute need of blood and related lack of time e.g. acute trauma.

Notwithstanding the above, the in our studies provided knowledge on the potency of several red cell antigens hopefully enables clinicians and blood bankers to prioritize which blood group antigens should be primarily matched between donor and recipient.

Optimizing red cell antigen matching: identifying the critical patient population

Next to exposure to high immunogenic non-self red cell antigens, alloimmunization requires a recipient´s immune system to be capable of mounting a significant adaptive immune response upon exposure. Currently, there is a limited understanding of what factors dictate and which immune cells and signals are essential to this specific immune response in humans. In this light, the factors we found associated with alloimmunization risk need to be placed into the complex string of events leading to alloimmunization.

Inflammation as a modulator of alloimmunization

Contrasting the processing of most microbial organisms in lymph nodes, senescent and damaged red cells are primarily sequestered in the red pulp of the spleen. Under non-inflammatory circumstances, splenic macrophages play a major role in clearing these cells from the circulation via their SIRPα interacting with CD47 on red cells.[22-25] Lower levels of clearance seem to take place in the liver, while essentially no clearance occurs in peripheral lymph nodes.[23] Mice experiments showed that in the absence of inflammation these animals only rarely form red cell alloantibodies following red cell transfusions,[26] possibly related to the functioning of red pulp macrophages during these circumstances. Indeed, red pulp macrophages have been implicated in both the induction of regulatory T cells and the inhibition of $CD4^+$ T cell responses.[24] As red pulp macrophages are important for clearing ageing autologous hematopoietic cells, they are rightly situated to have a regulatory function protecting against harmful autoimmune responses.

It is generally believed that for a more effective adaptive immune response, antigenic stimulation needs to be accompanied by additional, often cytokine mediated, 'danger signals' originating from e.g. pathogen-activated innate immune cells.[27-29] In line with this, we observed an association between a patient's inflammatory condition due to infection at the time of red cell transfusion and the development of red cell alloantibodies. In **chapter 3**, we demonstrate alloimmunization risk to be modulated by the type of infection, its intensity, and the patient's inflammatory response. In detail, patients with severe (tissue-invasive) bacterial and viral infections demonstrated higher alloimmunization incidences as compared to the general transfused patient population. In intriguing contrast, blood-borne infections with Gram-negative bacteria (known to express LPS on the outer surface of their cell membrane) coincided with an almost 2-fold reduction of alloimmunization risk. The available evidence supports the following hypotheses on the underlying immunological mechanisms:

First, in murine red cell transfusion models, exposure to several pro-inflammatory pathogen-associated molecular patterns (PAMPs) such as poly(I:C) and hypomethylated CpG-containing bacterial DNA unequivocally promoted red cell alloimmune responses.[23, 26, 30-32] Of interest, not all inflammatory triggers unanimously enhance alloantibody production, as lipopolysaccharide (LPS) pretreatment was associated with substantially suppressed alloimmunization rates in mice.[31]

These sharp contrasting outcomes on red cell alloantibody production with mono-administration of poly(I:C) versus LPS in mice underline that, within a similar inflammatory clinical phenotype, different intracellular signalling cascades and specific gene expression profiles can be activated depending on the specific interaction with their receptors on innate immune cells (pattern recognition receptors, PRRs). [33, 34] With a cell specific distribution of Toll-like receptors (TLRs),[33, 34] it seems reasonable to argue that a specific type of innate stimulus evokes a specific innate immune response.

Currently, dendritic cells (DCs) have been shown to be key players in the process of red cell alloimmunization as they are the important drivers of $CD4^+$ T cell responses. Following LPS administration, impairment of in vivo $CD4^+$ T cell proliferation via malfunctioning of a specific type of splenic conventional DCs (named "bridging channel $CD8^-$ $33D1^+$ DCs") was identified to underlie diminished alloimmunization with allogeneic red cells transfused.[27] The authors ascribed this phenomenon to a LPS-induced preactivation of these DCs leading to a lost capacity to present red cell alloantigens. Yet, in light of the above mentioned experiments, one might question whether and how these tolerance inducing mechanisms are trigger-specific, i.e. LPS causing a restraint of conventional DC maturation and skewing red blood clearance towards the macrophages, while poly(I:C) and CpG predominantly trigger DC activation.

A second interesting suggestion can be derived from etiologic mechanisms of some autoimmune diseases in which immune activation due to antigenic mimicry by several microorganism has been postulated. [35-37] Similarly, in experimental models, previous exposure to microbial T cell epitopes via a pathogen with small peptide homology to red cell antigens was demonstrated to significantly enhance primary alloantibody responses.[38] Whether potential existing similar mimicry between certain bacterial or viral epitopes could have played a causal role in our observations, can yet not be substantiated. However, the distribution of alloantibodies known to also occur 'naturally' (i.e. supposedly originating from red cell antigen-microbe mimicry) did not differ between patients with and without severe bacterial infections, disseminated viral infections, and Gram-negative bacteremia, suggesting an at most minor influence of mimicry.

Finally, we should consider our results to be at least partly influenced by the clinical conditions and treatments that necessarily follow the specific infections we studied. These sequel mediated associations may have been missed and as such not included in our multivariate regression analyses. In this respect, various antibiotics, including some types of cephalosporins commonly used for the treatment of Gram-negative bacterial infections, are known to suppress mitogenic responses of B and T lymphocytes.[39-41] Similarly, severely septic patients in addition to antibiotics often receive corticosteroids to diminish the potential harmful effects of extensive cytokine release. In consistency with our findings reported in **chapter 5**, such treatments likely also attenuate the alloimmune response in patients with Gram-negative sepsis.

Unlike murine ´single-stimulus´ experiments, real-life microbial infections in humans thus bring along exposure to a spectrum of simultaneous modulators. In addition to the treatments given, one microorganism may contain various components of which some might suppress (e.g. bacterial lipopeptides and LPS) and others might stimulate (e.g. hypomethylated CpG-containing bacterial DNA) adaptive immune responses.[42, 43] As such, it might well be possible that the presence of two different species, although belonging to the same microorganism family (e.g. Gram-negative enterobacteriae), disparately affect red cell alloimmunization. Unfortunately, the size of the current study

limits us to properly evaluate this. So far, except for one study demonstrating non-significant enhancement of red cell alloimmunization in polyoma virus infected mice,[38] the immune modulating potential of individual microorganism species with regard to red cell alloimmunization has not been assessed. Hopefully in the future, larger data sets could further detail and substantiate the in **chapter 3** observed associations between types of infections and red cell alloimmunization.

The spleen's critical role

As mentioned earlier, the spleen´s unique anatomy and location amidst the circulatory system allows an intimate contact between its resident immune cells and blood cells passing this organ.[24, 44, 45] The spleen is a preferential site for follicular B cell maturation and critical for the survival of IgM memory B cells, the latter being a unique B cell population in the marginal zone of the spleen producing natural IgM antibodies to e.g. polysaccharide structures.[46-49] In asplenic patients, IgM memory B cells are absent and these patients are at increased susceptibility to encapsulated bacterial infections.

Although T cell dependent B cell responses are generally preserved following splenectomy,[46] the spleen has been shown pivotal for antibody production against both autologous and allogeneic hematopoietic cell antigens.[50-55] Even in the presence of pro-inflammatory poly(I:C) stimulation, splenectomized mice showed a substantially attenuated antibody production against allogeneic red cells,[23] which at least was due to an impairment of priming and proliferation of antigen-specific $CD4^+$ T cells outside the splenic microenvironment.[27, 52, 55] So far not evaluated, splenectomy may also result into a restraint of B cell priming together with red cells shunting away towards the highly tolerogenic hepatic compartment.[55, 56] As such, removal of the spleen is used as a beneficial treatment for steroid-refractory autoimmune-mediated thrombocytopenia (ITP) and anemia (AIHA).[57-59]

In **chapter 4**, we evaluated the role of the spleen and, more specific, a history of splenectomy in transfusion-induced primary red cell alloimmunization. Alloimmunization following splenectomy was a highly unlikely event (relative risk (RR) 0.04, CI 0.01-0.55). Only one patient among an estimated number of 443 splenectomized patients (0.23%) developed red cell alloantibodies upon subsequent red cell transfusions, which is in sharp contrast with the 2.1% alloimmunization prevalence mentioned in **chapter 2**. Intriguing, splenectomy did not prevent the induction of an anti-M antibody, implicating a maintained IgM memory B cell response in this single patient. Thus, although of substantial influence, splenectomy is here demonstrated not to completely abrogate red cell alloimmune responses. We hypothesize some remaining splenic tissue or previously immunized B cells transferred via a concomitant transplantation of a solid organ to account for this single immunization.

Our results seem in contradiction with the few published cross-sectional studies in thalassemic and sickle cell patients of which some reported splenectomy to be associated

with antibody induction and others did not find any association.[60-62] None so far observed an abrogation of red cell alloantibody induction following splenectomy. However, one should recognize that thalassemic patients in need of splenectomy are often highly transfusion dependent. As most of these former studies did not correct for the cumulative numbers of red cells units received at the time of primary alloimmunization, potential exposure-related confounding should be considered. In addition, incomplete reporting of data, such as the receipt of extended matched and/or leukoreduced blood by some but not all analyzed patients, may have further compromised the validity of these studies. Finally, none of these studies comment on the timing of splenectomy in relation to the primary alloimmunization. Possibly, some of the splenectomized patients were already alloimmunized before this surgical intervention. As such, we cannot exclude that the different results and conclusions of these earlier studies as compared to our study are to be explained by the presence of (indication) bias..

Following our results, concerns for red cell alloantibody development in anatomic asplenic patients who are in need of high numbers of red cell transfusions seem unnecessary. As such, they do not need products matched beyond ABO/RhD. Future studies will need to (re-)evaluate whether this conclusion can also be extended to other asplenic patient populations e.g. patients with functional hyposplenism associated to celiac disease, autoimmune rheumatic disease, or caused by vaso-occlusive sickle cell disease crises.

Treatment-related immunosuppression

Finally, in **chapter 5 and 6**, we illustrate the strong protective role of immunosuppressive therapy in general. First, patients using corticosteroids and/or other immunosuppressive agents demonstrated a two-fold decreased risk of red cell alloimmunization (RR 0.55, CI 0.34-0.91). Second, patients with acute leukemia (either of myeloid or lymphoblastic origin), with mature (B or T cell) lymphomas, or patients post-autologous or -allogeneic stem cell transplantation, demonstrated a three-fold decreased incidence of clinically relevant antibodies against red cell alloantigens, which could similarly be ascribed to immunosuppressive (chemo-/ immuno)therapy.

These are the first large studies to support decreased alloimmunization risk in immunocompromised patients. As such, although not coming to a surprise, they are of importance to the transfusion medicine field.

Contrasting our results, previous studies concluded oncologic patients to have a similar or even an increased alloimmunization risk as compared to the general transfused population.[4, 7, 9, 63, 64] However, as the likelihood that one has formed alloantibodies increases with the number of exposures, patients who have formed alloantibodies will in general have been exposed to a higher number of red cell transfusions as compared to non-alloimmunized patients. These earlier studies thus roughly compared high-intensively transfused patients with less intensively transfused patients. As illustrated in **chapter 2**,

such an analysis will without doubt reveal an existing association of red cell alloimmunization with diseases that are in general supported with intensive red cell transfusions. Indeed, myelodysplastic syndrome (MDS) has so far been defined as a risk factor for alloimmunization and matching for high immunogenic antigens in this patient population reduces alloimmunization.[65] Yet, the observed association between MDS and red cell alloimmunization seems not due to intrinsic characteristics of the disease as we here demonstrate, but is primarily explained by the fact that MDS patients are often transfusion dependent and, consequently, exposed to a much higher transfusion burden. Thus, MDS patients not receiving treatment have a similar red cell alloimmunization risk *per transfusion event*, and are even protected from alloimmunization during treatment with chemotherapeutic agents. Thus, in general, one should take into account both the expected cumulative exposure as well as current treatments with immunosuppressive agents with regard to matching strategies for the individual hemato-oncological patient. Indeed, a recent study did not find any benefit of additional Rh/K matching in patients with acute leukemia and lymphoma as hardly any patient formed antibodies,[65] likely related to their immunosuppressive condition.

Our RRs do not take into account the dosing of and duration of immunosuppressive treatments, and whether or not agents were received as part of a combination regimen. It is rational, however, to regard patients receiving multiple dose-intensive immunosuppressive agents as more likely unresponsive to red cell alloantigen exposure. Also, patients at advanced stage of treatment i.e. patients still under treatment and already having received a large number of immunosuppressive treatments, might be considered more immunosuppressed as compared to patients who just initiated their treatment course. In line, following chemo- and/or immunotherapy for malignancies, the immune system remains dysfunctional for a certain period of time, depending on the intensity of the received treatment.[66-69]

Our findings support the notion that dose-intensive immunosuppressive therapy is the principal determinant of alloimmunization as non-treated patients with acute leukemia and mature lymphoma showed similar alloimmunization incidences to patients without these disease entities. However, we cannot exclude non-measured confounders associated with the likelihood of not receiving treatment (e.g. co-morbidities and disease stage) to have counteracted diminished immune responses. Intriguing, but only of speculative nature, the observed effects could be partly due to a direct interplay between the tumor and cells of the immune system. This process of host immune system subversion is a common hallmark of both hematological and solid tumors. Inflammatory signals from malignant cells initiate the recruitment of immune suppressor cells such as myeloid-derived suppressor cells and Foxp3 expressing regulatory T cells. Additionally, the production of effector cell suppressing cytokines (e.g. IL-10, TGF-β, and TNF-α), and polarization from a T helper 1 towards a T helper 2 response consequently result into the establishment of a tumor tolerant microenvironment.[70-74] If this mechanism would attribute to the observed

diminished alloimmunization incidences in patients with hematological malignancies, one would expect that especially patients with advanced stage of disease independent of the receipt of treatment would be protected from red cell alloimmunization. Unfortunately, the patient numbers in this study were too small to discriminate the alloimmunization risk per stage of disease.

Optimizing red cell antigen matching: future perspectives

The ultimate goal of the ongoing R-FACT study is to eventually establish an accurate alloimmunization prediction model, thereby enabling practical and risk-based clinical decision on extensive matching. In this perspective, the identified determinants of red cell alloimmunization (whether associated to induction or protection) serve as an important start for such a model. Yet, continuing research is needed to advance our understanding of immunobiological process of red cell alloimmunization, and identify other relevant determinants of this process. Ultimately, our efforts should lead to a prospective study on the feasibility and efficiency of extended matching for high-risk patients, with risk classifications based on the here and in future to be identified determinants.

Nature and Nurture

Future research focusing on other clinical determinants of alloimmunization should emphasize on 'nature' and 'nurture' as patient-based modulators of red cell alloimmunization.

Regarding nurture, it has repeatedly been suggested that certain environmental factors skew adaptive immune responses. For example, (early) exposure to bacterial commensals and helminthes infections have been implicated to modulate the immune system towards protection against various autoimmune diseases such as type 1 diabetes mellitus by selectively modulating the T helper 2 response and driving the regulatory arm of the immune system.[75-77] The latter also seems to explain the low propensity to develop allergic disorders observed in helminth-infected cohorts.[78] Vice versa, the dramatic increase in atopic diseases in the developed world might be a direct consequence of the eradication of helminth infections.[75] Thus, growing up in rural areas or keeping domesticated animals during early childhood (both associated with high microbial burden exposure), one's dietary contents (associated to the biomass and diversity of gut microbiota),[79] and use of antibiotics among other factors may all modulate the patient's response to allogeneic red cell antigens, similarly as they do for chronic inflammatory disorders.[80-82]

Next, taking a patient's genetic constitution ('nature') into consideration when deciding on matching seems another interesting approach. Although the costs and logistic challenges of high-throughput genotyping of blood group systems are still high, these tools will likely become available on a more routine base. Thus, genetic risk factor screening could

in future be easily added to these blood group gene arrays.[83] As we more extensively discussed in **chapter 1**, only a minor fraction of the genetic basis of red cell alloimmunization so far has been elucidated, with the majority of studies merely having focused on HLA gene polymorphism associations.[84-90] Knowledge of this topic can be extended by learning from related diseases e.g. evidence on the polygenic nature of inhibitor formation upon factor VIII administration in hemophilia A patients. This disease and its treatment has several features in common with red cell transfusions e.g. administration of the product via the blood stream, a risk to induce alloantibody formation upon exposure, and (at least in a subgroup of patients) the administration of a human-derived product. Over the last decade, the hemophilia research field has made several steps forward by linking a large number of single nucleotide polymorphisms (SNPs) in immunomodulating genes to inhibitor formation including SNPs in the CTLA-4, tumor necrosis factor (TNF)α, and interleukin-10 (IL-10) genes. [91-97] Second, a genome-wide association study on RhD allo-immunization by Sanquin Research in collaboration with Cambridge University just recently finished its sampling of DNA material from over 2,000 pregnant women and will soon start its analysis.[98] Obviously, results from this study might be translatable to allo-immunization to other red cell antigens and should help design future genetic research on transfusion-induced red cell alloimmunization.

Consistent with research on hemophilia A, studies performed in patients vaccinated against hepatitis B, measles, and influenza have demonstrated that variations in genes controlling adaptive immunity may predict vaccine efficiency.[99, 100] Although targeting a different antigenic processing pathway (due to their non-intravenous administration and a common use of conjugates or other adjuvants), microbial vaccines similarly to red cell alloimmunization aim to affect T cell dependent adaptive immunity. As such, the genetic background of vaccine non-responders might overlap with patients not forming red cell alloantibodies despite repeated allogeneic red cell exposure. In this regard, meta-analyses for hepatitis B responses found evidence that variants in class II HLA and IL-4 were significantly associated with humoral immune responses.[100-103] Other studies suggested associations with SNPs in cytokine genes, cytokine receptor genes, and toll-like receptor (TLR) genes. A comprehensive overview of these studies has been recently published by Newport et al.[99]

With the human genome sequence being completely elucidated and current techniques enabling high-throughput genome-wide analyses, it now seems feasible to extrapolate the above mentioned evidence into a large scale case-control study on genetic risk factors that modulate red cell alloimmunization. In this regard, we are currently planning to further expand our ongoing R-FACT study by prospectively sampling patient material at the time of red cell alloantibody detection from both antibody responders and non-responders. Subsequent analyses on immunomodulating genes should at least focus on polymorphisms in HLA class I and II genes (preferentially in relation to antigen-specific alloimmunization), and genes related to immune cell signaling e.g. chemo- and

cytokines and their receptors, toll-like receptors, molecules involved in costimulation, and nuclear transcription factors.

In addition to the above mentioned nurture and nature associated risk factors, biomarkers of the immune status of the patient may be predictive of red cell alloimmunization. In a first (retrospective) analysis on this subject which we discussed in **chapter 3**, we did not observe any association of the level of leukocytosis and CRP values as markers of inflammation with alloimmunization, possibly due to the multifactorial nature of these parameters. Other immune markers such as quantity and functionality of B, helper T, and regulatory T cell subsets might be better discriminative. Yet, such an analysis requires a complex study encompassing a substantially large cohort of patients being sampled at fixed time points over a considerable period of follow-up.

Finally, IgG immune responses to childhood vaccinations, as well as (age-adjusted) titers of naturally occurring IgM antibodies against antigen A and antigen B may predict the response to allogeneic donor red cells. Especially the latter seems intriguing as these IgM responses represent T cell independent immune responses.[104] Interestingly, the spleen and its proper functioning seems essential both for induction of T cell independent memory B cell responses [44, 105, 106] as well as IgG responses to red cell allogens (as discussed in **chapter 6**).

Benefits of matching

The studies presented in this thesis have tried to find an answer on questions starting with 'who' and 'what'. Who deserves to receive extensively matched donor red cell units? What red cell antigens should be taken into consideration when deciding to transfuse extended matched donor red cells?

The 'why' question has not received much attention so far. Yet, it is the driving force behind our studies.

Extensive matching in patients with sickle cell disease and thalassemia has proven to be effective, although most studies did not directly compare extended with non-extended matched patients.[61, 107] Preemptive extended matching for selected antigens (here: matched for the antigens c, C, E, K, Fy^a, Jk^a, and S) as compared to merely ABO/RhD matching reduced the primary alloimmunization rate by 5.3% (8.1% versus 2.9%) in a cohort of patients undergoing elective (cardiac) surgery.[108] In a post hoc analysis on patients who received merely red cell units, this absolute risk difference increased further to 8.0% (9.4% versus 1.4%; confidence interval (CI) 0.4-16.0). Indeed, patients who received platelets next to extended matched red cell units had comparable alloimmunization rates as compared to those receiving ABO/RhD matched units as they developed (non-D) Rh and K antibodies after cognate antigen exposure through platelet transfusions. Therefore, the few residual antigen incompatible red cells in platelet products can counteract the potential effect of extended red blood cell matching with regard to red cell alloimmunization prevention. Future studies should explore whether less antigen exposure by single donor

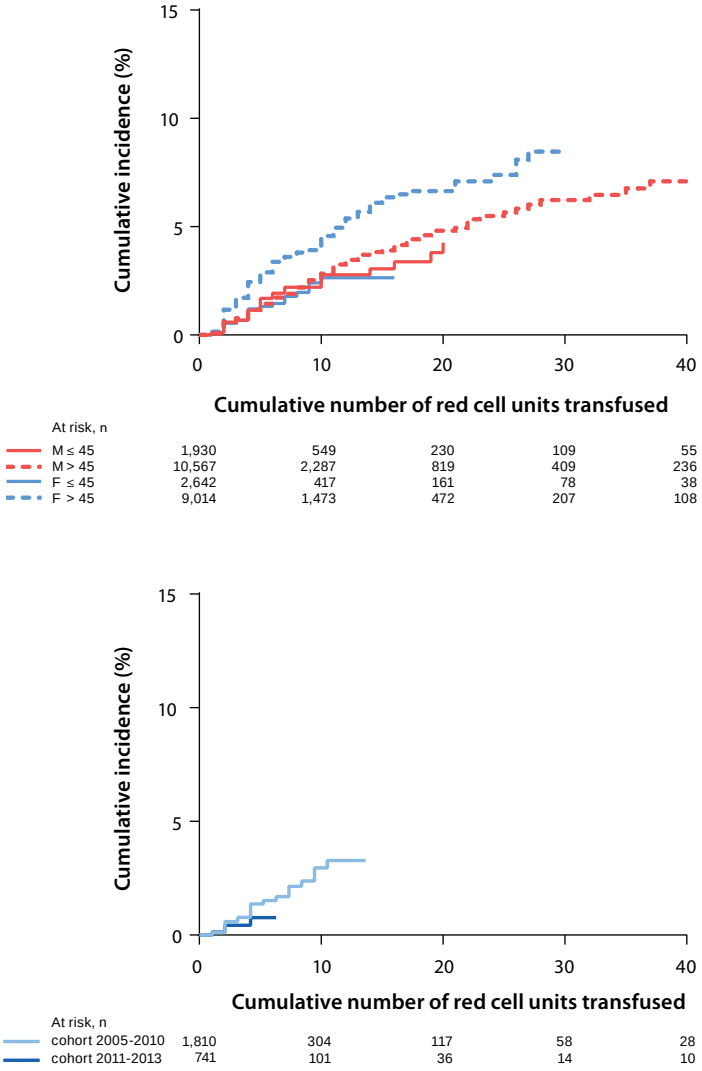
platelet apheresis products or even Rh/K matching with platelet transfusions could further reduce alloimmunization. Due to the low red cell antigenic load with platelet transfusions, we, however, estimate that the additive risk of non-matched platelet transfusions is negligible both for low immunogenic antigens and for the immunocompromised patient population.

In most cases, transfusions can be matched to existing antibodies thereby preventing antibody-antigen interactions and thus hemolytic transfusion reactions. Pregnancies, however, may be severely complicated by earlier alloimmunization due to ongoing maternal antigen exposure by the fetus. This especially accounts for earlier developed anti-K and anti-c.[109] For this reason, matching beyond ABO and RhD in women of childbearing age is nowadays routine practice in most European countries. In the Netherlands, women under 45 years of age receive K, and since 2011 additionally c and E, matched blood.[17] Although several reports have demonstrated an increased risk of fetal hemolytic complications for red cell alloimmunized pregnant women,[109, 110] no studies so far have assessed the beneficial effects of the introduction of K, c, and E matching in young women on alloimmunization incidences and its clinical consequences.

In this respect and in addition to our data reported in **chapter 2**, we observed significantly lower cumulative alloimmunization incidences among women under 45 years of age as compared to women above 45 years of age (4.4%, (CI 0.2-20.5) and 9.5% (CI 4.8-16.2) after 40 units received, log-rank p 0.013, Figure 3 upper panel). These differences seemed solely due to decreased Rh/K alloimmunizations in young women, since alloimmunization rates to non-Rh/non-K antigens did not differ between women under and above 45 years of age (Table 1). Furthermore, men under and above 45 years of age demonstrated similar alloimmunization incidences, excluding age as an explanatory factor. Of importance for accurate interpretation, the majority of the young women in our cohort received K, but not c and E, matched blood as this latter matching practice has only been nationwide established since 2011. Indeed, the 10 immunizations against c and E which we observed in women under 45 years of age all occurred before the introduction of matching for these antigens. In contrast, only one single anti-K immunization event occurred.

A preliminary analysis, which needs to be consolidated by extended follow-up, suggests a significant effect intensification from additional c and E matching (Figure 3, bottom panel). Only 4 of 741 (0.54%) women under 45 years of age consecutively transfused from January 2011 onwards as compared to 27 of 1,810 (1.49%) receiving red cell units between 2005 and 2010 developed alloantibodies. Due to the short follow-up and the consequently small cohort of women who received both K, c and E matched blood, differences fail to reach statistical significance (log-rank p 0.08). Notwithstanding the latter caveat, these findings strongly suggest that matching for E and c in addition to K is substantially effective in reducing alloimmunization rates.

Figure 3 Additional matching for K, c, and E protects against alloimmunization.



Upper panel: cumulative incidences of red cell alloimmunization according to age and sex. Lower panel: cumulative incidences of red cell alloimmunization in women under 45 years matched for K (cohort 2005-2010), and in addition also to c, and E (cohort 2011-2013). Only data from non-censored cohorts of at least 200 subjects are presented.

Additional antibody formation

Although the work presented in this thesis focuses on primary alloimmunization against a single antigen, it is well known that previously auto- or alloimmunized patients are at increased risk of developing additional antibodies with subsequent transfusions.[111, 112] As such, avoidance of exposure to antigens to which immunization has already occurred in addition to avoidance of exposure to high immunogenic antigens, is currently aimed for in these patients.

The risk to subsequently develop additional alloantibodies after primary immunization is patient-specific, i.e. resulting from a so-called high-responder phenotype to which nature and nurture associated factors contribute.[113, 114] In addition, immune activation by existing red cell alloantibodies themselves might play a role. In this regard, an immune response elicited to a particular high immunogenic antigen might enhance the response to weaker antigens. One potential mechanism, closely related to the earlier mentioned "non-exofacial polymorphic structures" (NEP) hypothesis (see **chapter 1**),[115] involves epitope spreading. Here, (an epitope of) antigen X appears in the context of a HLA class II molecule carried by a naive B cell as a result of this cell's phagocytic ability. When the B cell receptor (BCR) on this B cell is specific for antigen Y, subsequent activation of this B cell by a $CD4^+$ T cell which is sensitized against antigen X will lead to production of antibodies specific for antigen Y. Thus, immunization against antigen X may induce immunization against antigen Y.

Alternately, existing antibodies of IgG class can also suppress rather than enhance an immune response to non-cognate antigens.[116, 117] Here, phagocytosis of IgG opsonized allogeneic red cells via Fcγ receptors results into a rapid clearance of these cells from the circulation, thus preventing B cells from binding to other non-self red cell antigens. Additionally, IgG opsonized red cells may elicit inhibitory FcγRIIB signaling in B cells and as such prevent B cell activation. The earliest evidence for such an existing 'antibody-mediated immune suppression' (AMIS) was provided by the observation that ABO incompatibility between mother and child affords a degree of protection against Rh hemolytic disease of the fetus and newborn, because of anti-A or anti-B antibodies destroying the fetal red cells in the maternal circulation before immune system recognition.[118] Similarly, next to protecting against RhD immunization, prophylactic anti-D administration to women bearing a RhD-positive child was recently associated to an additional significant decreased risk to develop anti-E (personal communication, Zwiers, Koelewijn, van der Schoot et al, manuscript in preparation).

Current evidence of observational studies so far does not substantiate either epitope spreading nor AMIS to dominate immune responses in case of existing non-RhD antibodies. In this regard, in one study the type of first formed antibodies appeared not to be associated with the probability and type of the subsequent alloimmunization.[119] In agreement, we observed similar cumulative alloimmunization incidences to the non-matched non-Rh/K antigens in women under 45 years as compared to the rest of our incident new-user cohort (Table 1).

Table 1 Alloimmunizations to non-Rh/ K according to gender and age.

Number of transfused units	Men ≤45 yrs N=1,826	Men >45 yrs N=10,671	Women ≤45 yrs N=2,551	Women >45 yrs N=9,015
2	0.11 (0.00-18.19)	0.20 (0.01-1.82)	0.37 (0.00-5.47)	0.27 (0.01-2.02)
5	0.58 (0.00-9.00)	0.57 (0.08-2.39)	0.82 (0.02-6.59)	0.66 (0.09-2.75)
10	0.91 (0.01-9.76)	0.99 (0.20-3.22)	1.07 (0.02-8.89)	1.14 (0.18-4.22)
20	0.91 (0.01-9.76)	1.34 (0.23-4.66)	1.07 (0.02-8.89)	1.71 (0.26-6.21)
40	1.41 (0.01-15.87)	1.97 (0.30-7.07)	1.76 (0.01-18.31)	1.71 (0.26-6.21)

Women under 45 years of age demonstrated similar alloimmunization rates to non-Rh/K antigens as compared to older women.

Nevertheless, prevention of primary alloimmunization is of upmost importance both in a setting of epitope spreading as well as with AMIS. In case of high clinical relevance of epitope spreading, further prevention of alloimmunization (i.e. after a first antibody has already formed) would not suffice. Instead, absolute prevention of ($CD4^+$ T cell) sensitization, by primary avoidance of all or at least most allogeneic antigens would avoid both primary and subsequent additional immunization. In case the concept of protective AMIS proves to also exist beyond RhD antibodies, secondary matching to prevent (further) alloimmunization seems less needed. Again, primary matching for at least all high immunogenic antigens should deserve our main emphasis.

Yet, only a subset of the patient population can practically receive extended matched products due to financial costs and logistic feasibility, hereby once more underlining the high importance of accurate identification of the high-responder patient and high-risk conditions for alloimmunization. Accordingly, the studies and their outcomes presented in this thesis will serve future tailoring of preventive matching strategies by having identified respectively exposure to certain high immunogenic antigens, an infectious-disease related inflammatory condition, a treatment induced state of immunosuppression, and a functioning spleen to be ultimate determinants of red cell alloimmunization.

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