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

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# RED CELL ALLOIMMUNIZATON IN RELATION TO ANTIGENS' EXPOSURE AND THEIR IMMUNOGENICITY: A COHORT STUDY

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## Abstract

**Background**

Matching donor red cells on recipient antigens prevents alloimmunization. Knowledge about the immunogenicity of red cell antigens can help optimize risk-adapted matching strategies. We set out to assess the immunogenicity of red cell antigens.

**Methods**

In an incident new-user cohort of 21,512 previously non-transfused, non-alloimmunized Caucasian patients receiving non-extended matched red cell transfusions in six Dutch hospitals between 2006 and 2013, we determined the cumulative number of mismatched red cell units per patient. Missing antigen data were addressed using multiple imputation. Using Kaplan-Meier analysis, we estimated cumulative alloimmunization incidences per mismatched antigen dose as a measure of immunogenicity.

**Findings**

Alloantibodies occurred in 2.2% (474/21,512) of all transfused patients with cumulative alloimmunization incidences increasing up to 7.7% (95% confidence (CI) interval 4.9-11.2) after 40 units received. The antigens C, c, E, K, and $Jk^a$ were responsible for 78% of alloimmunizations in our cohort. K, E, and $C^w$ were the most immunogenic antigens (cumulative immunization incidences 2.3% (CI 1.0-4.8), 1.5% (CI 0.6-3.0), and 1.2% (CI 0.0-10.8) after 2 mismatched units). These antigens were 8.7, 5.4, and 4.6 times as immunogenic as $Fy^a$. This immunogenicity order was followed by e, $Jk^a$, and c (1.9, 1.9, and 1.6 as strong as $Fy^a$).

**Interpretation**

Red cell antigens vary in their potency to evoke a humoral immune response. Our findings highlight that donor-recipient red cell matching strategies will be most efficient when primarily focusing on prevention of C, c, E, K, and $Jk^a$ alloimmunization. Matching for $Fy^a$ is of lower clinical relevance. Ethnicity determined variations of antigen frequencies prevent extrapolating these conclusions to non-Caucasian populations.



**Funding**

This study was not externally funded.

## Introduction

Exposure to foreign red cell antigens may induce alloimmunization. Notwithstanding current ABO/RhD matching and stringent antibody screening policies, life-threatening hemolytic reactions resulting from boosting of previously induced alloantibodies still complicate red cell transfusions.[5,6] Moreover, previous alloimmunizations demand extensive laboratory efforts and can result in delays in finding compatible donor blood.

A complete antigenic donor-recipient phenotype match would theoretically eliminate all transfusion induced alloimmunizations, however, meets countless logistical and financial challenges. The next best alternative is to select donor units matched at least on the most immunogenic antigens for patients at high risk. In line with this, patients with myelodysplastic syndrome, and auto- and/or alloimmunized patients in the Netherlands are advised to receive CcEe and K matched blood, while patients with hemoglobinopathy additionally receive $Fy^a$ and preferentially $Jk^b$ and Ss matched blood as well.[7] As alloimmunization can severely complicate subsequent pregnancies, women under 45 years of age receive cE and K matched blood.[7] These matching strategies are based on broad expert consensus on the antigens' immunogenicity i.e. their intrinsic potency to stimulate humoral immune responses. RhD is without doubt the most immunogenic antigen and is followed by K.[1] However, data on the relative immunogenicity of several other antigens is conflicting,[1-3] requiring additional observational evidence.

We set out to quantify antigen-specific alloimmunization rates in relation to the cumulative number of mismatched transfusions per patient as a measure of the intrinsic immunogenicity of red cell antigens. This knowledge will enable an evidence-based design of optimizing matching strategies, balancing benefits against costs and logistic aspects.

## Methods

### Study design and setting

We performed an incident new-user cohort study among patients consecutively transfused in three university hospitals and three non-university hospitals in the Netherlands. We included all previously non-transfused and non-alloimmunized patients who received at least one red cell transfusion during the study period, provided the availability of at least one pre- and post-transfusion antibody screen. The study period varied per hospital according to the electronic availability of necessary data: January 1, 2005 to December 31, 2010 at Leiden University Medical Center (Leiden), September 6, 2006 to December 31, 2013 at University Medical Center Utrecht (Utrecht), November 19, 2011 to December 31, 2013 at VU University Medical Center (Amsterdam), May 1, 2007 to April 30, 2013 at Catharina Hospital (Eindhoven), July 1, 2005 to December 31, 2013 at Jeroen Bosch Hospital ('s Hertogenbosch), and October 5, 2008 to December 31, 2013 at Haga Teaching Hospital (The Hague).

We used the following safeguards. First, patients alloimmunized within seven days of a mismatched transfusion were excluded as they more likely presented boosting rather than primary alloimmunization. Second, the records of alloimmunized patients were consulted against the nationwide Dutch alloimmunization registry (TRIX)[8] for earlier alloantibody detection in other hospitals. Third, allo- rather than auto-immunization was verified based on the patient's phenotype. Fourth, as CcEe and K phenotypes for over 99% of donor blood units were available, we excluded CcEe and/or K alloimmunized patients with no identifiable mismatched transfusion. Finally, we set out to exclude all patients who received more than only routinely (ABO/RhD) matched units. To that aim, we excluded women below 45 years of age who, in line with Dutch guidelines,[7] receive c, E, and K compatible units. Auto-immunized patients without alloimmunization, and hemoglobinopathy patients were excluded as they usually receive extended matched units as well.[7] In addition, ethnicity determined differences in antigen distribution between hemoglobinopathy patients and the Dutch, generally Caucasian, donor population would have led to unrepresentative alloimmunizations, further compromising the validity of antigen immunogenicity estimates. We did not exclude patients with myelodysplastic syndrome since, despite Dutch guideline advises,[7] they do not receive extended matched blood (unpublished personal data). Infants under 6 months of age were excluded as poor antibody responses during the first months of life are reported.[9]

The study protocol was approved by the Ethical Review Board in Leiden and by the local board of each participating center.

## Detection of red cell alloantibodies

At a maximum of 72 hours prior to red cell transfusion, patients in the Netherlands are routinely screened for red cell alloantibodies. According to the Dutch transfusion guideline, commercially available screening cells are required to be homozygous positive for D, C, c, E, e, K, $Fy^a$, $Fy^b$, $Jk^a$, $Jk^b$, M, S, and s. The K antigen needs to be present minimally heterozygously. The presence of $C^w$, $Lu^a$, $Wr^a$, and $Kp^a$ is not mandatory on commercially available screening cells.[7]

Screening was performed using a 3-cell panel including an indirect antiglobulin test (LISS Diamed ID system (Bio-Rad, Cressier, Switzerland), or Ortho Biovue ID system (Ortho Clinical Diagnostics, Raritan NJ, United States)) and subsequent antibody identification. The antigen $C^w$ was present on 93% of all 3-cell panels used during the time frame of this study.

## Data acquisition

Routinely stored data on red cell transfusion dates, product unique identification number, dates and results of antibody screens, antibody specificity, and patient's date of birth and sex were gathered from the hospitals' electronic laboratory information systems.

Available antigen phenotypes for all transfused products were delivered by Sanquin Blood Supply, Amsterdam, The Netherlands.

## Statistical analyses

### Overall and antigen-specific cumulative incidence of alloimmunization

All included patients were followed up and labelled as 'alloimmunized' upon a first-time alloantibody identification or as 'non-alloimmunized' if the alloantibody screen remained negative.

Cumulative numbers of transfused red cell units corresponded to the total number of units received up to the last available negative screen for non-alloimmunized patients and up to the last verified or presumed antigen-mismatched unit that preceded the first positive screen for alloimmunized patients (Figure S1). Consequently, transfusions received after these screens were not taken into account. Using Kaplan-Meier survival tables, we then calculated overall cumulative alloimmunization incidences and subsequently cumulative incidences per antigen.

Cumulative incidences with 95% confidence intervals (CI) were calculated with Graphpad Prism version 6, using the exponential Greenwood formula. The association between sex and alloimmunization incidences was assessed with the log-rank test using SPSS version 20.0. P-values <0.05 were considered to be statistically significant.

### Immunogenicity of red cell antigens

To evaluate the immunogenicity of various red cell antigens, we calculated cumulative alloimmunization incidences per antigen according to the total number of mismatched (i.e. antigen-positive) units a patient had received (Figure S1). For this purpose, only those at risk for alloimmunization against a given antigen should be considered, i.e. patients lacking expression of this antigen. As antigen phenotyping of non-alloimmunized patients is limited to ABO and RhD in the Netherlands, we established 'antigen-negative cohorts' comprising all (per definition antigen-negative) patients alloimmunized to a given antigen plus a randomly sampled subgroup of non-alloimmunized patients. Although patients in these sampled subgroups not necessarily all lacked expression of the corresponding antigen, they only functioned as a representative for the true antigen-negative, non-alloimmunized individuals in the source population. We based the sampling sizes of these subgroups on known antigen frequencies in the Caucasian population.[10] Thus, as 29% of the Caucasian population express the E antigen,[10] 71% of our source population lack E expression. Given an extensive size of the source population, the numbers of E-positive units received by the randomly sampled 71% will closely correspond to the numbers received by the true E-negative, non-alloimmunized individuals in the source population. This sampling method is not likely liable to selection bias as expression of a given antigen is not associated with the amount of alloantigen exposure in non-extended matched individuals.

To address missing donor phenotypes, we used multiple imputation to complete the dataset. Details on frequencies of missing data and the used method are presented in table S1.

We then calculated cumulative alloimmunization incidences for each antigen according to the total number of mismatched units, except for antigens with over 50% missing data.

Finally, we compared antigen-specific cumulative alloimmunization incidences with those of $Fy^a$. Previously, comparisons with K were made.[1-4] However, the rate of censoring between baseline and *N* transfusions may compromise the validity of estimated cumulative incidences (for illustration, see Supplementary Box 1). Consequently following the antigen's low frequency, the reliability of estimated cumulative anti-K incidences decreases with the number of K-positive units exposed as only a few patients are repeatedly exposed to K. As in former reports $Fy^a$ immunogenicity was in the middle of the extremes[1-4] and as (except for S) the probability that a random individual both does not express a given antigen and is exposed to this antigen is the highest for $Fy^a$,[10] we chose $Fy^a$ as the reference. Due to the above mentioned issues regarding censoring, we only calculated cumulative alloimmunization incidences per antigen for antigen-negative cohorts containing at least 200 non-censored patients.

### Role of the Funding Source

This study was not externally funded. The corresponding author had full access to all of the data and the final responsibility to submit for publication.

## Results

A total of 54,347 patients received their first red cell transfusion during the study period, of which 21,512 patients fulfilled the inclusion criteria. Figure 1 presents numbers of patients per exclusion criterion. The majority of the 32,835 patients deemed ineligible were excluded while no antibody screen was performed after their single transfusion episode (N=25,037).

Table 1 presents patient demographics. Patients received a median of 4 (interquartile range (IQR) 2-8) units of red cells during a median follow-up period of 86 (IQR 14-395) days. In 474/21,512 patients (2.2%), 536 first formed alloantibodies were detected, the majority being against C, c, E and K antigens (table 2). In 51 patients, the alloantibody was detected within the second week after the first documented antigen-mismatched transfusion.

**Figure 1** Study flow diagram.

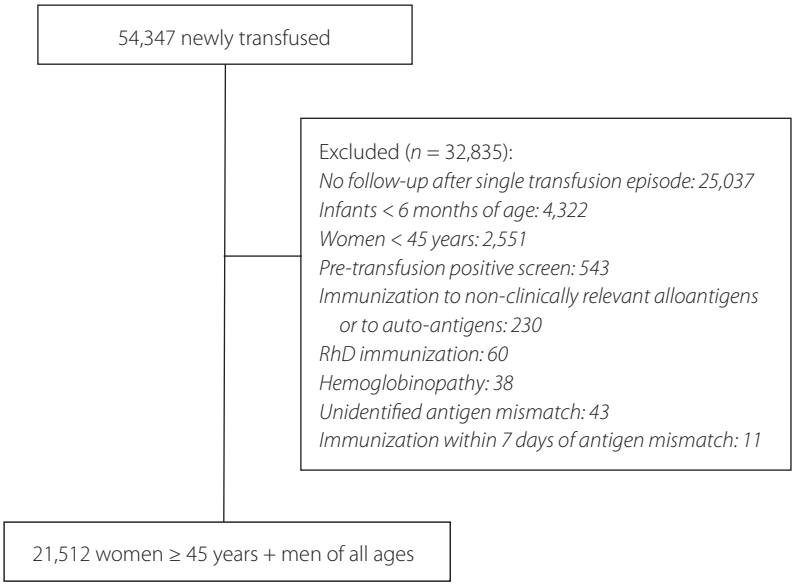



**Table 1** Patient demographics of the study population.

| | **N=21,528** |
|---|---|
| Men (N, %) | 12,511 (58.1) |
| Age in years at 1st transfusion, median (range / IQR) | 67.7 (0.5-107.2 / 57.5-76.9) |
| Cumulative units | 153,429 |
| Units per patient, median (range / IQR) | 4 (1-462 / 2-8) |
| Follow-up in days, median (range / IQR) | 86 (1-3155 / 14-395) |
| Alloimmunized patients | 474 |
| Alloimmunization frequency (%) | 2.2 |

Follow-up period = period in days from 1st red cell transfusion up to the last negative antibody screen for non-alloimmunized patients, and up to the first positive alloantibody screen for alloimmunized patients.
IQR = interquartile range.

**Table 2** Specificity and frequency of first-time formed clinically significant alloantibodies (N, %).

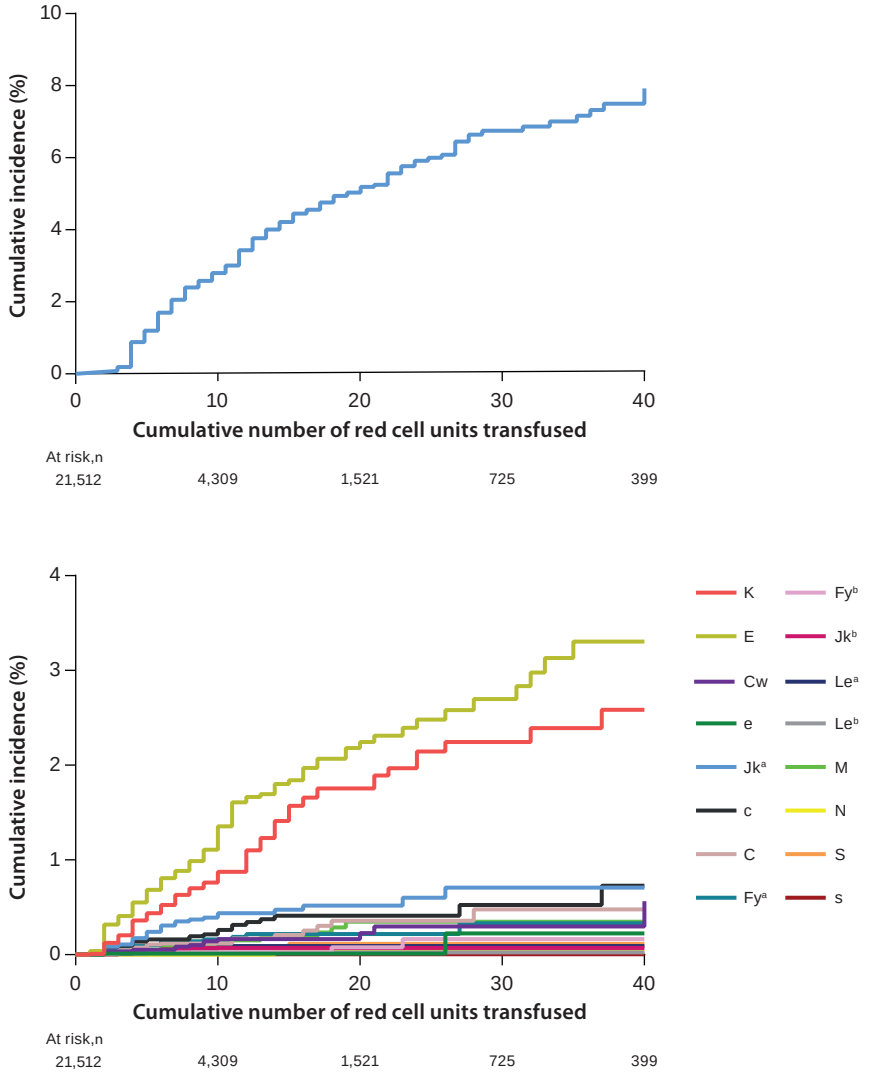
| Alloantibody specificity | All N=21,512 | RhD pos N=18,191 (84.6%) | RhD neg N=3,321 (15.4%) |
|---|---|---|---|
| anti-C | 22 (0.10) | 18 (0.10) | 4 (0.12) |
| anti-c | 37 (0.17) | 37 (0.20) | 0 (0) |
| anti-E | 177 (0.82) | 173 (0.95) | 4 (0.12) |
| anti-e | 4 (0.02) | 4 (0.02) | 0 (0) |
| anti-K | 122 (0.57) | 95 (0.52) | 27 (0.81) |
| anti-$C^w$ | 19 (0.09) | 18 (0.09) | 1 (0.03) |
| anti-$Fy^a$ | 24 (0.11) | 21 (0.12) | 3 (0.09) |
| anti-$Fy^b$ | 5 (0.02) | 4 (0.02) | 1 (0.03) |
| anti-$Jk^a$ | 50 (0.23) | 41 (0.23) | 9 (0.27) |
| anti-$Jk^b$ | 7 (0.03) | 7 (0.04) | 0 (0) |
| anti-$Lu^a$ | 31 (0.14) | 29 (0.16) | 2 (0.06) |
| anti-$Lu^b$ | 0 (0.0) | 0 (0) | 0 (0) |
| anti-$Le^a$ | 8 (0.04) | 6 (0.03) | 2 (0.06) |
| anti-$Le^b$ | 3 (0.01) | 3 (0.02) | 0 (0) |
| anti-M | 18 (0.08) | 14 (0.08) | 4 (0.12) |
| anti-N | 1 (0.01) | 1 (0.01) | 0 (0) |
| anti-S | 8 (0.04) | 7 (0.04) | 1 (0.03) |
| anti-s | 0 (0) | 0 (0) | 0 (0) |
| All antibodies | 536 | 478 | 58 |
| Number of cases | 474 (2.20) | 419 (2.30) | 55 (1.66) |

## Overall cumulative incidence of alloimmunization

The overall cumulative alloimmunization incidence increased to 7.7% (CI 4.9-11.2) after 40 red cell units transfused (figure 2 upper panel). Antibodies to E and K were formed at the highest rates (figure 2 bottom panel). Table 3 presents cumulative alloimmunization incidences against various sets of antigens referenced to the overall cumulative alloimmunization incidence after 40 units transfused. Hence, in this ABO/RhD matched patient cohort, 78.6% of alloimmunizations were due to immunizations against C, c, E, K, or $Jk^a$.

Frequencies of anti-c and anti-E mirrored *RHD* and *RHCE* gene linkage. In this respect, anti-c was only formed by RhD-positive patients and absence of RhD expression led to significantly less E alloimmunizations with cumulative allo-E incidences of 1.7% (CI 0.0-32.0) and 3.7% (CI 1.4-7.9) after 40 red cell units received for RhD-negative and RhD-positive patients, respectively (log-rank $p<0.0001$). RhD phenotype did not modulate the risk of immunization against other, non gene-linked, red cell antigens (figure S2).

**Figure 2** Cumulative alloimmunization incidences in the general population (upper panel) and according to antigen (lower panel).




Cumulative alloimmunization incidences as a function of cumulative red cell units exposed. Antibodies to E and secondary to K were formed at the highest incidence rates.

**Table 3** Cumulative alloimmunization incidences (%) against various sets of antigens referenced to the overall cumulative alloimmunization incidence.

| | Cumulative alloimmunization incidence (%)* | Proportion of all antibodies (%) |
|---|---|---|
| All antibodies | 7.66 (4.89-11.24) | 100 |
| E | 3.32 (1.20-7.25) | 43.3 |
| cE | 3.59 (1.40-7.47) | 46.9 |
| cEK | 5.20 (2.71-8.87) | 67.9 |
| cEeK | 5.39 (2.84-9.12) | 70.4 |
| CcEK | 5.74 (3.15-9.42) | 74.9 |
| cEK+Jk$^a$ | 5.67 (3.15-9.23) | 74.0 |
| CcEK+Jk$^a$ | 6.02 (3.46-9.55) | 78.6 |
| CcEK+Jk$^a$+Fy$^a$ | 6.24 (3.66-9.76) | 81.5 |
| CcEK+Jk$^a$+Fy$^a$+C$^w$ | 6.39 (3.81-9.88) | 83.4 |

***** Cumulative alloimmunization incidences as calculated for 40 red cell units transfused.

Among patients over 45 years of age, women showed higher Rh (i.e. CcEe and C$^w$) and K alloimmunization incidences compared to men (7.9% (CI 3.2-15.3) versus 5.2% (CI 2.0-10.9) after 40 units received, log-rank p<0.0001, figure S3). Alloimmunization to non-Rh/non-K antigens did not differ between male patients under and above 45 years of age (log-rank p=0.705).

## Immunogenicity of red cell antigens

The antigen's specific immunogenicity was derived from cumulative alloimmunization incidences according to cumulative antigen mismatched units received. Substantial missing antigen data for Le$^b$, Lu$^a$, and Lu$^b$ prevented immunogenicity calculations for these antigens (table S1).

Cumulative alloimmunization incidences after exposure to only two antigen-positive units were 2.3% (CI 1.0-4.8), 1.5% (CI 0.6-3.0), and 1.2% (CI 0.0-10.8) for K, E, and C$^w$ respectively. Less extensive responses were observed for e, Jk$^a$, and c (figure 3 upper panel, table 4). Following a similar amount of E exposure, anti-E formation did not differ between RhD-negative and RhD-positive patients (log-rank p 0.44).

The calculated relative immunogenicity of K, E, and C$^w$ was 8.7, 5.4, and 4.6 times higher than Fy$^a$ after only two antigen-positive units. For e, Jk$^a$, and c these rates were 1.9, 1.9, and 1.6, respectively. Relative immunogenicity rates were lower for the other antigens (figure 3 bottom panel).

**Table 4** Adjusted cumulative red cell alloimmunization incidences according to numbers of antigen-mismatched units received in the antigen-negative cohorts.

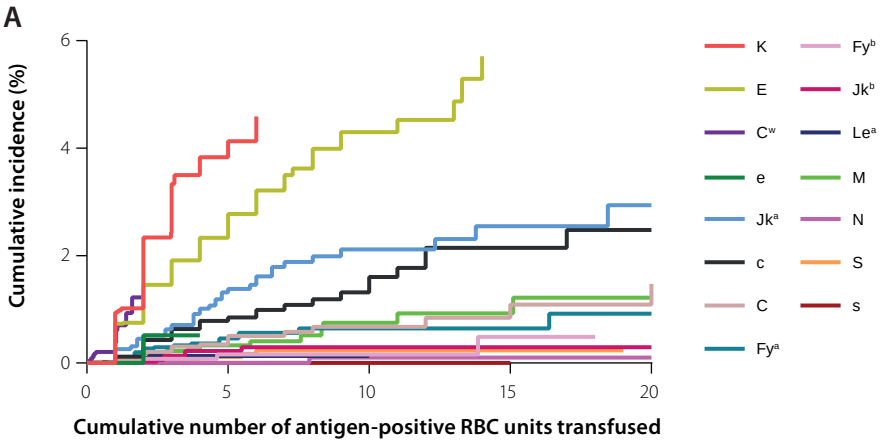
| | Adjusted cumulative alloimmunization incidence, % (CI) | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mismatched units (N) | C | c | E | e | K | $C^w$ | $Fy^a$ | $Fy^b$ | $Jk^a$ | $Jk^b$ | $Le^a$ | M | N | S | s |
| 1 | 0.05 (0.00-8.78) | 0.12 (0.00-6.10) | 0.73 (0.21-2.00) | 0.00 (0.00-0.00) | 0.94 (0.27-2.53) | 0.38 (0.00-5.42) | 0.09 (0.00-4.33) | 0.00 (NC) | 0.26 (0.00-3.40) | 0.00 (NC) | 0.06 (0.00-4.74) | 0.09 (0.00-7.25) | 0.00 (NC) | 0.04 (0.00-7.91) | 0.00 (NC) |
| 2 | 0.21 (0.00-3.66) | 0.43 (0.01-3.55) | 1.46 (0.61-2.99) | 0.51 (0.00-26.76) | 2.34 (0.95-4.84) | 1.23 (0.02-10.78) | 0.27 (0.01-2.87) | 0.08 (0.00-16.81) | 0.51 (0.03-3.24) | 0.02 (0.00-30.92) | 0.10 (0.00-3.84) | 0.18 (0.00-5.02) | 0.00 (NC) | 0.08 (0.00-5.34) | 0.00 (NC) |
| 5 | 0.50 (0.01-4.20) | 0.85 (0.07-4.24) | 2.77 (1.34-5.07) | | 4.13 (1.53-8.80) | | 0.46 (0.02-3.50) | 0.16 (0.00-14.66) | 1.38 (0.24-4.79) | 0.23 (0.00-6.72) | 0.13 (0.00-4.40) | 0.33 (0.00-5.14) | 0.00 (NC) | 0.12 (0.00-6.13) | 0.00 (NC) |
| 10 | 0.67 (0.02-5.11) | 1.60 (0.18-6.68) | 4.30 (1.96-8.06) | | | | 0.64 (0.03-4.36) | 0.16 (0.00-14.66) | 2.11 (0.46-6.29) | 0.30 (0.00-6.88) | 0.13 (0.00-4.40) | 0.75 (0.01-7.34) | 0.10 (0.00-37.40) | 0.24 (0.00-7.21) | 0.00 (NC) |
| 15 | 0.84 (0.02-6.81) | 2.15 (0.28-8.17) | | | | | 0.64 (0.03-4.36) | 0.49 (0.00-26.04) | 2.55 (0.51-7.83) | 0.30 (0.00-6.88) | | 0.94 (0.02-8.28) | 0.10 (0.00-37.40) | 0.24 (0.00-7.21) | 0.00 (NC) |
| 20 | 1.48 (0.02-12.15) | 2.47 (0.29-9.55) | | | | | 0.92 (0.01-9.23) | | 2.94 (0.49-9.65) | 0.30 (0.00-6.88) | | 1.22 (0.02-10.47) | 0.10 (0.00-37.40) | | |

Only data from non-censored cohorts of at least 200 subjects are presented. CI = 95% confidence interval. NC = non-computable due to the absence of events.

When only $C^w$ alloimmunized patients with a verified $C^w$ mismatched transfusion were included in the above analysis (N=10, those with assumed $C^w$ mismatched transfusions excluded), $C^w$ alloimmunization incidences did not change substantially (1.2% (CI 0.0-10.8 versus 1.0% (CI 0.0-12.8) after 2 $C^w$ positive units transfused, log-rank p=0.10).

As an additional sensitivity analysis, results of antigen immunogenicity calculations repeated in only men were identical (figure S4).

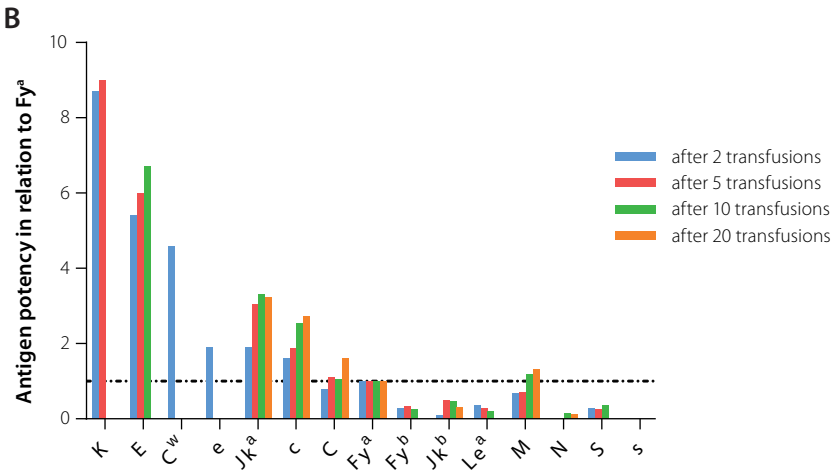
**Figure 3** The relative immunogenicity of specific antigens presented by cumulative alloimmunization incidences as a function of antigen exposure.




| At risk,n | | | | | |
|---|---|---|---|---|---|
| K | 19,274 | 324 | 35 | 7 | 2 |
| E | 15,122 | 1,759 | 506 | 195 | 95 |
| $C^w$ | 20,648 | 20 | 3 | 1 | 0 |
| e | 433 | 172 | 91 | 52 | 36 |
| $Jk^a$ | 4,890 | 1,665 | 718 | 378 | 227 |
| c | 4,247 | 1,516 | 683 | 372 | 229 |
| C | 6,758 | 1,923 | 774 | 403 | 259 |
| $Fy^a$ | 7,181 | 2,193 | 897 | 445 | 255 |
| $Fy^b$ | 3,583 | 1,278 | 560 | 290 | 172 |
| $Jk^b$ | 5,482 | 1,766 | 725 | 363 | 209 |
| $Le^a$ | 16,427 | 1,001 | 222 | 70 | 24 |
| M | 4,648 | 1,610 | 890 | 372 | 225 |
| N | 5,895 | 1,798 | 753 | 407 | 234 |
| S | 9,479 | 2,096 | 746 | 350 | 184 |
| s | 2,317 | 893 | 410 | 209 | 141 |

(**A**) Antigen-specific cumulative alloimmunization incidences according to number of antigen-positive red cell units received by the antigen-negative patient cohorts, and (**B**) as referenced to $Fy^a$. K, E, $C^w$, and to a lesser degree e, $Jk^a$, and c, are the most immunogenic antigens.
Numbers at risk correspond to total number of patients within the corresponding antigen-negative cohort exposed to at least *N* antigen-positive red cell units. Only data from non-censored cohorts of at least 200 subjects are presented.

**Figure 3** Continued.




## Discussion

In this study covering 21,512 newly transfused patients, we established estimates of dose-specific red cell alloimmunization risks. In agreement with previous reports, K is most potent in stimulating humoral alloimmune responses. E demonstrates the second highest immunogenicity, and the order of antigen immunogenicity is then followed by $C^w$, e, $Jk^a$, and c.

The here established alloimmunization rate of 0.9% after one K-positive unit is five times lower than historically assumed.[1] Moreover, as previous studies did not take into account the cumulative mismatched transfusion burden, contradictory conclusions regarding the immunogenic potency of other red cell antigens, including c and E, have been reported.[1-4] Indeed, the seemingly flattening of the antigens' alloimmunization risk curves illustrates that the chance to alloimmunization diminishes with subsequent antigen exposure. We therefore specifically set out to properly estimate the cumulative number of antigen-mismatched units each patient had received. Sampling specific-sized 'antigen-negative cohorts' for each antigen enabled us to estimate the cumulative antigen exposure within the true antigen-negative individuals. Selection bias did not interfere with this sampling method as the prevalence of a given antigen is not associated with the likelihood of exposure in non-alloimmunized patients.

The validity of our assessment is confirmed by the constant immunogenicity, though diverse alloimmunization rates against c and E, between RhD-negative and RhD-positive patients, as was expected based on the known *RHD* and *RHCE* gene linkage. Transfusing RhD compatible blood has long since been routine practice as this high immunogenic

antigen induces allo-D in around 30% of D-negative transfused patients and in up to 80% of healthy volunteers.[11,12] Due to this gene linkage, RhD matching not only prevents anti-D formation, but in addition effectively protects against E alloimmunization. That is, as only 6% of the Caucasian RhD-negative population express the E antigen,[10] RhD-negative patients are rarely exposed to E. Conversely, as 67% of RhD-positive individuals lack E,[10] 23% of RhD-positive patients risk E alloimmunization with routine RhD matching. Moreover, the comparable anti-E formation after non-self E exposure between RhD-negative and RhD-positive patients mirrors the fact that RhD phenotype does not influence E-immunogenicity, rather that alloimmunization rates reflect (linkage dependent) exposure. Similarly, anti-c was only identified in the RhD-positive patient cohort as a RhD-negative phenotype is approximately always accompanied by expression of c. However, the chance of a RhD-positive patient to be exposed to c being a non-self antigen is 17%.[10]

Current Dutch matching regimens might seem strict compared to those of other Western countries. Despite, for prior auto- and/or alloimmunized patients, as well as for multi-transfused patients, better targeted matching will likely further reduce the risk of (additional) alloimmunization. As our data seem broadly applicable to populations of Caucasian origin, they enable further optimization as well as unification of current nationwide evidence-based guidelines. In this regard, matching seems most profitable for those antigens with relatively strong immunogenicity, moderate frequency, and potential clinical consequences. Hence, red cell transfusions limited to donor units compatible with the high to moderate immunogenic antigens C, c, E, K, and $Jk^a$ would have reduced alloimmunization incidences by 78%. In line with this, and as anti-$Jk^a$ is notorious for causing delayed hemolytic transfusion reactions,[6,13] additional $Jk^a$ matching for the mentioned risk groups seems advisable. Due to its frequency in the Caucasian population and intermediate immunogenicity, matching for $Fy^a$ should be considered optional. The need to additionally match for $C^w$ seems debatable as, although this antigen is highly immunogenic, severe hemolysis by anti-$C^w$ is rare[14] and the chance of subsequent exposure after primary immunization small (2%).[10]

In agreement with recent data from a 15% overlapping patient cohort,[15] we found higher cumulative alloimmunization incidences in women compared to men over 45 years of age, attributed to higher Rh and K alloimmunization rates. This finding is not easily accounted for by boosting of pregnancy-induced alloantibodies as we used several safeguards to exclude previously alloimmunized women. Moreover, despite (pregnancy-induced) alloantibodies commonly disappearing,[16,17] boosting seems an insufficient explanation as non-RhD alloantibodies form in only 0.33% of first trimester pregnancies[18] amounting for 30 of our 238 (12.6%) alloimmunized post-fertile women. Indeed, others have suggested estrogen or even persisting feto-maternal chimerism to modulate alloimmune responses in women.[15,19]

Several factors and possible limitations of our results require discussion.

First, the time needed for an antibody to develop to serologically detectable levels potentially differs per antigen, but these 'lag periods' are currently unknown. Additionally, titers of previously formed alloantibodies can decrease over time to a degree that prior alloimmunizations are no longer detectable by serological tests. Subsequent exposure to the antigen might boost these 'evanescent' alloantibodies. Indeed, 25 to 40 percent of formed alloantibodies will become undetectable over time, with the highest rates reported for anti-Jk$^a$ and anti-C$^w$.[16,17] However, as evanescence rates largely depend on the time since exposure (for illustration, see Supplementary Box 2), we were not able to estimate the underestimating effect of antibody evanescence on our immunogenicity calculations.

Second, anti-E, anti-C$^w$, anti-Le$^a$, anti-Le$^b$, anti-Lu$^a$, and anti-M can also occur 'naturally', i.e. secondary to environmental antigen exposure.[10] One might thus wonder whether the high immunogenicity of C$^w$ should not be explained by high numbers of naturally formed anti-C$^w$. A sensitivity analysis, including only C$^w$ alloimmunizations which were verified (rather than assumed) to be preceded by C$^w$ mismatched transfusions, confirmed our conclusions. In fact, C$^w$ immunogenicity may well have been underestimated as 7% of used screening cell panels did not present C$^w$ and therefore could not detect anti-C$^w$. Next, some identified anti-M alloantibodies might have been only of IgM class. Although according to Dutch guidelines a reference laboratory should determine whether identified anti-M antibodies are due to warm-reacting IgG antibodies,[7] this procedure has not been routinely followed in the Netherlands so far.

Third, we set out to exclude all previously transfused and alloimmunized patients. Eleven patients presented with a positive screen within seven days after the first antigen-mismatched transfusion and were excluded as these might have reflected boosting. Nevertheless, while boosting periods can extend seven days and might even differ per antigen, we might not have excluded all previously alloimmunized patients. In this regard, 51 of 474 patients (10.8%) tested positive for alloantibodies within the second week after a first mismatched transfusion, while only a subset of those (N=31) were also tested (negative) during the first week after transfusion. Next, due to some unavailable non-Rh/K donor phenotypes, it remains possible that we assumed a few of those phenotypes to be antigen-positive while in fact the alloimmunized individual was never exposed by transfusion. Finally, we cannot exclude that included patients received some transfusions in other hospitals prior or during the study period. Consequently, some overestimation of the antigens' immunogenicity has to be reckoned.

Fourth, we used multiple imputation addressing missing donor antigen phenotypes while, contrasting nearly complete CcEe and K phenotyping, expression of other minor red cell antigens is less extensively determined among Dutch donors. Several reports have emphasized the superiority of multiple imputation over the traditional missing data techniques.[20-22] Considering data missing at random (MAR), limiting analyses to antigens

with less than 50% missing values, and the substantial sample size we performed this method in, our followed approach will likely have produced unbiased and rather accurate estimates of missing values.[22] Our conservative approach, however, consequently disabled us from presenting any estimations on the immunogenicity of $Le^b$, $Lu^a$, and $Lu^b$. While anti-$Lu^a$ antibodies represented 5.7% (31/537) of all detected antibodies, we cannot exclude $Lu^a$ to be of importance in alloimmunization.

Fifth, hemoglobinopathy patients, often frequently transfused but from non-Caucasian background, were not included in the study. While nearly all donors are of Caucasian origin,[23] around 12% of the Dutch population is of origin other than Caucasian.[24] This discordance may have led to a minor deviation of our estimated mismatched transfusions in the non-alloimmunized patients. Reported antigen immunogenicities may thus be slightly overestimated. In general, due to antigenic, immunological, and genetic differences between ethnicities,[25,26] our results should not be extrapolated to populations of other ethnic backgrounds.

Sixth, although anti-D was detected in 60 of our newly-transfused patient population, we were unable to analyse and confirm the previously reported high immunogenicity of the D antigen[11,12] for several reasons. Due to routine RhD matching, only a very small minority of our RhD-negative patients received D-mismatched units. Next, while the cause of anti-D was often not documented, these antibodies could have been due to either unmatched transfusions or recent anti-D administration.

Seventh, alloantibody responses may differ between various patient cohorts e.g. immunosuppressed versus immune activated patients.[25,27,28] This, however, does not affect any of our conclusions as we only compared the immunogenicity of red cell antigens with one another within the same population. Nevertheless, alloimmunization risks will differ between patient cohorts and the here presented incidences should therefore not be generalized to populations other than general transfused patients. Studies in humans aimed at identifying factors of influence on immunization risks are in progress.[28,29]

Finally, we did not adjust for homo- versus heterozygous donor genotypes as a variable of antigen dose. For most antigens, patients will have received mainly heterozygous donor units. As an example, the observed high immunogenicity of E and K is not distorted by minor cumulative dose differences as the homozygous prevalence rates are only 2.4% and 0.21%, respectively.[30] Moreover, we previously did not find an association between massive versus dispersed transfusions on the risk of alloimmunization.[31]

Though beyond the scope of the present study, a few related and relevant subjects should be mentioned. As an optimal preventive matching strategy demands a comprehensively typed donor cohort, high-throughput genotyping might better facilitate rapid and complete typing in the near future.[32,33] Next, antibody formations needs both sensitization of a B cell as well as priming of a naive $CD4^+$ T cell. Thus, in some of our non-alloimmunized patients, alloreactive B cells to a given blood group antigen might

have been present, yet they lacked specific T cells recognizing a peptide as part of this blood group antigen in the context of human leucocyte antigen (HLA). Finally, knowledge on factors modulating subsequent alloimmunization (such as the type of first-time formed alloantibody possibly determining the rate and type of subsequent alloimmunization) might benefit the already alloimmunized patient.

In conclusion, the risk of red cell alloimmunization is related to both antigen exposure and the antigen's immunogenicity. In this to our knowledge largest Caucasian cohort to date with a defined follow-up reaching an eight year period, we determined the immunogenic order of red cell antigens and quantified dose-based immunization risks with K and E being the most immunogenic antigens, followed by $C^w$, e, $Jk^a$, and c. Based on the likelihood of alloantigen exposure, the antigens' immunogenicity, and the potential detrimental consequences of anti-$Jk^a$ boosting, we recommend adding $Jk^a$ matching to current CcEe and K based matching strategies, whenever possible and especially in high-risk patients. Matching for $Fy^a$ can be considered, but, as compared to $Jk^a$, seems of lesser clinical significance. Due to antigenic frequency differences, these conclusions are not generalizable to patients of non-Caucasian background.

## References


1. Giblett ER. A critique of the theoretical hazard of inter vs. intra-racial transfusion. Transfusion. 1961;1:233-8.
2. Schonewille H, van de Watering LM, Loomans DS, Brand A. Red blood cell alloantibodies after transfusion: factors influencing incidence and specificity. Transfusion. 2006;46:250-6.
3. Tormey CA, Stack G. Immunogenicity of blood group antigens: a mathematical model corrected for antibody evanescence with exclusion of naturally occurring and pregnancy-related antibodies. Blood. 2009;114:4279-82.
4. Winters JL, Pineda AA, Gorden LD, et al. RBC alloantibody specificity and antigen potency in Olmsted County, Minnesota. Transfusion. 2001;41:1413-20.
5. TRIP Hemovigilance report 2014. Accessible at: https://www.tripnet.nl/pages/nl/documents/TRIP2014H-emovigilantie.pdf.
6. SHOT report 2014. Accesible at: http://www.shotuk.org/shot-reports/report-summary-supplement-2014.
7. CBO Blood Transfusion Guideline 2011. English version accessible at: http://www.sanquin.nl/repository/documenten/en/prod-en-dienst/287294/blood-transfusion-guideline.pdf.
8. TRIX register. Dutch transfusion register for irregular antibodies. Further information via website (in Dutch): http://www.sanquin.nl/producten-diensten/diagnostiek/trix.
9. Strauss RG, Cordle DG, Quijana J, Goeken NE. Comparing alloimmunization in preterm infants after transfusion of fresh unmodified versus stored leukocyte-reduced red blood cells. J Pediatr Hematol Oncol. 1999;21:224-30.
10. Reid ME, Lomas-Francis C, Olsson ML. The blood group antigen factsbook, second edition. San Diego: Elsevier Academic Press; 2004.
11. Frohn C, Dümbgen L, Brand JM, Görg S, Luhm J, Krichner H. Probability of anti-D development in D- patients receiving D+ RBCs. Transfusion. 2003;43:893-8.
12. Gunson HH, Stratton F, Cooper DG, Rawlinson VI. Primary immunization of Rh-negative volunteers. Br Med J. 1970;1:593-5.
13. Vamvakas EC, Pineda AA, Reisner R, Santrach PJ, Moore SB. The differentiation of delayed hemolytic and delayed serologic transfusion reactions: incidence and predictors of hemolysis. Transfusion. 1995;35:26-32.
14. Bowman JM, Pollock J. Maternal CW alloimmunization. Vox Sang. 1993;64:226-30.
15. Verduin EP, Brand A, Middelburg RA, Schonewille H. Female sex of older patients is an independent risk factor for red blood cell alloimmunization after transfusion. Transfusion. 2015;55:1478-85.
16. Schonewille H, Haak HL, van Zijl AM. RBC antibody persistence. Transfusion. 2000;40:1127-31.
17. Tormey CA, Stack G. The persistence and evanescence of blood group alloantibodies in men. Transfusion. 2009;49:505-12.
18. Koelewijn JM, Vrijkotte TG, van der Schoot CE, Bonsel GJ, de Haas M. Effect of screening for red cell antibodies, other than anti-D, to detect hemolytic disease of the fetus and newborn: a population study in the Netherlands. Transfusion. 2008;48:941-52.
19. Lang TJ. Estrogen as an immunomodulator. Clin Immunol. 2004;113:224-30.
20. Wayman JC. Multiple imputation for missing data: what is it and how can I use it? Paper presented at the 2003 Annual Meeting of the Maerican Educational Research Association, Chicago, IL.
21. Schafer JL, Graham JW. Missing data: our view of the state of the art. Psychol Methods. 2002;7:147-77.
22. Donders AR, van der Heijden GJ, Stijnen T, Moons KG. Review: a gentle introduction to imputation of missing values. J Clin Epidemiol. 2006:59;1087-91.
23. Atsma F, Veldhuizen I, de Vegt F, Doggen C, de Kort W. Cardiovascular and demographic characteristics in whole blood and plasma donors: results from the Donor InSight study. Transfusion. 2011;51:412-20.
24. Statline, Statististics, Heerlen/The Hague The Netherlands. Accesible at: http://statline.cbs.nl.
25. Yazdanbakhsh K, Ware RE, Noizat-Pirenne F. Red blood cell alloimmunization in sickle cell disease: pathophysiology, risk factors, and transfusion management. Blood. 2012;120:528-37.
26. Zimring JC, Stowell SR, Johnsen JM, Hendrickson JE. Effects of genetic, epigenetic, and environmental factors on alloimmunization to transfused antigens: Current paradigms and future considerations. Transfus Clin Biol. 2012;19:125-31.
27. Zimring JC, Hendrickson JE. The role of inflammation in alloimmunization to antigens on transfused red blood cells. Curr Opin Hematol. 2008;15:631-5.

28. Zalpuri S, Zwaginga JJ, van der Bom JG. Risk Factors for Alloimmunization after red blood Cell Transfusions (R-FACT): a case cohort study. BMJ Open. 2012;2:e001150.
29. Zalpuri S, Evers D, Zwaginga JJ, Schonewille H, de Vooght KM, le Cessie S et al. Immunosuppressants and allo-immunization against red blood cell transfusions. Transfusion. 2014;54:1981-7.
30. Geoff Daniels. Human Blood Groups, 3rd ed. Chichester UK: Wiley-Blackwell; 2013.
31. Zalpuri S, Middelburg RA, Schonewille H, et al. Intensive red blood cell transfusions and risk of alloimmunization. Transfusion. 2014;54:278-84.
32. Anstee DJ. Goodbye to agglutination and all that? Transfusion. 2005;45:652-3.
33. Avent ND, Martinez A, Flegel WA, Olsson ML, Scott ML, Nogués N et al. The BloodGen project: toward mass-scale comprehensive genotyping of blood donors in the European Union and beyond. Transfusion. 2007;47:40S-46S.

## Supplementary material

**Supplementary Box 1** The effect of censoring on estimated cumulative incidences.

We calculated cumulative alloimmunization incidence rates only for antigen-negative cohorts containing at least 200 non-censored patients. This cut-off was chosen as the rate of censoring between baseline and *N* transfusions can compromise the validity of estimated cumulative incidences. In case of a low-frequent antigen (i.e. K), only a few patients will repeatedly be exposed to this antigen and the contribution of random error to the estimated cumulative incidence thus could become unacceptably large. With 200 patients under analysis, one random additional alloimmunization event will increase the cumulative alloimmunization rate by maximally 0.5%, which, in our opinion, justifies comparison of one risk curve to another.

For this same reason, we compared cumulative antigen-specific alloimmunization incidences with alloimmunization incidences of $Fy^a$. Previously, comparisons with K have been made.[1-4] However, the reliability of estimated anti-K cumulative incidences significantly decreases with the number of K-exposed red cell units as only a minority of patients receive multiple K-positive units due to its low antigen frequency (being 9% in the Dutch donor population).[5] As in former reports $Fy^a$ immunogenicity was in the middle of the extreme[1-4] and as the probability of exposure to a non-self red cell antigen for a random individual is highest (except for S) for $Fy^a$,[5] we chose $Fy^a$ as the reference.

Finally, we present our data as 'adjusted cumulative risk curves'. As alloimmunized patients are censored at the time of immunization, the size of the population at risk is reduced as a result of alloimmunization. Alloimmunization can thereby lead to an overestimation of the cumulative risk, mainly when a large number of immunization events take place. We therefore calculated 'adjusted' numbers of alloimmunized patients, equalling the number of alloimmunized patients that would have received *N* transfusions had they not been censored at the time of alloimmunization. We used these numbers for Kaplan-Meier analysis.

The examples A-C illustrate that the distribution of events and the rate of censoring can have dramatic effects on final cumulative incidences.

**A** The effect of event distribution on the cumulative risk.

| | | Early immunizations | | Late immunizations | |
|---|---|---|---|---|---|
| **Transfused units** | **Not censored (N)** | **Immunization events (N)** | **Cumulative risk (%)** | **Immunization events** | **Cumulative risk (%)** |
| 1 | 10,000 | 50 | 0.50 | 0 | 0 |
| 2 | 9,000 | 50 | 1.05 | 0 | 0 |
| 3 | 8,100 | 50 | 1.66 | 0 | 0 |
| 4 | 7,290 | 50 | 2.33 | 0 | 0 |
| 5 | 6,560 | 50 | 3.08 | 0 | 0 |
| 6 | 5,910 | 0 | 3.08 | 50 | 0.85 |
| 7 | 5,310 | 0 | 3.08 | 50 | 1.78 |
| 8 | 4,780 | 0 | 3.08 | 50 | 2.81 |
| 9 | 4,300 | 0 | 3.08 | 50 | 3.94 |
| 10 | 3,870 | 0 | 3.08 | 50 | 5.18 |

Fictitious baseline cohort of 10,000 patients, censoring rate 10% per transfused red cell unit. The same number of events induces a higher cumulative risk when these events occur in a smaller cohort.

**B** The effect of censoring rates on the cumulative alloimmunization risk.

| | | Censoring rate 10% | | Censoring rate 50% | |
|---|---|---|---|---|---|
| **Transfused units** | **Immunization events** | **Not censored (N)** | **Cumulative risk (%)** | **Not censored (N)** | **Cumulative risk (%)** |
| 1 | 10 | 10,000 | 0.10 | 10,000 | 0.10 |
| 2 | 10 | 9,000 | 0.21 | 5,000 | 0.30 |
| 3 | 10 | 8,100 | 0.33 | 2,500 | 0.70 |
| 4 | 10 | 7,290 | 0.47 | 1,250 | 1.49 |
| 5 | 10 | 6,560 | 0.62 | 625 | 3.07 |
| 6 | 10 | 5,910 | 0.79 | 313 | 6.17 |
| 7 | 10 | 5,310 | 0.97 | 156 | 12.18 |
| 8 | 10 | 4,780 | 1.18 | 78 | 23.44 |
| 9 | 10 | 4,300 | 1.41 | 39 | 43.07 |
| 10 | 10 | 3,870 | 1.67 | 20 | 71.54 |

Fictitious baseline cohort of 10,000 patients with a fixed number of 10 alloimmunization events per transfused red cell unit. Censoring rates of 10% versus 50% per transfused red cell unit. A high censoring rate induces a disproportionally increase of the cumulative risk.

**C** The effect of censoring of immunized patients at time of alloimmunization.

| Transfused units | Immunization events (N) | Not censored cohort (N) | Cumulative risk (%) | Adjusted not censored cohort (N) | Adjusted cumulative risk (%) |
|---|---|---|---|---|---|
| 1 | 100 | 10,000 | 1.00 | 10,000 | 1.00 |
| 2 | 100 | 9,000 | 2.10 | 9,090 | 2.09 |
| 3 | 100 | 8,100 | 3.31 | 8,274 | 3.27 |
| 4 | 100 | 7,290 | 4.63 | 7,538 | 4.56 |
| 5 | 100 | 6,560 | 6.09 | 6,879 | 5.94 |
| 6 | 100 | 5,910 | 7.68 | 6,293 | 7.44 |
| 7 | 100 | 5,310 | 9.42 | 5,752 | 9.05 |
| 8 | 100 | 4,780 | 11.31 | 5,277 | 10.77 |
| 9 | 100 | 4,300 | 13.37 | 4,848 | 12.61 |
| 10 | 100 | 3,870 | 15.61 | 4,467 | 14.57 |

Fictitious baseline cohort of 10,000 patients, censoring rate 10% per red cell transfusion. Not adjusting for censored alloimmunized patients overestimates the cumulative risk after 10 red cell transfusions by 7.1% ((15.61-14.57) / 14.57) * 100.

**Supplementary Box 2** Estimated evanescence rates are dependent on the time following antibody induction.

Reported rates of evanesced antibodies[6,7] so far have been based on prevalences (i.e. the frequency of evanesced alloantibodies at a certain time point) rather than on incidences (i.e. the frequency of evanesced alloantibodies according to the time following exposure). These studies reported evanescence rates as high as 25 to 40% for anti-$Jk^a$ and anti-$C^w$.

When considering these numbers, one should realize that the frequency of evanesced antibodies increases significantly with increasing time since antibody induction. Alloantibodies against antigens of moderate frequency will in general form rather early during the transfusion history as with every transfusion the likelihood to be exposed to this alloantigen is rather high. In contrast, induction of alloantibodies against low or high frequent antigens will be more evenly distributed along the transfusion history. Thus, even though two types of antibodies evanescence at the same rate, the observed frequency of evanesced antibodies will diverge with the number of red cell units exposed as illustrated in a fictitious example for anti-A and anti-B here below. In conclusion, reported rates of evanesced antibodies are highly dependent on the chance of alloantigen exposure and thus on antigen population frequencies. Addressing evanescence into incidence-based alloimmunization calculations is only possible with data on serological follow-up at multiple fixed times after antigen exposure being available.

| time event | anti-A (N) | evanesced anti-A | Cumulative (non-evanesced) anti-A | Persistence rate (%) |
|---|---|---|---|---|
| 1 | 20 | 0 | 20 | 100 |
| 2 | 10 | 10 | 20 | 66.7 |
| 3 | 3 | 10 | 13 | 39.4 |
| 4 | 2 | 6.5 | 8.5 | 24.3 |
| 5 | 0 | 4.25 | 4.25 | 12.1 |
| **time event** | **anti-B (N)** | **evanesced anti-B** | **Cumulative (non-evanesced) anti-B** | **Persistence rate (%)** |
| 1 | 7 | 0 | 7 | 100 |
| 2 | 7 | 3.5 | 10.5 | 75.0 |
| 3 | 7 | 5.25 | 12.25 | 58.3 |
| 4 | 7 | 6.13 | 13.13 | 46.9 |
| 5 | 7 | 6.53 | 13.56 | 38.7 |

Fictitious example of a transfused cohort in which 35 patients formed alloantibodies against a moderately frequent antigen A, and a low-frequent antigen B. Allo-anti A and allo-anti-B both disappear at a rate of 50% per time period.

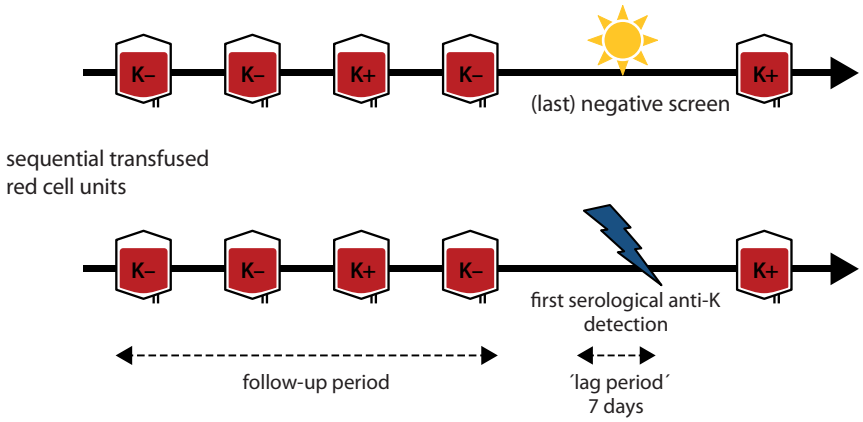
Due to a relatively high likelihood of (non-self) A exposure per time event, most of these anti-A's will be formed early. Thus, in this example, 88% of the 35 formed anti-A's will not be detected after a follow-up of 5 time events. In contrast, as anti-B forms at lower rates due to a lower chance of encountering this (non-self) antigen per time event, only 61% of the 35 formed anti-B's will not be detected after a follow-up of 5 time events.

**Table S1** Overview of missing donor antigen data and the use of multiple imputation.

| Antigen | Missing data (%) |
|---|---|
| D | 0.0 |
| C | 0.5 |
| C | 0.4 |
| E | 0.4 |
| E | 0.5 |
| $C^w$ | 44.7 |
| K | 0.5 |
| $Fy^a$ | 35.0 |
| $Fy^b$ | 45.8 |
| $Jk^a$ | 19.3 |
| $Jk^b$ | 19.9 |
| $Le^a$ | 48.9 |
| $Le^b$ | 57.7 |
| $Lu^a$ | 78.5 |
| $Lu^b$ | 90.6 |
| M | 26.5 |
| N | 38.0 |
| S | 21.5 |
| S | 38.7 |

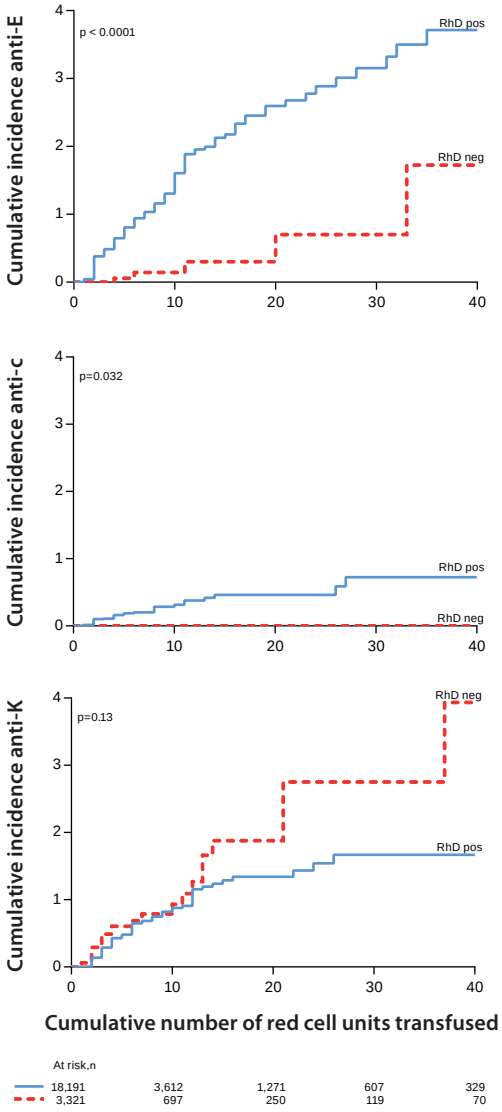
Percentages of missing antigen values in 152,412 red cell units transfused to 21,512 patients.
To address missing donor phenotypes, multiple imputation was used thereby creating five imputed datasets. Here, we assumed randomness of missing data (i.e missing values depended on observed data, but not on the value of the missing variable itself).[8,9] Imputation was only performed for antigen-negative cohorts with less than 50% missing antigen data. Consequently, $Le^b$, $Lu^a$, and $Lu^b$ were excluded from antigen immunogenicity calculations.
Predictor variables for the imputation model included transfusion center, age under or above 45 years, sex, allo-immunization status, and known red cell antigen phenotypes of the blood product (i.e. other, non-missing antigens).

**Figure S1** Illustration of calculations for cumulative numbers of transfused (antigen-mismatched) red cell units.



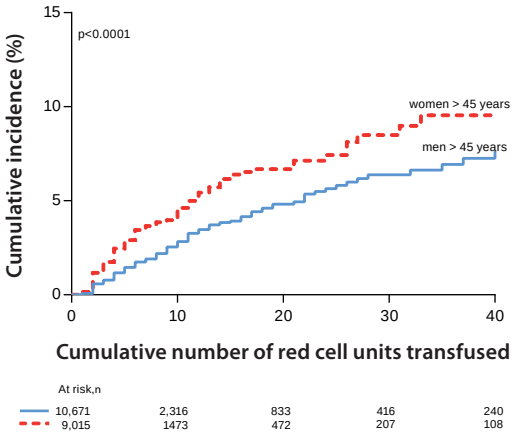

Non-alloimmunized patients were followed-up until their last available negative screen and alloimmunized patients were followed-up until the first positive screen. Alloimmunizations detected within seven days of a mismatched transfusion were excluded from analyses as they most likely represented boosting from previous induced alloimmunization rather than primary alloimmunization.
In this example, one non-immunized K-negative patient and one K-immunized patient both received four red cells units and one K-mismatched unit during their follow-up.

**Figure S2** RhD-negative individuals do not form anti-c and rarely anti-E.




Due to linkage of the *RHD* and *RHCE* gene, approximately all RhD-negative patients express the c antigen and cannot form allo-anti-c. As only 2.9% of RhD-negative Dutch donors are E-positive, RhD matching strongly reduces the risk of allo-anti-E formation in RhD-negative patients. RhD phenotype is not associated to the risk of alloimmunization against other red cell antigens as here demonstrated for anti-K.

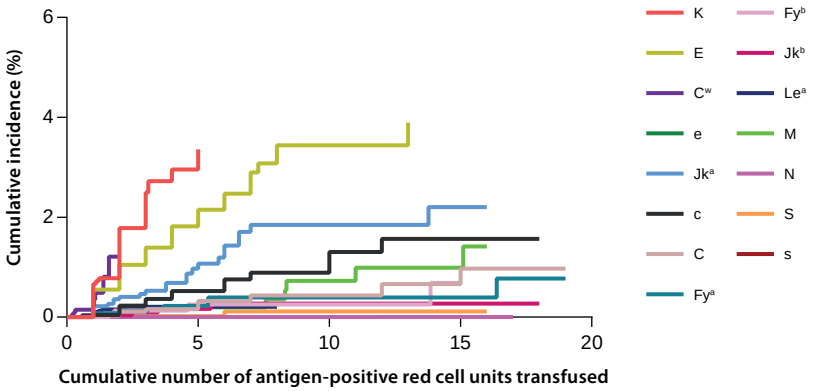
**Figure S3** Cumulative alloimmunization incidences according to sex in patients aged 45 years and above.




| | Cumulative alloimmunization incidences, % (CI): | | | |
|---|---|---|---|---|
| | against all antigens | | against the antigens CcEe, $C^w$, and K | |
| Number of transfused units | Men >45 yrs N=10,671 | Women >45 yrs N=9,015 | Men >45 yrs N=10,671 | Women >45 yrs N=9,015 |
| 5 | 1.41 (0.56-3.01) | 2.91 (1.59-4.88) | 0.88 (0.24-2.48) | 2.24 (1.11-4.19) |
| 10 | 2.73 (1.35-4.94) | 4.62 (2.70-7.30) | 1.85 (0.66-4.17) | 3.46 (1.80-6.07) |
| 20 | 4.60 (2.45-7.77) | 6.69 (3.96-10.37) | 3.52 (1.51-6.90) | 5.00 (2.57-8.62) |
| 40 | 7.08 (3.44-12.49) | 9.51 (4.80-16.23) | 5.23 (1.97-10.90) | 7.91 (3.24-15.31) |

Women as compared to men over 45 years of age showed statistically significant higher alloimmunization incidences (figure) due to higher Rh and K immunization rates (table).
CI = 95% confidence interval.

**Figure S4** Cumulative alloimmunization incidences as a function of antigen exposure in the male population.




At risk, n

| | | | | | |
|---|---|---|---|---|---|
| K | 11,215 | 242 | 21 | 5 | 2 |
| E | 8,787 | 1,171 | 363 | 150 | 85 |
| $C^w$ | 12,025 | 18 | 4 | 1 | 0 |
| e | 246 | 106 | 54 | 29 | 16 |
| $Jk^a$ | 2,847 | 1,074 | 461 | 249 | 145 |
| c | 2,469 | 988 | 480 | 273 | 166 |
| C | 3,935 | 1,227 | 574 | 321 | 181 |
| $Fy^a$ | 4,178 | 1,386 | 584 | 316 | 188 |
| $Fy^b$ | 2,090 | 758 | 353 | 206 | 135 |
| $Jk^b$ | 3,194 | 1,094 | 503 | 284 | 176 |
| $Le^a$ | 9,571 | 664 | 156 | 52 | 18 |
| M | 2,709 | 1,029 | 465 | 245 | 148 |
| N | 3,432 | 1,124 | 491 | 261 | 159 |
| S | 5,520 | 1,316 | 512 | 247 | 134 |
| s | 1,347 | 542 | 259 | 159 | 105 |

| | Male cohort Cumulative incidence, % (CI) | | | | |
|---|---|---|---|---|---|
| **Mismatched units (N)** | **E** | **K** | **$C^w$** | **$Fy^a$** | **$Jk^a$** |
| 1 | 0.55 (0.06-2.53) | 0.66 (0.07-3.05) | 0.24 (0.00-11.68) | 0.08 (0.00-10.03) | 0.22 (0.00-6.42) |
| 2 | 1.05 (0.23-3.24) | 1.78 (0.40-5.27) | 1.21 (0.00-16.81) | 0.12 (0.00-8.11) | 0.41 (0.00-5.22) |
| 5 | 2.15 (0.63-5.44) | 3.36 (0.67-10.01) | | 0.23 (0.00-7.31) | 1.07 (0.06-6.34) |
| 10 | 3.44 (1.03-8.35) | | | 0.39 (0.00-7.60) | 1.43 (0.11-7.02) |
| 15 | | | | 0.39 (0.00-7.60) | 1.85 (0.20-7.73) |

The relative antigen immunogenicity and the antigen potency order observed in male did not differ from the entire cohort.
CI = 95% confidence interval. Only data from non-censored cohorts of at least 200 subjects are presented.

## References of supplementary material


1. Giblett ER. A critique of the theoretical hazard of inter vs. intra-racial transfusion. Transfusion. 1961;1:233-8.
2. Tormey CA, Stack G. Immunogenicity of blood group antigens: a mathematical model corrected for antibody evanescence with exclusion of naturally occurring and pregnancy-related antibodies. Blood. 2009;114:4279-82.
3. Schonewille H, van de Watering LM, Loomans DS, Brand A. Red blood cell alloantibodies after transfusion: factors influencing incidence and specificity. Transfusion. 2006;46:250-6.
4. Winters JL, Pineda AA, Gorden LD, et al. RBC alloantibody specificity and antigen potency in Olmsted County, Minnesota. Transfusion. 2001;41:1413-20.
5. Reid ME, Lomas-Francis C, Olsson ML. The blood group antigen factsbook, second edition. San Diego: Elsevier Academic Press; 2004.
6. Tormey CA, Stack G. The persistence and evanescence of blood group alloantibodies in men. Transfusion. 2009;49:505-12.
7. Reverberi R. The persistence of red cell alloantibodies. Blood Transfus. 2008;6:225-34.
8. Schafer JL, Graham JW. Missing data: our view of the state of the art. Psychol Methods. 2002;7:147-77.
9. Wayman J.C. Multiple imputation for missing data: what is it and how can I use it? Paper presented at the 2003 Annual Meeting of the American Educational Research Assocation, Chicago, IL; 2003.