INSTITUTO POLITÉCNICO DE LISBOA

ESCOLA SUPERIOR DE SAÚDE DE LISBOA

# RED BLOOD CELL ALLOIMMUNIZATION:

# IMPACT OF POST-TRANSFUSION FOLLOW-UP

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**Master`s in Clinical-Laboratory Technology**

*Lisbon, 2025*

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**Master's in Clinical-Laboratory Technology**

(this version includes suggestions made by the jury)

*Lisbon, 2025*

*When my timing is off, I have trouble;*

*when it ain`t, I don`t.*

Hank Aaron

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## Declaration of Authorship and Copyright

I hereby declare that this master`s thesis entitled “Red Blood Cell Alloimmunization: Impact of Post-Transfusion Follow-Up” is an original work and has not been submitted elsewhere for any degree or qualification. This work does not involve any conflicts of interest of a personal, academic, political, or financial nature, nor did it receive any financial support for the research that led to its development.

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## Acknowledgments

This was never just a thesis. It was a journey, and like all journeys, it had its highs and lows, moments of doubt and moments of uncertainty. One thing, however, remained constant: none of this would have been possible without the people who walked alongside me, and continue to do so.

Cristina, my wife, thank you for everything,

For your patience and encouragement when I doubted myself, and for carrying so much more than your share so I could finish this project.

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Thank you for reminding me every day what really matters. I owe you time, hugs, and stories…

To my parents and parents-in-law,

Your constant presence gave me space to grow.

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Thank you for the trust you placed in me to revisit two decades of transfusion data. Without your confidence, this thesis would not have been possible.

Doutor. António Robalo Nunes and Doutor. Edna Carneiro,

Thank you for being honest, and generous with your time and knowledge. This work is so much better because of your guidance.

And finally, I would like to thank myself,

For having the healthy "madness" to embark on this master's degree. For seeking discomfort instead of staying where it was safe. For always searching for something, even when most of the time I wasn't entirely sure what I was looking. For learning not just how to write a thesis, but how to be patient, to stay focused, and to be kinder with my own limits.

*«O fim de uma viagem é apenas o começo de outra.*
*É preciso ver o que não foi visto, ver outra vez o que se viu já,*
*ver na primavera o que se vira no verão, ver de dia o que se viu de noite (...)*
*É preciso recomeçar a viagem. Sempre.»*

*José Saramago*

# Abstract

**Introduction:** Alloantibody formation against red blood cell (RBC) antigens is a common complication in immunohematology. It can delay or complicate transfusions by reducing the availability of compatible RBC units and increasing the risk of delayed hemolytic transfusion reactions.

**Objectives:** Estimate the prevalence, identify associated risk factors, and evaluate the impact of post-transfusion indirect antiglobulin test (IAT) timing in RBC alloimmunization.

**Methodology**: Retrospective study which included 20,841 adult patients who received 94,689 RBC units at a Portuguese hospital between 2002 and 2022. Patients were organized into four cohorts. Three included individuals sensitized through transfusion with Rh/Kell antigen-positive RBC units for which they were antigen-negative, monitored by IAT performed at different intervals: 30–180 days (ideal), >180 days (delayed), or not performed (no follow-up). A fourth cohort included non-sensitized or early-tested patients (<30 days).

**Results:** Alloantibodies were identified in 527 patients (2.53%), with higher prevalence in females (68.9%) despite representing 53.8% of transfused cases. Rh and Kell antibodies were accounted for 69.7%. The most common were anti-D (25.0%), anti-K (17.3%), and anti-E (13.3%). Alloimmunization rates were higher in patients tested within 30-180 days (11.1%) than after 180 days (4.6%) ($p$<0.05). Timing significantly affected detection of anti-C, anti-E, and anti-K ($p$<0.05). In females, all three showed statistical significance; in males, only anti-E did. Female sex, Rh negativity, and number of transfusions were associated with increased immunogenicity. A logistic regression model estimated undetected alloimmunization in 2,790 patients without follow-up.

**Discussion/Conclusion:** Detection was strongly dependent on timing. Failure to monitor within the 30–180 day window likely underestimates true prevalence due to antibody evanescence. Structured post-transfusion follow-up significantly improves antibody detection and could enhance transfusion safety, especially in high-risk populations.



**Keywords**: Red blood cells, Transfusion, Alloimmunization, Immunogenicity, Timing.

# Resumo

**Introdução:** A formação de aloanticorpos contra antigénios eritrocitários é uma complicação comum em imuno-hemoterapia. Pode atrasar ou dificultar transfusões ao reduzir a disponibilidade de unidades de concentrado eritrocitário (CE) compatíveis e aumentar o risco de reações hemolíticas tardias.

**Objetivos:** Estimar a prevalência, identificar fatores de risco e avaliar o impacto do tempo de pesquisa pós-transfusional na deteção de aloanticorpos.

**Metodologia:** Estudo retrospetivo que incluiu 20.841 pacientes adultos que foram transfundidos com 94.689 unidades de CE num hospital português entre 2002 e 2022. Os pacientes foram organizados em quatro coortes. Três incluíram indivíduos sensibilizados por transfusão com CE Rh/Kell antigénio-positivas, para os quais eram antigénio-negativos, monitorizados por pesquisa de anticorpos irregulares (PAI) pós-transfusional realizada em diferentes momentos: 30–180 dias (período ideal), >180 dias (período não-ideal) ou sem seguimento. O quarto incluiu doentes não sensibilizados ou testados precocemente (<30 dias).

**Resultados:** Foram identificados aloanticorpos em 527 pacientes (2,53%), com maior prevalência no sexo feminino (68,9%), apesar de representarem 53,8% da população transfundida. Anticorpos Rh e Kell corresponderam a 69,7%. Os mais frequentes foram anti-D (25,0%), anti-K (17,3%) e anti-E (13,3%). As taxas de aloimunização foram mais elevadas entre 30–180 dias (11,1%) do que após 180 dias (4,6%) ($p$<0,05). O timing afetou significativamente a deteção de anti-C, anti-E e anti-K ($p$<0,05). No sexo feminino, os três apresentaram significância estatística; no masculino, apenas o anti-E. Sexo feminino, Rh negativo e número de transfusões estiveram associados a maior imunogenicidade. Um modelo de regressão logística procurou estimar a aloimunização em 2.790 doentes sem seguimento.

**Discussão/Conclusão**: A deteção de anticorpos é significativamente afetada pelo período da PAI pós-transfusional. A ausência de monitorização no intervalo de 30–180 dias subestima a prevalência real devido à evanescência dos anticorpos. O seguimento estruturado pós-transfusional melhora significativamente a deteção de anticorpos e pode aumentar a segurança dos pacientes, especialmente em populações de risco

**Palavras-chave**: Concentrado eritrocitário, Transfusão, Aloimunização, Imunogenicidade, Período ideal

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# Abbreviations List

**APC** – Antigen Presenting Cells
**BSA –** Bovine Serum Albumin
**CE –** *Concentrado Eritrocitário*
**DAT –** Direct Antiglobulin Test
**DC –** Dendritic Cells
**DHTR** - Delayed Hemolytic Transfusion Reactions
**DTT –** Dithiothreitol
**EFSAM -** *Établissement Français du Sang Alpes-Méditerranée*
**ESSL –** *Escola Superior de Saúde de Lisboa*
**GDPR –** General Data Protection Regulation
**HDFN** - Hemolytic Disease of the Fetus and Newborn
**HTR** - Hemolytic Transfusion Reactions
**IAI –** Irregular Antibody Identification
**IAT** – Indirect Antiglobulin Test
**IPL** – *Instituto Politécnico de Lisboa*
**IPST –** Instituto Português do Sangue e Transplantação
**IHS** - Immunohematology Service
**ISBT –** International Society of Blood Transfusion
**LISS –** Low Ionic Strength Solution
**MDS –** Myelodysplastic Syndrome
**MHC** - Major Histocompatibility Complex
**RBC** - Red Blood Cells
**RhIG** - Rh Immunoglobulin
**PAI –** *Pesquisa de Anticorpos Irregulares*
**PBM –** Patient Blood Management
**PEG –** Polyethylene Glycol
**PPH** – Postpartum Hemorrhage
**SCD** - Sickle Cell Disease
**SPSS -** Statistical Package for Social Sciences
**TACO** - Transfusion Associated Circulatory Overload
**TRALI** - Transfusion Related Acute Lung Injury
**TRIX -** Transfusion Register of Irregular Antibodies and Cross-Match Problems
**ULSAR** – *Unidade Local de Saúde do Arco Riberiinho*
**USA -** United States of America
**WBIT –** Wrong Blood in Tube

# Chapter 1. Introduction

## 1.1 Contextualization

Blood transfusions are a standard part of modern medicine and are widely used for the management of acute haemorrhage, chronic anaemia, haematological malignancies, and surgical interventions[1]. They are also essential in obstetric emergencies such as complicated deliveries and severe postpartum haemorrhage (PPH), which are still among the leading causes of maternal morbidity and mortality worldwide[2 3].

This paper aims at assessing the current state of transfusion medicine in Portugal, with a focus on the challenges and risks associated with blood transfusions, as well as exploring potential solutions to improve the safety and efficiency of transfusion practices. Every year, millions of people worldwide receive this life-saving therapy; however, transfusion medicine is not without risks, and one of the most significant complications is red blood cell RBC alloimmunization – a process in which a recipient's immune system forms antibodies against foreign antigens expressed on donor RBCs[4]. These alloantibodies can complicate future transfusions, delay life-saving treatments, and increase the risk of Hemolytic Transfusion Reactions (HTR)[5 6 7 8].

The steady decrease in blood donations in combination with the growing demand for transfusion services brought about by population aging, and the rising incidence of chronic diseases, creates an environment of high risk for transfusion practices[9]. Furthermore, the incorporation of ethnically diverse populations in the Portuguese healthcare system has introduced the problem of lack of antigenically matched blood from a predominantly European donor population[10].

## 1.2 Problem Statement. Proposed Solution.

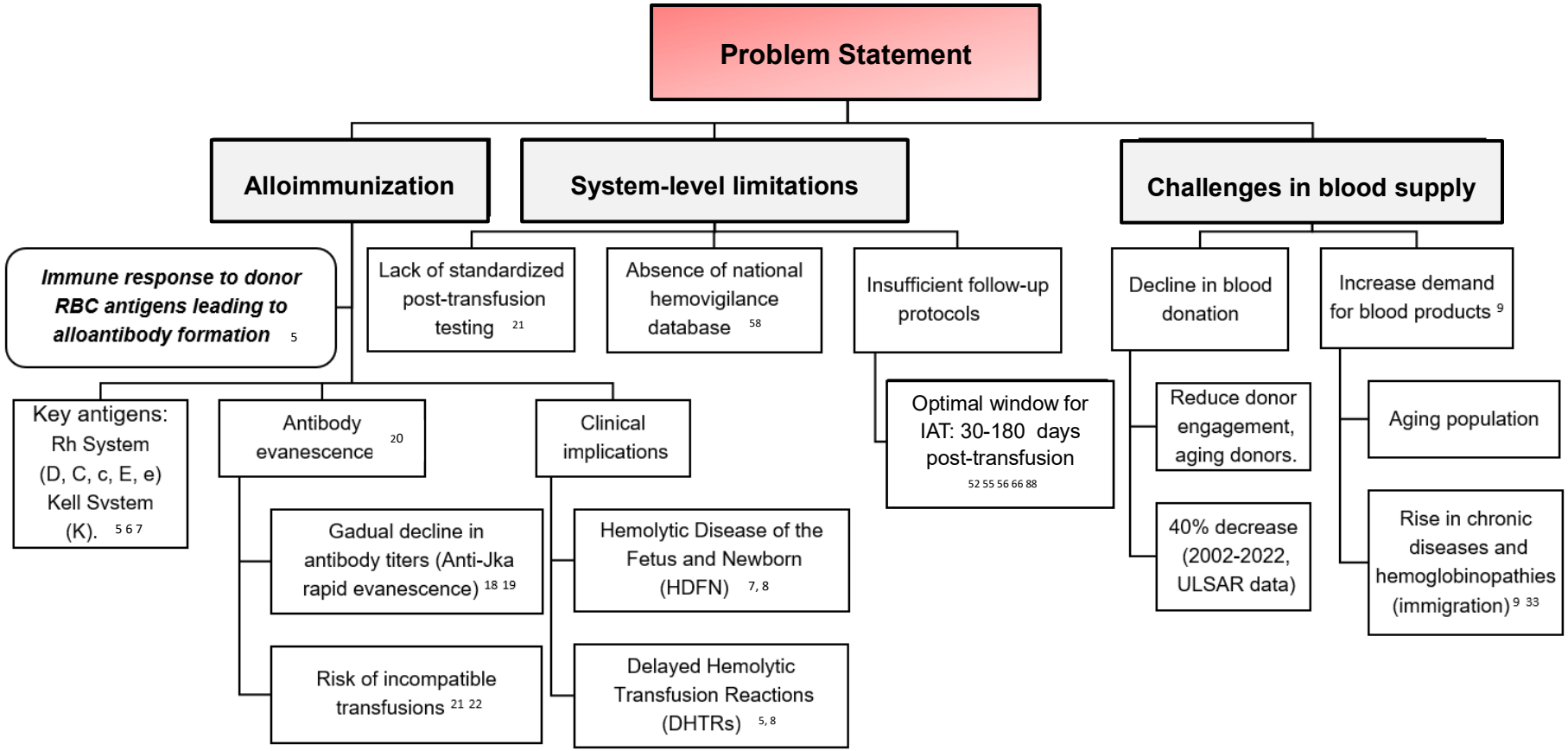


**Figure 1.1**. Proposed Statement flowchart. This flowchart presents the key challenges addressed in this study. It serves as an overview of the interconnections between clinical, systemic and demographic factors that justify the relevance of the research. Original flowchart.

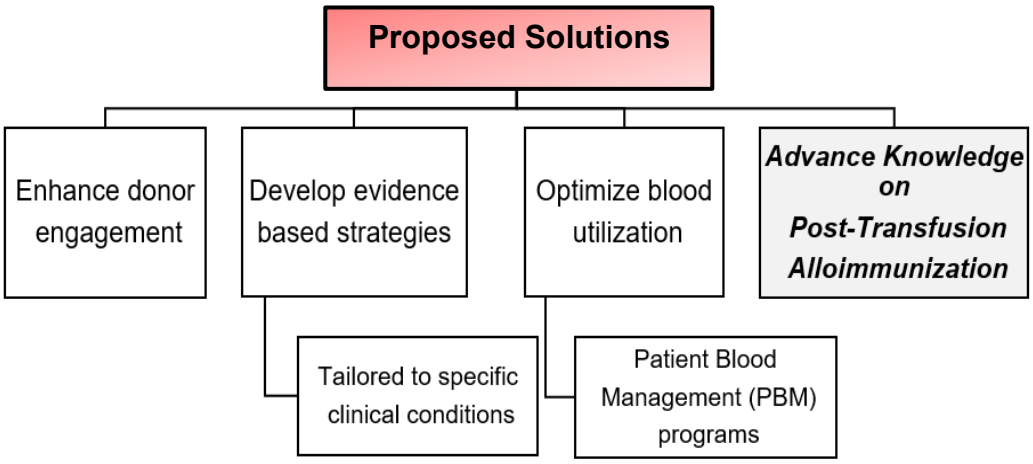


**Figure 1.2.** Proposed Solutions Flowchart. This flowchart outlines the key solutions proposed in this study to address the challenges related to transfusion medicine. The highlighted solution, "Advance Knowledge on Post-Transfusion Alloimmunization", represents the primary focus of the thesis, emphasizing its contribution to advancing research and improving clinical practices. Original flowchart.

## 1.3 Justification and Relevance

The current study was designed with the aim to tackling some of the most important issues in transfusion medicine, such as RBC alloimmunization and its clinical implications[5 6]. The demand for blood has increased while the supply of blood donations has been declining, putting pressure on healthcare systems worldwide, particularly in the *Unidade Local de Saúde do Arco Ribeirinho* (ULSAR), where there has been a 40% annual reduction in blood collections from 2002 to 2022, as can be observed in Figure 1.3**.**

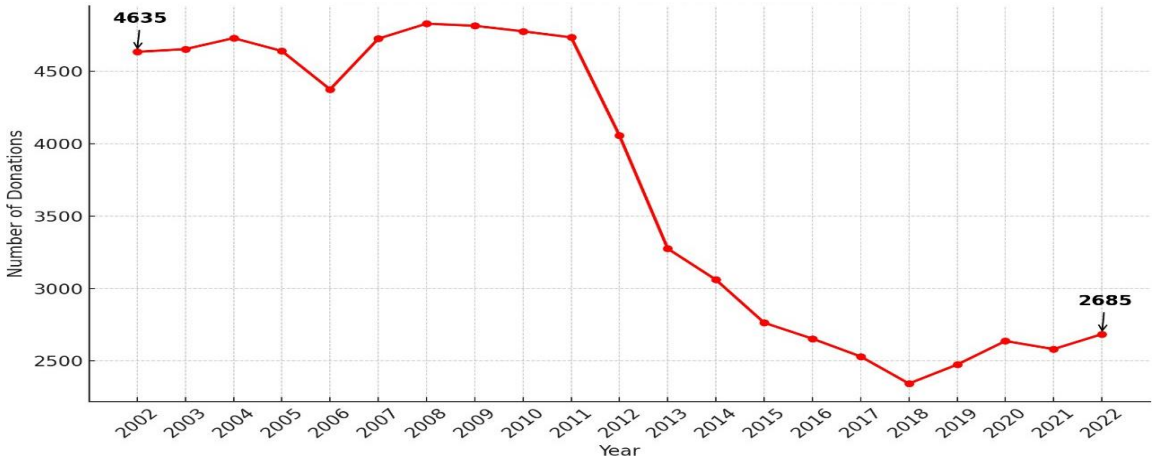


**Figure 1.3**. Annual Blood Donations at ULSAR (2002–2022). Blood donation numbers remained relatively stable from 2002 to 2011. Between 2012 and 2018, donations declined, reaching the lowest point in 2018. Thereafter, a gradual recovery was observed, with 2,685 donations recorded in 2022 – approximately 40 % below 2002 levels. *Adapted from: AsisWeb, ULSAR Immunohematology Service.*

Several factors that exacerbate the growing shortfall in supply versus demand are key drivers of the problem [1]. They include an aging population[11], the increasing burden of chronic diseases[12], changes in immigration leading to a rise in hemoglobinopathies[13], and an increase in unmonitored pregnancies, especially among immigrant women, which often result in complicated deliveries that call for urgent blood transfusions[14 15]. In addition, the expanding application of monoclonal antibody therapies in oncology also increases the demand for transfusions since these therapies may interfere with the pre-transfusion testing and require additional blood support[16 17].

The major contributors to alloimmunization events are still the disproportionate immunogenicity of certain RBC antigens, particularly those in the Rh (D, C, c, E, e) and Kell systems[6]. These are alloantibodies that complicate future transfusions, delay the provision of compatible blood and increase the risk of DHTR[8]. However, the issue of antibody evanescence wherein the alloantibody titres appear undetectable at one time and the patient is therefore at risk of receiving incompatible RBC in a subsequent transfusion is a critical diagnostic challenge also. This is particularly a problem with certain antibodies, including anti-JK$^a$, which – with anti-E and anti-K – are three of the most common causes of DHTRs[18 19]. Unlike anti-E and anti-K, however, anti-JK$^a$ is distinguished by its propensity for more rapid evanescence[20]. The transient nature of these antibodies leaves patients at increased risk of receiving incompatible RBC units in subsequent transfusions, especially when post-transfusion follow-up protocols are lacking[21 22]. These reasons have made alloimmunization to become the third leading cause of transfusion associated fatalities in the United States of America (USA). The causes of death included Transfusion Associated Circulatory Overload (TACO) 32%, Transfusion Related Acute Lung Injury (TRALI) and possible TRALI 21%, and HTR due to non-ABO incompatibilities (14%)[15].

The issues are also due to the fact that Portugal has ethnic diversity, particularly in the ULSAR region which has populations of African descent – who are more likely to be Fy(a–b–) – underrepresented in the blood donor pool[23]. This discrepancy renders transfusion strategies difficult, most especially for patients with Sickle Cell Disease (SCD) and other hemoglobinopathies who often need transfusion therapy[24 25]. These systematic problems need solutions that can ensure that antigen-compatible blood is available and alloimmunization risks are minimized [26]. These systematic problems highlight the importance of optimizing post-transfusion follow-up to ensure that clinically significant alloantibodies are detected in a timely and reliable manner. Nevertheless, evidence remains limited regarding how the timing of such follow-up testing influences the detection of red cell alloantibodies in real-world clinical settings.

**Research question.** *Does the timing of post-transfusion antibody screening (within 30–180 days vs after 180 days) influence the detection rate of clinically significant red cell alloantibodies in transfused patients?*

### 1.4 Research objectives

To address the research question, this study pursued the following specific objectives:

1. **Compare Alloantibody Detection Across Follow-Up Cohorts:**
   To determine whether different post-transfusion testing intervals (30–180 days vs. >180 days) result in distinct alloantibody detection rates.
2. **Identify Predictors of Alloimmunization:**
   To assess the association of age, sex, ABO/Rh group, and transfusion burden with the risk of RBC alloimmunization, using merged cohorts to increase statistical power.
3. **Develop a Predictive Model of Alloimmunization:**
   To train and validate a model using data from monitored cohorts to infer probable alloimmunization outcomes among patients without documented follow-up.
4. **Estimate the Overall Prevalence of Irregular RBC Antibodies:**
   To describe the distribution and frequency of clinically significant alloantibodies in the study population.
5. **Support Evidence-Based Transfusion Strategies:**
   To provide data that inform optimized post-transfusion monitoring practices and contribute to safer and more sustainable transfusion policies

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## 2.1. Introduction to Alloimmunization in Transfusion Medicine

Alloimmunization is a major immunological event in transfusion medicine, where RBC transfusion recipients produce antibodies against nonself-antigens on donor erythrocytes. This makes future transfusions difficult, prolongs the availability of compatible units and creates a risk for serious complications like HTR[4]. Although advances in transfusion protocols have minimized risks, there are still challenges ahead of alloimmunization to prevent, especially in populations of patients receiving multiple blood transfusions[27].

The effects of alloimmunization extend beyond the generation of alloantibodies and have a direct impact on the mechanisms of hemolysis in adverse transfusions. The two pathways of hemolysis (intravascular and extravascular) by which alloantibodies bound to donor RBC initiate hemolysis are shown in Figure 2.1, which highlights the need for antigen-matched early detection to prevent such morbidities.

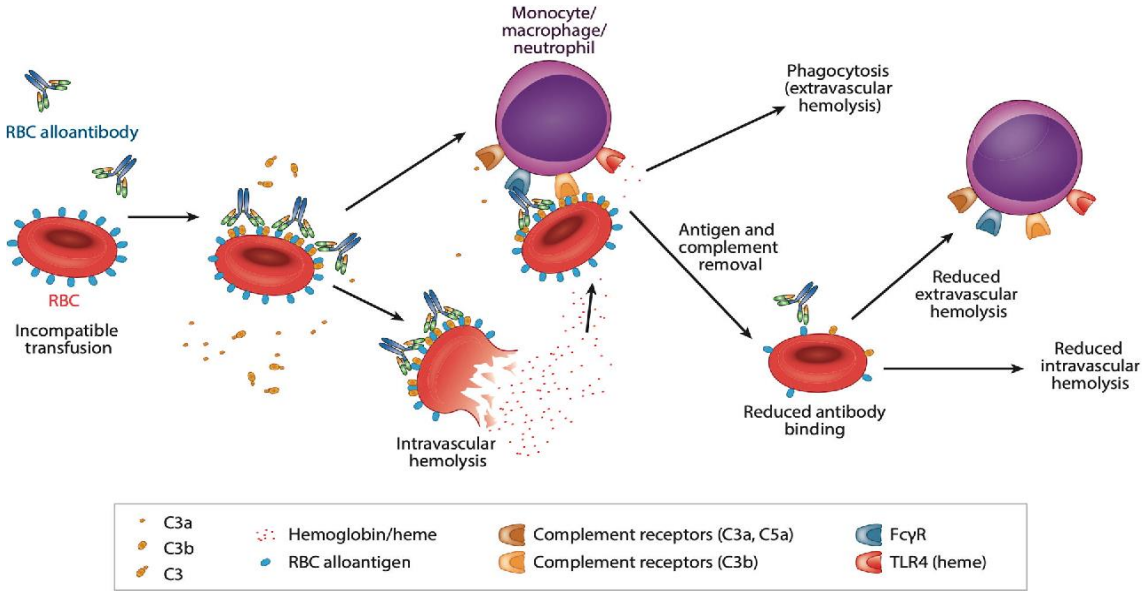


**Figure 2.1.** Hemolysis Pathways in RBC Alloimmunization. The figure demonstrates the mechanisms of hemolysis following an incompatible RBC transfusion in an alloimmunized patient. Alloantibodies bind to donor RBC antigens, activating two primary pathways. Intravascular Hemolysis: Complement activation leads to the destruction of RBCs within the vasculature, releasing hemoglobin and heme, which trigger systemic inflammation. Extravascular Hemolysis: Monocytes, macrophages, and neutrophils recognize antibody-coated RBCs via Fcγ receptors, phagocytosing and degrading them in the spleen or liver. *Adapted from* Arthur, C., & Stowell, S. (2022).

### 2.1.1 RBC Antigens and Antibody Classes

RBC antigens are molecular markers that line the surface of every erythrocyte. Made of proteins, glycoproteins, or glycolipids, they define our blood group and act like immunological fingerprints. When someone receives blood carrying antigens they don't have, the immune system may perceive them as foreign and react accordingly. The International Society of Blood Transfusion (ISBT) has identified over 360 such antigens, grouped into 43 systems[28].

In response to these foreign antigens, the body produces antibodies – with IgM and IgG being the most relevant in transfusion settings. IgM is the first line of defence: a large, pentameric molecule (~900 kDa) that responds quickly and powerfully, often triggering immediate intravascular hemolysis via activation of the complement system. ABO mismatches are classic examples of IgM-mediated reactions. IgG, by contrast, is more nuanced. It's smaller (~150 kDa), monomeric, and able to cross the placenta. Instead of destroying red cells directly, it tags them for removal, leading to extravascular hemolysis in organs like the spleen or liver. These structural and functional distinctions between IgM and IgG are illustrated in Figure 2.2, which highlights their differing sizes, binding sites, and immunological behaviours. Not all IgG antibodies behave the same way. IgG1 and IgG3 are the most potent in clinical settings - they bind tightly to Fc receptors, activate complement, and are the main drivers of serious hemolytic reactions and HDFN. IgG2 and IgG4 play a quieter role, with limited complement activation and generally milder clinical consequences[5]. Understanding how these antibodies function, and how they interact with red cell antigens, helps explain why some patients mount strong immune responses while others do not. It also lays the groundwork for understanding how and why alloimmunization occurs.

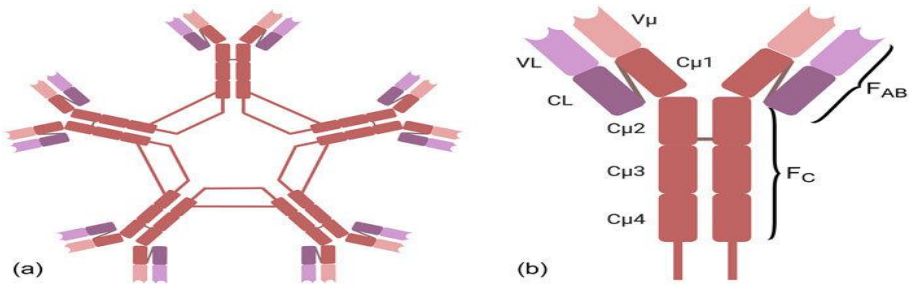


**Figure 2.2.** Immunoglobulin structure. (a) IgM structure is comprised of five monomers with ten binding sites. (b) IgG structure is comprised of one monomer with two binding sites. The binding sites are located at the end of the FAB region of the monomer, in the variable region. *Adapted from* Soler et al., 2020.

## 2.2. Mechanisms of Alloimmunization

Alloimmunization occurs when the immune system identifies donor RBC antigens as foreign, triggering an immune cascade. The process begins with antigen-presenting cells (APC), such as dendritic cells (DC) and macrophages, engulfing donor RBCs. These APC process and present alloantigens to CD4+ T-helper cells via Major Histocompatibility Complex (MHC) class II molecules, initiating the adaptive immune response. Activated CD4+ T-helper cells then stimulate B cells to differentiate into plasma cells that secrete alloantibodies. These antibodies, upon subsequent transfusions, can lead to hemolysis in alloimmunized patients. However, some individuals – referred to as nonresponders – do not generate alloantibodies despite repeated antigen exposure, whereas others, called responders, do. Genetic and immune factors likely influence these differences[5] [29]. The mechanisms underlying this process are illustrated in Figure 2.3, which depicts the pathways through which APC activate CD4+ T-helper cells, leading to the production of alloantibodies in responders and highlighting the lack of such responses in nonresponders.

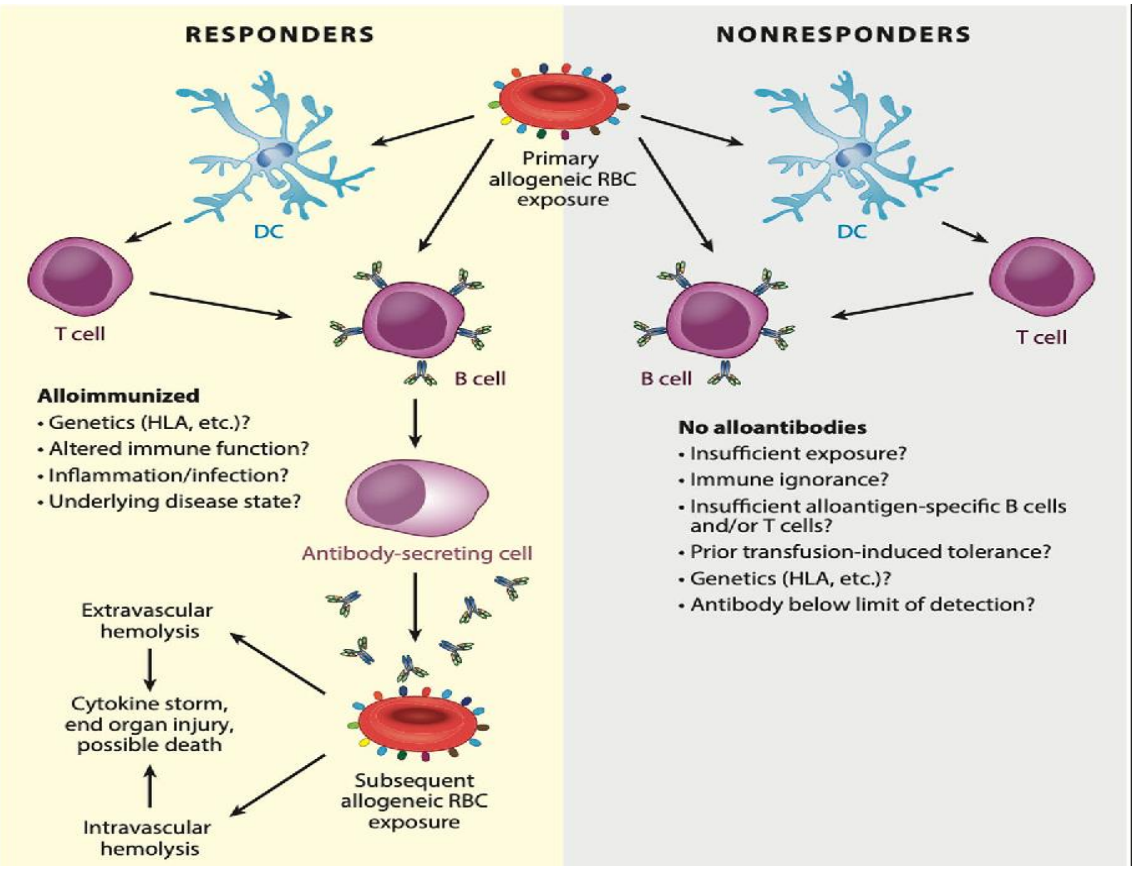


**Figure 2.3.** Mechanisms of RBC Alloimmunization**.** *Factors such as HLA genetic diversity, immune status, and donor unit variability*. *Adapted* from Arthur, C., & Stowell, S. (2022).

### 2.3. History and Evolution of Red Cell Compatibility Testing

The history of red cell compatibility testing has evolved significantly over the past century, driven by continuous scientific advancements aimed at improving transfusion safety. These key milestones are summarized in Figure 2.4, which provides a chronological overview of the major developments that have shaped modern compatibility testing and optimized donor-recipient matching.

#### 2.3.1. Early Discoveries and Initial Developments

The success of blood transfusions fundamentally depends on the identification of red cell agglutination caused by antigen-antibody interactions. In 1900, Karl Landsteiner observed that mixing blood from different individuals could result in agglutination, highlighting the presence of distinct blood groups. This discovery culminated in the identification of the ABO blood group system in 1901[30]. The adoption of ABO blood typing as a mandatory pre-transfusion procedure was first advocated by Minot[31]. Building on this advancement, Ottenberg and Hektoen introduced the concept of crossmatching, establishing the foundational framework for modern compatibility testing[32].

The recognition of non-ABO antibodies, likely targeting Rh system antigens, was first reported by Unger in 1921. He emphasized the necessity of grouping and directly testing a patient's blood against that of a prospective donor prior to transfusion. This recommendation gained further credibility through documented cases of HTR, particularly in previously transfused patients[33].

#### 2.3.2. Mid-20th Century Advances: Serological Testing and Enhanced Safety

The introduction of the antiglobulin test by Coombs, Mourant, and Race in 1945 revolutionized transfusion medicine. This advancement facilitated the development of more sensitive crossmatching protocols, including the antiglobulin test, thereby enhancing transfusion safety[34].

Enhancements in agglutination techniques were achieved through the use of reagents such as Bovine Serum Albumin (BSA),[35] Low Ionic Strength Saline (LISS),[36] and Polyethylene Glycol (PEG),[37] which increased antibody detection sensitivity and reduced incubation times. The application of enzyme-treated red cells, utilizing proteolytic enzymes like papain or ficin, further improved the detection of irregular antibodies[38].

### 2.3.3. Pre-Transfusion Testing: Methods and Technological Advances

In Portugal, Decree-law 185/2015 defines the legal framework for the administration of blood and blood components[39]. Modern advancements, such as the use of gel-based methods, have significantly improved the efficiency and standardization of compatibility testing. These techniques support antibody screening and identification through multi-cell panels, enabling precise detection of clinically significant alloantibodies and aligning with best practices in immunohematology[40].

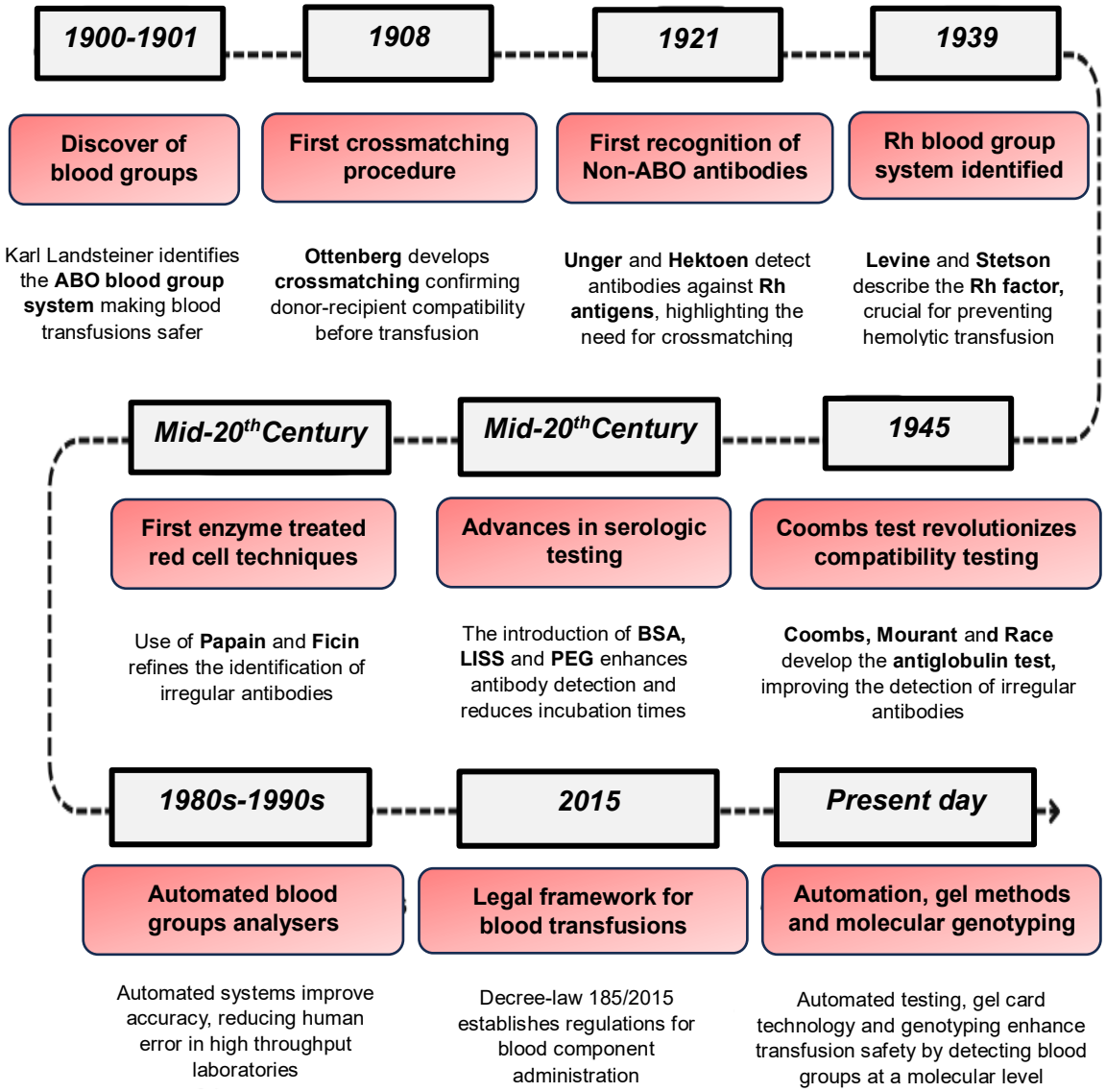


**Figure 2.4.** Timeline of Key Milestones in Red Cell Compatibility Testing. Original work.

## 2.4. Clinical Significance of Blood Group Systems

The RBC blood group systems are of clinical importance well beyond routine transfusion practice. Their antigenic structure, immunogenicity and role in clinical complications makes transfusion medicine a complex science and each of these systems contribute to this complexity. Although this thesis focuses on immune-mediated RBC alloimmunization and does not address naturally occurring antibodies such as those of the ABO system, it is important to acknowledge that ABO incompatibility remains the most clinically critical in transfusion medicine. ABO antibodies, primarily of the IgM class, can cause immediate and severe intravascular hemolysis, making ABO matching a non-negotiable prerequisite for any transfusion[28]. Therefore, the following section will focus on other blood group systems that, although less immediately lethal, pose significant risks in the context of repeated transfusions, pregnancy, and alloimmunization For a comprehensive overview that provides information on the most clinically relevant blood group systems, the most clinically significant, including Rh, Kell, Kidd, Duffy, MNS and others, are summarized in Appendix A[41 42 43 44].

### 2.4.1. The Rh Blood Group System

The Rh blood group system, made up of five major antigens (D, C, c, E, and e), is clinically the second most important blood group system after the ABO system. Of these, the RhD antigen is the most immunogenic and commonly associated with hemolytic complications including HDFN and DHTR. The immunogenicity of RhD is due to its distinct structure with, amino acid differences of over 400 between the two RhCE proteins. This degree of polymorphism produces a large number of epitopes that can interact with a broad variety of MHC molecules, enhancing the immune response in RhD negative individuals reacting to exposure to RhD positive RBC[7 42].

Although less immunogenic than RhD, anti-C and anti-E alloantibodies are clinically significant, especially in multiply transfused patients. The presence of these antibodies can make transfusion compatible matches more complicated, particularly if no full antigen match is possible[22].

Appendix B illustrates how RhD, RhCE, and RhAG can be distinguished structurally in a transmembrane manner. The structural features depicted that contribute to the high immunogenicity of RhD (i.e., the extracellular epitopes) and those that are also the basis for extensive antigenic variations in RhCE molecules (i.e., C/c and E/e) are highlighted.

### 2.4.2 The Kell Blood Group System

The Kell system is among the most clinically significant after Rh, primarily due to the high immunogenicity of the K antigen. Anti-K alloantibodies are associated with severe hemolysis, both in transfusion settings and during pregnancy.

- **Anti-K and Fetal Anemia**: Anti-K antibodies suppress fetal erythropoiesis, leading to profound fetal anemia independent of hemolysis. This unique pathophysiological mechanism necessitates close monitoring and specialized management in pregnant women with anti-K alloantibodies[7].
- **Immunogenic Potential**: The K antigen, a glycoprotein with enzymatic functions, exhibits structural features that enhance its immunogenicity. Additionally, low-frequency Kell antigens, such as $Kp^a$ and $Js^a$, can also induce clinically significant alloantibodies, although their rarity limits their overall clinical impact[45].

### 2.4.3 The Kidd and Duffy Blood Group Systems

The Kidd and Duffy systems are notable for their intermediate immunogenicity and the unique clinical challenges they present.

- **Kidd System ($JK^a$, $JK^b$)**: Kidd antigens are moderately immunogenic, yet the antibodies they elicit, such as anti-$JK^a$, are clinically significant due to their rapid evanescence. This transient nature often results in false-negative IAT, increasing the risk of undetected DHTR[18 27]. Kidd antibodies are also implicated in severe, albeit rare, hyperhemolysis syndromes.
- **Duffy System ($Fy^a$, $Fy^b$)**: The Duffy system holds particular relevance in ethnically diverse populations. The Fy(a-b-) phenotype, common in individuals of African descent, confers resistance to Plasmodium vivax malaria but complicates transfusion practices due to its prevalence among recipients and scarcity among donors in predominantly European donor pools[46]. As shown in Figure 2.5 the global distribution of the Duffy antigen highlights the frequency of the Fy(a-b-) phenotype in different regions. Anti-$Fy^a$ and anti-$Fy^b$ antibodies, while less immunogenic than RhD or Kell, pose significant challenges in multi-transfused populations, including patients with SCD[47 48].

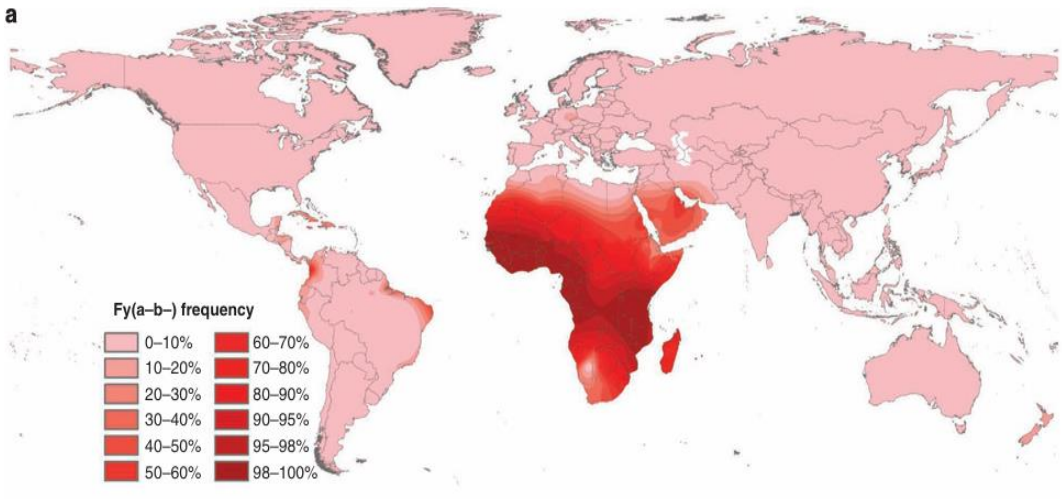


**Figure 2.5:** World Map showing the global distribution of Duffy Antigen. *Adapted* from Howes et al., 2011.

### 2.4.4 The MNS and Minor Blood Group Systems

The MNS, Lewis, Lutheran, and other minor blood group systems contribute to the nuanced landscape of transfusion medicine.

- **MNS System (M, N, S, s)**: Anti-S and anti-s antibodies are clinically significant due to their ability to cause hemolysis,[49] while anti-M and anti-N are typically weaker and less clinically relevant unless reactive at 37°C[50].
- **Lewis ($Le^a$, $Le^b$)**: The Lewis antigens, which are adsorbed rather than intrinsically expressed on RBC membranes, rarely elicit clinically significant alloantibodies. However, when present, anti-$Le^a$ and anti-$Le^b$ can cause mild hemolysis, particularly in enzyme-enhanced serological testing[51].
- **Lutheran ($Lu^a$, $Lu^b$)**: Anti-$Lu^a$ antibodies are generally mild, whereas anti-$Lu^b$ can cause severe hemolysis in rare cases. The low expression of Lutheran antigens contributes to their limited clinical relevance[7].

## 2.5. Immunogenicity

RBC alloimmunization is impacted by various molecular and host-related factors. Molecular factors that drive this include antigen polymorphisms, amino acid substitutions and high antigen density, all of which collectively increase immunogenicity[5] [7] [42]. Host-related factors include genetic predispositions, chronic inflammation, previous antigenic experience, and immunosuppressed states, all of which play a role in the immune response[4] [5] [22]. See Appendix C (Table 2) for a more detailed description of these factors and their clinical implications. The analysis demonstrates the interdependence of molecular characteristics with host conditions in determining the alloimmunization risk.

## 2.6. RBC Alloimmunization: Challenges in Diagnosis and Monitoring

The diagnosis and monitoring of RBC alloimmunization present significant clinical challenges, stemming from both biological and methodological limitations. Understanding and addressing these challenges is essential to improving transfusion safety.

### 2.6.1. Antibody Evanescence

One of the greatest challenges in detecting alloimmunization is antibody evanescence, the gradual decline in circulating alloantibody levels until they become undetectable. This phenomenon is especially problematic for antibodies against antigen systems such as Kidd ($JK^a$, $JK^b$) and Duffy ($Fy^a$, $Fy^b$), which are known for their transient nature. For instance, Kidd antibodies are among the most common contributors to DHTR, but their rapid disappearance from circulation often results in false-negative results during standard serological testing.

The implications of antibody evanescence extend beyond diagnostic difficulties. Patients who have developed alloantibodies that later become undetectable are at an increased risk of HTR if re-exposed to the corresponding antigen. This underscores the importance of robust follow-up protocols to monitor alloimmunization over time.

Quantitative studies, such as those conducted by Roberto Reverberi using Kaplan-Meier survival curves, have assessed the persistence of antibodies over time[52]. Figure 2.6 illustrates that approximately 50% of antibodies disappear within 1300 days (3 years and

7 months), highlighting the significant temporal limitations of current serological monitoring practices.

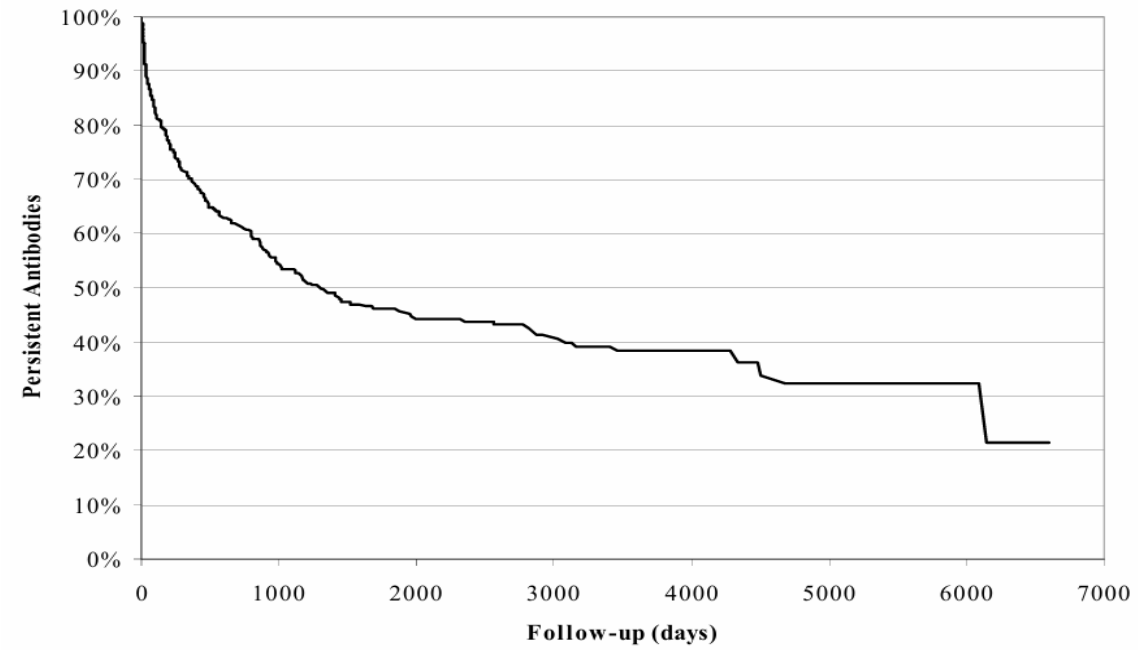


**Figure 2.6.** The Persistence of Antibodies. Kaplan-Meier estimate of the survival curve of all antibodies (N=673), showing that 50% of antibodies disappear within approximately 1300 days (3 years and 7 months). This highlights the phenomenon of antibody evanescence and the importance of timely post-transfusion monitoring. *Adapted from* Roberto Reverberi (2008)

### 2.6.2. Anamnestic Responses

Anamnestic responses add another layer of complexity to the diagnosis and monitoring of RBC alloimmunization. These responses, also known as secondary immune responses, occur when a previously sensitized individual is re-exposed to an antigen. This leads to a rapid and pronounced increase in alloantibody levels. Unlike primary immune responses, which may take weeks to produce detectable levels of antibodies, anamnestic responses can develop within hours to days[4]. This accelerated timeline increases the likelihood of DHTR, particularly in patients who receive antigen-positive transfusions before their immune response is recognized. To illustrate this progression, Figure 2.7 presents a schematic diagram showing the stages of primary antigen exposure, the subsequent decline (or evanescence) of antibody levels over time, and the abrupt rise in antibody production triggered by secondary exposure (anamnestic response)[53].

The clinical implications of anamnestic responses are significant. For example, a patient with a history of anti-JK$^a$ antibodies may test negative for this alloantibody during routine

pre-transfusion screening but could rapidly produce high titers upon re-exposure to JK$^a$-positive RBC. Such scenarios highlight the limitations of one-time pre-transfusion testing and emphasize the need to maintain detailed and comprehensive transfusion histories to ensure patient safety.

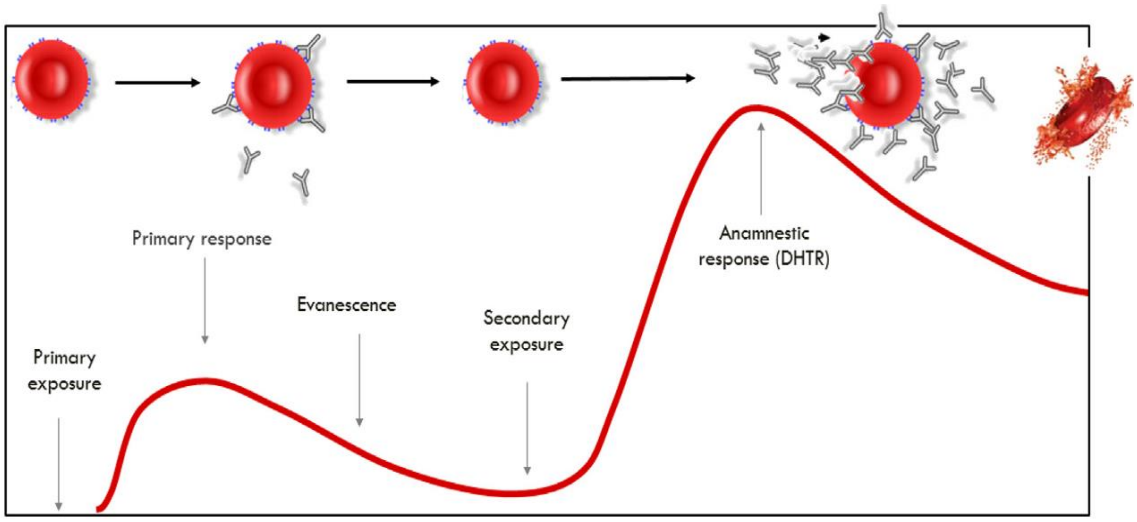


**Figure 2.7.** Anamnestic response. Schematic timeline depicting the transition from a primary immune response against RBC antigens to the rapid surge upon re-exposure. *Adapted* from Fasanoa et al., 2019.

### 2.6.3. Detection Limitations

Current diagnostic methods often fail to detect clinically significant alloantibodies, further complicating transfusion management. Stack et al. (2016) reported that only 30% of clinically significant alloantibodies are detected during routine screening, due in part to the following limitations:

- **Card-Based Agglutination Techniques**: Widely used due to their simplicity and cost-effectiveness, these methods have reduced sensitivity for low-titer or transient antibodies, particularly those targeting Kidd and Duffy antigens[21].
- **Timing of Testing**: Antibody screens conducted within 30 days post-transfusion often fails to detect newly formed alloantibodies. Research indicates that the optimal window for alloantibody detection lies between 30 and 180 days post-transfusion[54 55 56].
- **Lack of Follow-Up Protocols**: Many clinical settings lack standardized post-transfusion antibody identification protocols. As a result, alloantibodies that develop outside routine testing intervals frequently go undetected[57 58].

## 2.7. RBC Alloimmunization – Clinical Implications

### 2.7.1 Hemoglobinopathies

Patients with hemoglobinopathies, such as SCD and thalassemia, frequently require chronic RBC transfusions to manage anemia, vaso-occlusive crises, and related complications[59]. As shown in Figure 2.8, the distinctive sickling of RBC in SCD can obstruct microcirculation, exacerbating tissue ischemia and hemolysis. This pathophysiological process necessitates regular transfusions, thereby increasing the exposure to foreign antigens and heightening the risk of alloimmunization. Indeed, studies indicate alloimmunization rates ranging from 19–43% in patients with SCD and 5–45% in those with transfusion-dependent thalassemia[4].

Chronic inflammation in these patient populations further amplifies their immunologic response, as pro-inflammatory cytokines and other mediators upregulate antigen presentation, making alloimmunization more likely[60]. To mitigate these risks, extended phenotypic matching is recommended for highly immunogenic antigens, particularly in ethnically diverse populations where donor-recipient mismatches are more prevalent. Studies have shown that extended blood typing decreases alloimmunization in SCD patients, though it is not universally adopted[61]. Additionally, molecular genotyping can complement traditional serological methods to ensure more precise compatibility, especially in individuals with a known history of alloimmunization[4].

In the Duffy blood group system, the Fy(a-b-) phenotype, common in populations with African ancestry, is often associated with a GATA-box mutation in the FYB promoter region. This mutation silences $Fy^b$ antigen expression on RBC but retains its expression in other tissues, allowing individuals to tolerate $Fy^b$+ transfusions without risk of alloimmunization. However, $Fy^a$ antigen remains immunogenic and must be avoided in transfusions for these patients. Distinguishing between a true Fy(a-b-) phenotype due to the absence of both FYA and FY*B alleles and the Fy(a-b-) phenotype caused by GATA-box mutation is critical, as genotyping can significantly expand the pool of compatible donors for patients with the latter[62] [63] [64].

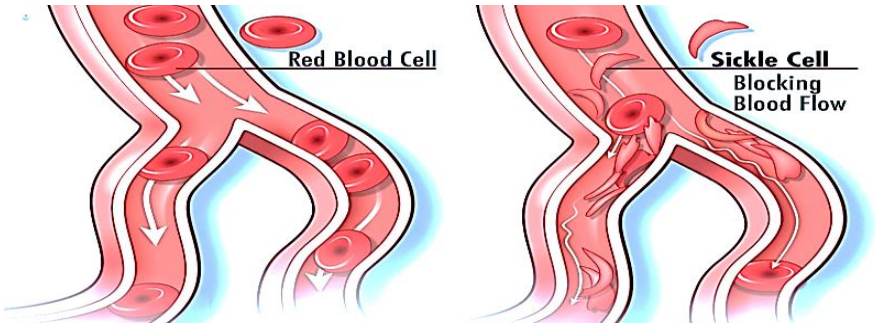


**Figure 2.8.** Comparison of Normal and Sickled RBCs. The panel on the left illustrates normal, biconcave RBCs flowing unobstructed through a blood vessel, whereas the panel on the right shows elongated, sickled RBCs blocking blood flow. This sickling phenomenon underlies vaso-occlusive crises and chronic hemolysis in SCD, driving the need for frequent transfusions and thereby increasing the risk of alloimmunization. *Adapted from* U.S. National Library of Medicine

## 2.7.2 Alloimmunization in Pregnant Women

Pregnancy is a key sensitizing event for alloimmunization. Maternal exposure to fetal RBC antigens inherited from the father can lead to antibody formation, posing risks for both current and future pregnancies. The most clinically significant antibodies include anti-D, anti-K, and anti-C, which are major causes of HDFN[65].

### 2.7.2.1. Anti-D and Rh Immunoglobulin (RhIg)

The introduction of prophylactic anti-D immunoglobulin (RhIg) has substantially decreased the incidence of anti-D alloimmunization by selectively removing fetal RhD-positive erythrocytes from the maternal bloodstream, thereby averting sensitization. Notwithstanding this success in mitigating RhD-related HDFN, alloimmunization against other antigens – particularly Kell – remains problematic, as RhIg does not confer protection beyond the D antigen[66 67 68 69].

To illustrate the immunological mechanism behind RhIg prophylaxis, Figure 2.9 is included in the main body of this thesis. It depicts how passive anti-D immunoglobulin prevents maternal B cells from mounting an endogenous response against fetal RhD-positive RBC. Moreover, because adherence to standardized dosing schedules is vital to prophylaxis, a structured flowchart detailing recommended RhIg doses and administration windows (e.g., 1,500 IU at 28 weeks of gestation and within 72 hours postpartum) has been provided in Appendix D**:** RhIg Prophylaxis. This algorithm underscores the pivotal role of timely prophylaxis in minimizing maternal sensitization and subsequent HDFN.

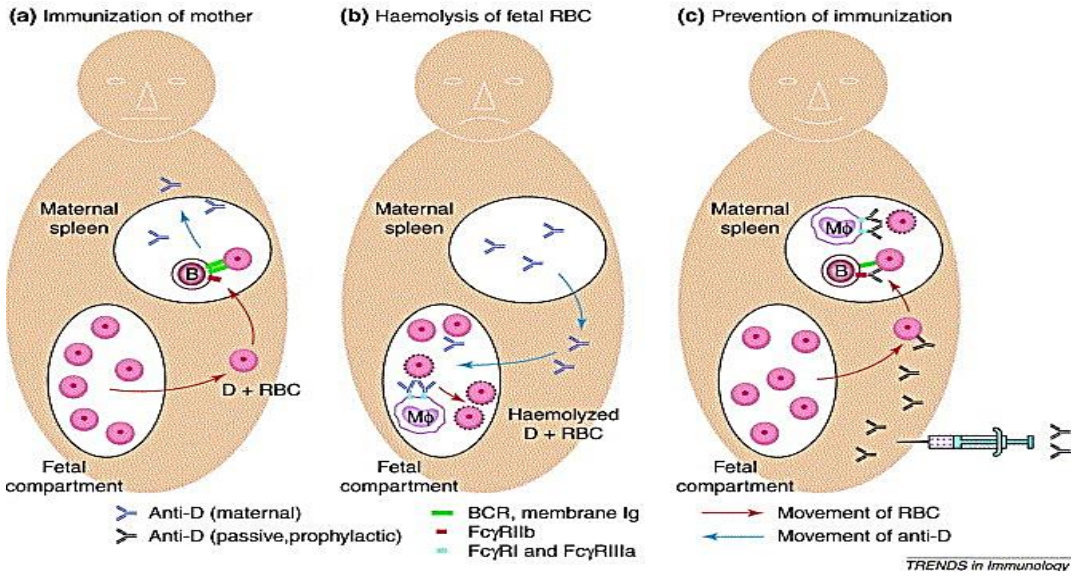


**Figure 2.9.** Mechanism of Anti-D–Mediated Immune Suppression**.** This schematic depicts three stages: (a) maternal immunization upon exposure to fetal RhD-positive RBCs, (b) hemolysis of fetal RBCs when maternal anti-D antibodies cross the placenta, and (c) prophylactic anti-D administration that prevents maternal B cells from responding to RhD antigens. *Adapted from* Kumpel BM, Elson CJ. (2001)

### 2.7.2.2 Hemolytic Disease of the Fetus and Newborn (HDFN)

HDFN is a severe alloimmune condition that occurs when maternal IgG antibodies cross the placenta and target paternally inherited antigens on fetal RBC. This immune response leads to hemolysis, fetal anemia, and, in severe cases, life-threatening complications such as hydrops fetalis and stillbirth[66].

Historically, the RhD antigen has been the most common cause of HDFN. The introduction of RhIg prophylaxis has significantly reduced its incidence, underscoring the success of targeted prevention strategies[70] [71]. However, HDFN remains clinically significant due to the role of other antigens, including Rh system antigens (C, c, E, e) and non-Rh antigens such as Kell[72]. Additionally, rarer antibodies, including those from the Duffy ($Fy^a$, $Fy^b$), MNS (M, S, s, U), and Gerbich (Ge3) systems, can also contribute, albeit less frequently[73] [74].

### 2.7.2.2.1. Major Mechanisms of HDFN

#### RhD-Mediated HDFN

RhD-mediated HDFN is characterized by maternal anti-D antibodies binding to fetal RhD-positive RBCs, leading to their destruction via extravascular hemolysis in the fetal spleen. This classic mechanism results in progressive fetal anemia if untreated[75]. The introduction of RhIg has drastically decreased the prevalence of RhD-mediated HDFN by preventing maternal sensitization (Figure 2.10).

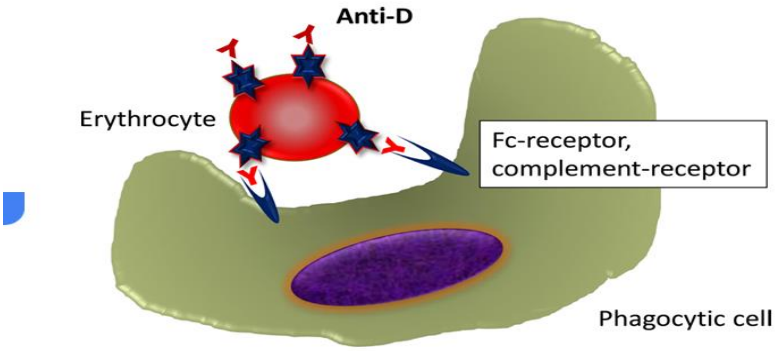


**Figure 2.10.** Pathophysiology of RhD-Mediated HDFN: *Maternal anti-D antibodies bind to RhD-positive fetal RBCs, leading to extravascular hemolysis in the spleen. This process can result in severe anemia and, in advanced cases, hydrops fetalis. Adapted from* Ohto et al., 2020.

#### Kell-Mediated HDFN

The Kell system represents the most clinically significant non-Rh antigen associated with HDFN. Unlike RhD-mediated disease, anti-Kell antibodies exert a dual mechanism:

1. ***RBC Hemolysis***: Anti-Kell antibodies opsonize fetal RBCs for phagocytosis.
2. ***Suppression of Erythropoiesis:*** Anti-Kell targets erythroid progenitor cells in the fetal bone marrow, leading to ineffective erythropoiesis and worsening anemia.

This dual mechanism often results in disproportionately severe anemia compared to the degree of hemolysis observed in peripheral RBCs[74]. Figure 2.11. illustrates this pathophysiology[73].

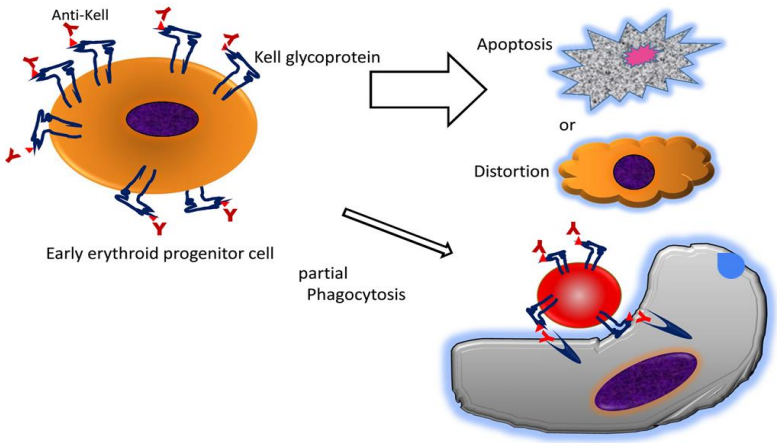


**Figure 2.11. Kell-Mediated Fetal Anemia.** Anti-Kell antibodies suppress fetal erythropoiesis by targeting erythroid progenitor cells while also inducing RBC hemolysis. This mechanism leads to severe anemia and increased risk of hydrops fetalis. Adapted from Ohto et al., 2020.

#### 2.7.2.2.2. Other Antibodies and HDFN

While Rh and Kell systems account for most cases of HDFN, rarer antibodies from systems such as Duffy, MNS, Gerbich, and Vel have also been implicated. These antibodies, although significantly less common, can cause HDFN through distinct mechanisms. The table in Appendix E summarizes their mechanisms of action and associated clinical outcomes.

### 2.7.3 Oncology and Immunomodulatory Therapies

Oncology patients undergoing transfusion therapy present distinct challenges regarding RBC alloimmunization, influenced by the type of malignancy (solid vs. liquid tumors), disease pathology, and treatment regimens. Despite frequent transfusions, the overall incidence of alloimmunization in oncology patients is relatively low, ranging from 0.3% to 4%, largely due to the immunosuppressive effects of chemotherapy and corticosteroids, which attenuate alloimmune responses[76] [77]. However, these effects also complicate the detection of alloantibodies, increasing the risk of DHTR[78] [79].

Differences between solid and liquid tumors (Hematologic Malignancies**)** significantly impact the dynamics of RBC alloimmunization.

#### 2.7.3.1. Solid Tumors

These are cancers of solid organs such as the breast, colon and other organs. Patients with such cancers often need blood transfusions occasionally for chemotherapy induced anemia or for blood loss operating. Sporadic exposure to RBC antigens, together with moderate levels of immunosuppression, reduce the chance of alloimmunization[80 81 82].

#### 2.7.3.2. Liquid tumors (Hematologic Malignancies)

Patients with hematologic malignancies, such as leukemia or lymphoma, frequently experience severe pancytopenia due to the disease or its treatment. This leads to regular RBC transfusions and higher cumulative antigen exposure, increasing the risk of alloimmunization. Additionally, therapies such as hematopoietic stem cell transplantation or monoclonal antibodies (e.g., rituximab) deeply modulate immune function, which can both mitigate and obscure alloimmunization risks[83 84].

#### 2.7.3.3. Daratumumab and Serological Challenges

Monoclonal antibody therapies like daratumumab, used in treating multiple myeloma, introduce further complexities. Daratumumab binds to CD38 antigens on RBCs, producing panreactivity in serological tests and obscuring clinically significant alloantibodies (Figure 2.12). Specialized techniques such as dithiothreitol (DTT) treatment are required to overcome this interference and ensure accurate antibody identification (Figure 2.13)[85 86].

#### 2.7.3.4. Strategic Implications

Extended antigen matching is particularly beneficial in patients with hematologic malignancies due to their higher transfusion burden and increased risk of alloimmunization. This approach minimizes complications such as DHTR and ensures the availability of compatible units, even in the context of challenges like daratumumab interference. However, even for solid tumors, strategies such as rigorous post-transfusion monitoring and comprehensive transfusion histories remain critical to ensuring safety[77 87]. In conclusion, while overall rates of RBC alloimmunization are lower in oncology patients compared to other transfusion-dependent populations, significant differences exist between solid and liquid tumors. These distinctions underscore the need for individualized transfusion strategies, incorporating tailored antigen matching and enhanced serological practices, to optimize transfusion outcomes and minimize complications.

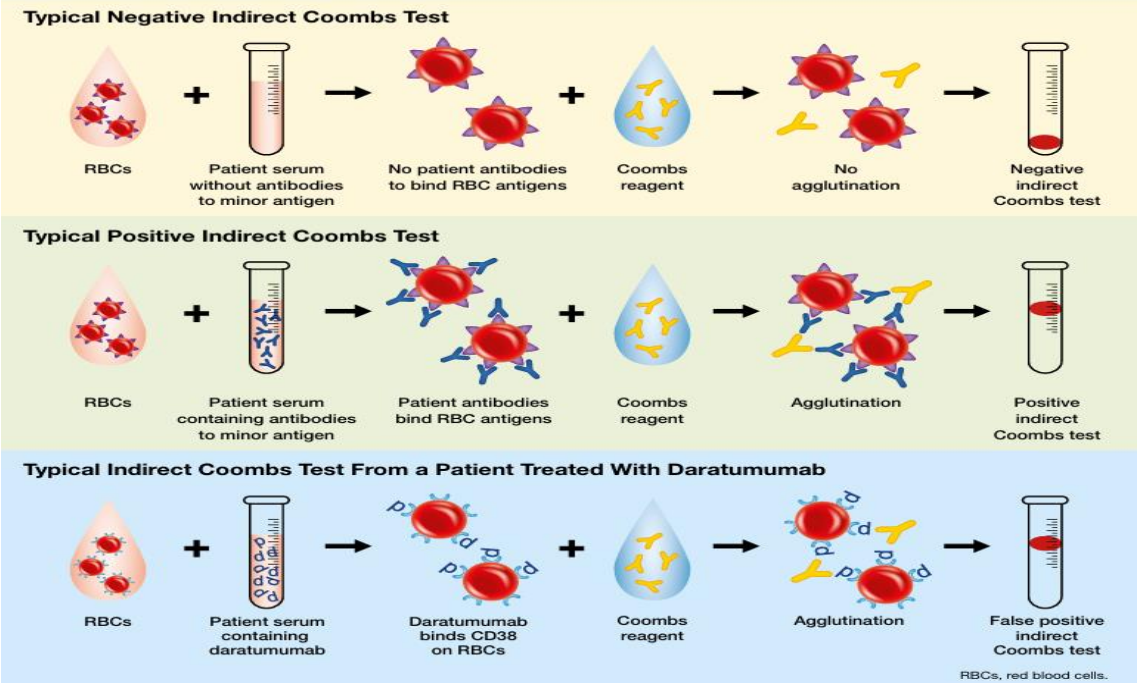


**Figure 2.12.** Typical Results of the Indirect Coombs Test, Including Interference by Daratumumab This figure demonstrates the outcomes of the Indirect Coombs Test under three different scenarios. Daratumumab, a human monoclonal antibody, binds to CD38, a protein expressed at low levels on RBCs. This binding may mask the detection of alloantibodies in the patient's serum, interfering with routine pre-transfusion compatibility tests, including antibody screening and crossmatching. *Adapted from* NHS Blood and Transplant

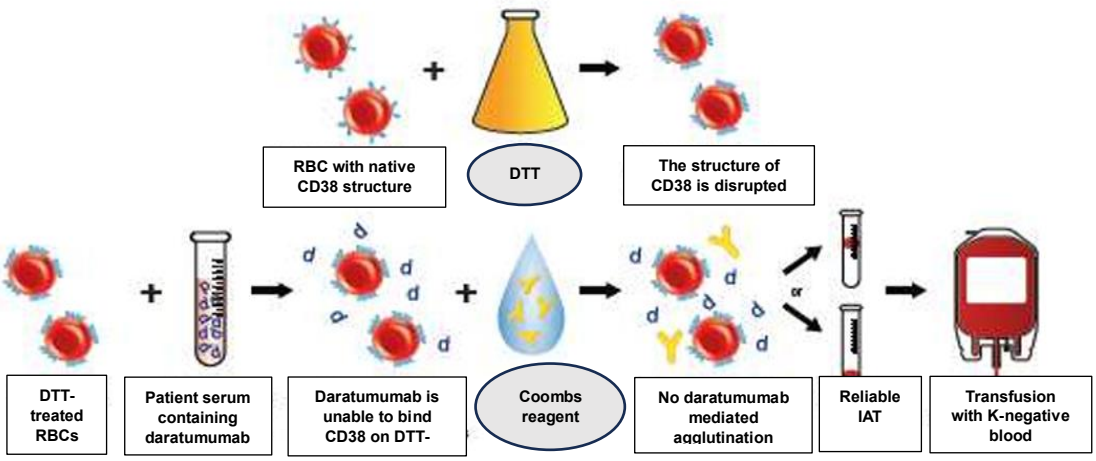


**Figure 2.13.** Resolving Daratumumab Interference in Pre-Transfusion Testing. RBCs treated with DTT lose their CD38 structure, preventing daratumumab-mediated panreactivity and allowing accurate detection of clinically significant alloantibodies. This process is critical for daratumumab-treated patients to ensure compatibility. As Kell antigens are also sensitive to DTT, transfusion units must be matched for K-negative or k-negative phenotypes to prevent alloimmunization risks. *Adapted from* NHS Blood and Transplant.

# Chapter 3. Methodology

This chapter outlines the methodological framework employed to investigate RBC alloimmunization.

## 3.1 Study Setting

The study was conducted at the ULSAR IHS. This institution is the sole provider of immunohematology care for the municipalities of Barreiro, Moita, Alcochete, and Montijo, covering an estimated population exceeding 220,000 individuals (Annex 1).

## 3.2 Study Design

This is a retrospective, observational, and analytical study. The records of all patients (*n*=20,841) who received RBC units were examined retrospectively by searching the computer database from ASIS, over a 20-year period, between 2002 and 2022. The study examines IHS records to analyse patient sensitization to RBC antigens, focusing on the immunogenicity of Rh (C, c, E, e) and Kell (K) antigens. Additionally, it evaluates the prevalence of irregular antibodies, including those detected in patients who were transfused by the IHS and/or arrived at the IHS already immunized (from prior transfusions or other exposures).

## 3.3 Immunohematology testing

Upon the first blood sample collection for transfusion purposes/blood donation, ABO and Rh(D) typing was performed for both patients and blood donors, followed by Rh and Kell phenotyping. This process ensured that all transfusion recipients and donors had their antigen profiles recorded, improving transfusion safety and alloimmunization risk assessment. Additionally, for each new blood sample received from a previously typed patient/blood donor, ABO and Rh(D) confirmation was systematically conducted to verify blood group identity and minimize the risk of misidentification.

Blood samples from patients submitted to blood transfusions were screened for RBC alloantibodies using a selected three-cell set of reagent RBCs for antibody detection.

- **Dia cell I** contains the following antigens: D, C, e, Cw, k, $Kp^b$, $Fy^b$, $Le^a$, P, N, S, s, Lub and $Xg^a$.
- **Dia cell II** contains the following antigens: D, E, c, k, $Kp^b$, $Fy^b$, $JK^a$, $Le^b$, M, S, $Lu^a$, $Lu^b$ and $Xg^a$.
- **Dia cell III** contains the following antigens: c, e, K, k, Kpb, $Fy^a$, $JK^b$, P1, M, N, s, Lub and $Xg^a$.

The technique for antibody detection involved the use of 25 µL of serum/plasma and 50 µL of 0.8-percent RBC in LISS gel tests.

In case of a positive screen, antibody identification was accomplished with commercial panels of cells – which consists of 11 different group O red cells, each having variable antigens of Rh, Kell, Duffy, Kidd, Lewis, P, MNS, Lutheran and Xg blood group system (D, C, E, c, e, Cw, K, k, $Kp^a$, $Kp^b$, $Js^a$, $Js^b$, $Fy^a$, $Fy^b$, $JK^a$, $JK^b$, $Le^a$, $Le^b$, P1, M, N, S, s, $Lu^a$, $Lu^b$, $Xg^a$) -, tested by similar methods or additional techniques (e.g., polyethylene glycol and enzyme) whenever needed. Finally, according to presented pattern of each panel, type of specific antibody against each antigen was determined. An auto control was also put simultaneously to determine the presence of autoantibody. If specificity could not be clearly determined, the blood sample was sent to our reference immunohematology laboratory – *Instituto Português do Sangue e Transplantação* (IPST) - for further analysis. The results of both antibody screening and antibody identification were valid for 72 hours (a transfusion episode). Complete crossmatching, including an indirect antiglobulin phase, was performed.

### 3.4 Population and Sample

#### 3.4.1 Target Population

The target population includes all patients who received RBC transfusions at ULSAR between January 1, 2002, and December 31, 2022.

#### 3.4.2 Sample Selection

The study sample comprises four databases, each representing a distinct patient scenario (Table 3.1).

**Table 3.1.** Overview of Databases and Patient Characteristics

| Database | Number of patients | Patient characteristics | *IAT Status* |
|---|---|---|---|
| **Ideal timing** | 1,131 | Patients sensitized to Rh and/or Kell antigens | IAT performed between 30-180 days post-transfusion |
| **Delayed timing** | 831 | Patients sensitized to Rh and/or Kell antigens | IAT performed strictly more than 180 days post-transfusion, allowing for the detection of potential antibody evanescence. |
| **No follow-up** | 2,790 | Patients sensitized to Rh and/or Kell antigens | No IAT performed after transfusion |
| **Non-included cases** | 16,089 | Patients sensitized to Rh and/or Kell antigens.<br>Patients not sensitized. | Not sensitized or with IAT performed <30 days post-transfusion (excluded to control for early anamnestic response bias and outside defined follow-up windows). |

### 3.5 Sampling Method

Convenience sampling was employed due to the retrospective nature of the study. The dataset includes all patients meeting the inclusion criteria, ensuring a comprehensive analysis of the available data and covering diverse patient scenarios within the study period.

### 3.6 Eligibility Criteria

#### 3.6.1 Inclusion Criteria

1. Patients transfused with RBC units carrying Rh (C, c, E, e) and Kell antigens, distinct from the patient's phenotype (ideal timing, delayed timing, no follow-up).

#### 3.6.1.1 Justification for the Inclusion Window of 30–180 Days

Post-transfusion IAT conducted too early may miss the primary immune induction phase, while delayed testing increases the risk of overlooking antibodies that have undergone evanescence and fallen below detectable levels. Stack and Tormey, building on the prospective observations of Redman et al., demonstrated increasing detectability from 30 to 112 days, showing that routine testing outside this range may fail to capture a substantial proportion of new antibodies [21 55]. Alves et al., in a prospective cohort, found that all clinically significant alloantibodies were detected between one and six months post-transfusion, with subsequent titer decline being common – reinforcing the rationale for the 30–180-day window [56]. Beyond this period, persistence becomes variable and antibody loss increasingly frequent.

In a study by Tormey and Stack, approximately half of hospital-acquired alloantibodies that became undetectable over time did so within six months of their initial identification, while longitudinal analyses by Schonewille and Reverberi reported 25–40% antibody evanescence over time, particularly among low-titer specificities [52 66].

Anti-D kinetic modeling indicates that the vast majority of alloantibodies develop within six months, reinforcing 180 days as an appropriate upper limit for follow-up testing [88].

Collectively, these data define 30-180 days as the optimal analytical horizon for alloantibody detection – broad enough to encompass primary induction yet narrow enough to precede significant evanescence.

### 3.6.2 Exclusion Criteria

1. Patients < 18 years
2. Patients with incomplete transfusion records or missing antibody screening data.
3. Women with passive anti-D antibodies following RhIg prophylaxis*

* Women with isolated anti-D antibodies were excluded from the prevalence analysis when passive anti-D was confirmed in clinical records following antenatal RhIg prophylaxis. All such cases occurred in women under 45 years of age, consistent with previous reports distinguishing obstetric RhIg-induced antibodies from true transfusion alloimmunization[52]. According to national guidelines, RhIg is administered at 28 weeks to all Rh-negative pregnant women not previously sensitized, and the IAT should occur beforehand [89]. However, in practice, IAT is sometimes performed after prophylaxis, which may lead to passive antibody detection. To minimize misclassification, these cases were excluded.

### 3.7 Variables

The study variables were classified as dependent or independent according to their role in the analysis. Dependent variables represented alloantibody-related outcomes, while independent variables included patient and transfusion factors potentially influencing those outcomes.

#### 3.7.1 Dependent Variables

1. **Alloantibody presence:** binary outcome indicating whether a clinically significant antibody was detected (Yes/No).
2. **Alloantibody specificity:** categorical variable identifying the antigen target (Anti-D, Anti-C, Anti-c, Anti-E, Anti-e, Anti-K).
3. **Alloantibody detection rate:** continuous variable expressed as frequency (%) within each cohort.

#### 3.7.2 Independent Variables

1. **Age:** continuous (years).
2. **Sex:** categorical (male/female)
3. **ABO/Rh group:** categorical (A, B, AB, O / Rh-positive or Rh-negative).
4. **Number of transfused RBC units:** continuous (count of units received).
5. **Timing of antibody screening:** categorical (ideal: 30–180 days vs. delayed: >180 days).

### 3.8 Data Collection Instruments

Data was extracted from the AsisWeb database, the electronical record system used by IHS. The database includes: patient demographic information (age and sex), patient blood group and phenotype (ABO, Rh, Kell, extended phenotype), IAT, antibody identification records, and detailed transfusion history (dates, donor unit antigen profiles).

All data were recorded and anonymized by the author using Microsoft Excel, organized into the four previously mentioned anonymized databases.

To ensure data accuracy, the irregular antibodies identified in the ASIS database were cross-verified with the original paper-based antibody panels stored at the IHS. This

verification process ensured consistency between the digital records and the manually interpreted results.

### 3.9 Statistical Methodology

This section outlines the procedures used to address the study's primary objectives, including an additional prevalence assessment of all irregular antibodies detected over the 20-year period. All analyses were conducted using IBM SPSS Statistics.

#### 3.9.1 Descriptive Statistics

A descriptive analysis of the variables was conducted using frequency distribution tables for categorical variables and summary statistics including means, standard deviations, minimum, and maximum values for quantitative variables.

#### 3.9.2 Overall Prevalence of Irregular Antibodies (All databases)

A review was conducted to determine the overall prevalence and variety of irregular antibodies identified by the service across all databases from 2002 to 2022. This encompassed any clinically relevant specificities (e.g., Rh system, Kell, Kidd, Duffy), providing a broader perspective on alloimmunization trends during the two-decade study period.

#### 3.9.3 Comparative Analysis of Immunogenicity (Ideal Timing and Delayed Timing)

Chi-square tests were performed to compare proportions of immunogenicity according to transfusional follow-up timing (ideal vs delayed). The same tests were utilized to compare immunogenicity proportions across sex, age groups, blood groups, and categories of the number of transfused RBC units (ideal and delayed). Fisher's exact test was applied for 2x2 contingency tables when expected frequencies were < 5; otherwise, chi-square tests were used.

### 3.9.4 Assessment of "Lost Detection" (No follow-up)

Database 3 (no follow-up) includes patients known to be sensitized to Rh or Kell antigens who did not undergo subsequent IAT testing. Because there was no follow-up, it remains unclear whether their developed antibodies, if they stayed detectable or if their levels fell below the diagnostic threshold. For predicting immunogenicity, logistic regression models were employed to examine the relationship between explanatory variables and the immunogenic response. Initially, the models were fitted using data from patients with detailed follow-up records, allowing the estimation of regression coefficients. Variable selection within each model was based on the statistical significance of regression coefficients, assessed through Wald tests, ensuring that only statistically significant predictors were retained. After model construction and validation, these estimated coefficients were applied to the patient group lacking follow-up, enabling immunogenicity prediction in this subset. The quality of model fit was assessed through the percentage of correctly classified cases, measuring the accuracy of the model in predicting immunogenic response. A significance level of 5% ($p < 0.05$) was used as the threshold for rejecting the null hypothesis.

## 3.10 Ethical Considerations

The study was approved by the Ethics Committee of the ULSAR, the ULSAR Clinical Research Unit, and the Ethics Committee of the *Escola Superior de Saúde de Lisboa* (ESSL). Approvals are documented in the Annexes (Annex B, C and D). All patient data were anonymized in accordance with General Data Protection Regulation (GDPR) guidelines, and access to sensitive information was restricted to authorized personnel only. Ethical safeguards ensured that no patient-identifiable information was disclosed in publications or report.

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# Chapter 4. Results Presentation

This chapter is divided into two complementary sections. The first part focuses on the overall characterization of the alloimmunized cohort, including the prevalence, distribution, and antibody profiles observed between 2002 and 2022. These findings provide a contextual foundation for the second, core section of the results: the immunogenicity analysis and impact of post-transfusion follow-up timing.

## 4.1 Patient Cohort Characterization and Alloimmunization Prevalence (2002–2022)

In accordance with transfusion protocols, all transfused patients underwent routine pre-transfusion antibody screening using the IAT as part of standard compatibility testing.
Between January 2002 and December 2022, a total of 20,841 adult patients (≥18 years) receiving RBC transfusions were identified within the ULSAR AsisWeb system. Of these, 9,624 (46.2%) were male and 11,217 (53.8%) were female, yielding a male-to-female ratio of 1:1.16. During this period, a total of 94,689 RBC units were transfused. Out of the total cohort, 591 patients had a positive IAT, corresponding to a crude positivity rate of 2.83%. However, 64 women with passive anti-D antibodies following RhIg prophylaxis, as documented in records, were excluded from the antibody prevalence analysis since these cases reflected *passive immunization* rather than true transfusion-related alloimmunization. The final cohort for alloimmunization analysis therefore consisted of 527 patients, yielding a true alloimmunization prevalence of 2.53% (Figure 4.1).
Among these 527 patients:164 were male (31.1%), and 363 were female (68.9%) resulting in a male-to-female ratio of 1:2.21. A total of 588 irregular antibodies were identified: 186 (31.6%) in male patients, and 402 (68.4%) in female patients. Most patients developed a single antibody (n=473; 89.7%), while 47 patients (8.9%) presented with two antibodies, and only 7 patients (1.4%) developed three or more.

### 4.1.1 Antibody Distribution by Blood Group System

Analysis of the 588 antibodies by blood group system revealed a clear predominance of the Rh system, accounting for 52.4% of all identified antibodies. This was followed by the Kell system (17.3%), MNS (8.2%), Kidd (6.8%), and Duffy (6.5%). Antibodies from less frequent systems—including Lewis, Lutheran, P1, Chido and others—accounted for the remaining 7.1%, grouped under “Other systems.” Additionally, 6 autoantibodies (1%) and 4 unidentified antibodies (0.7%) were detected (Figure 4.2).

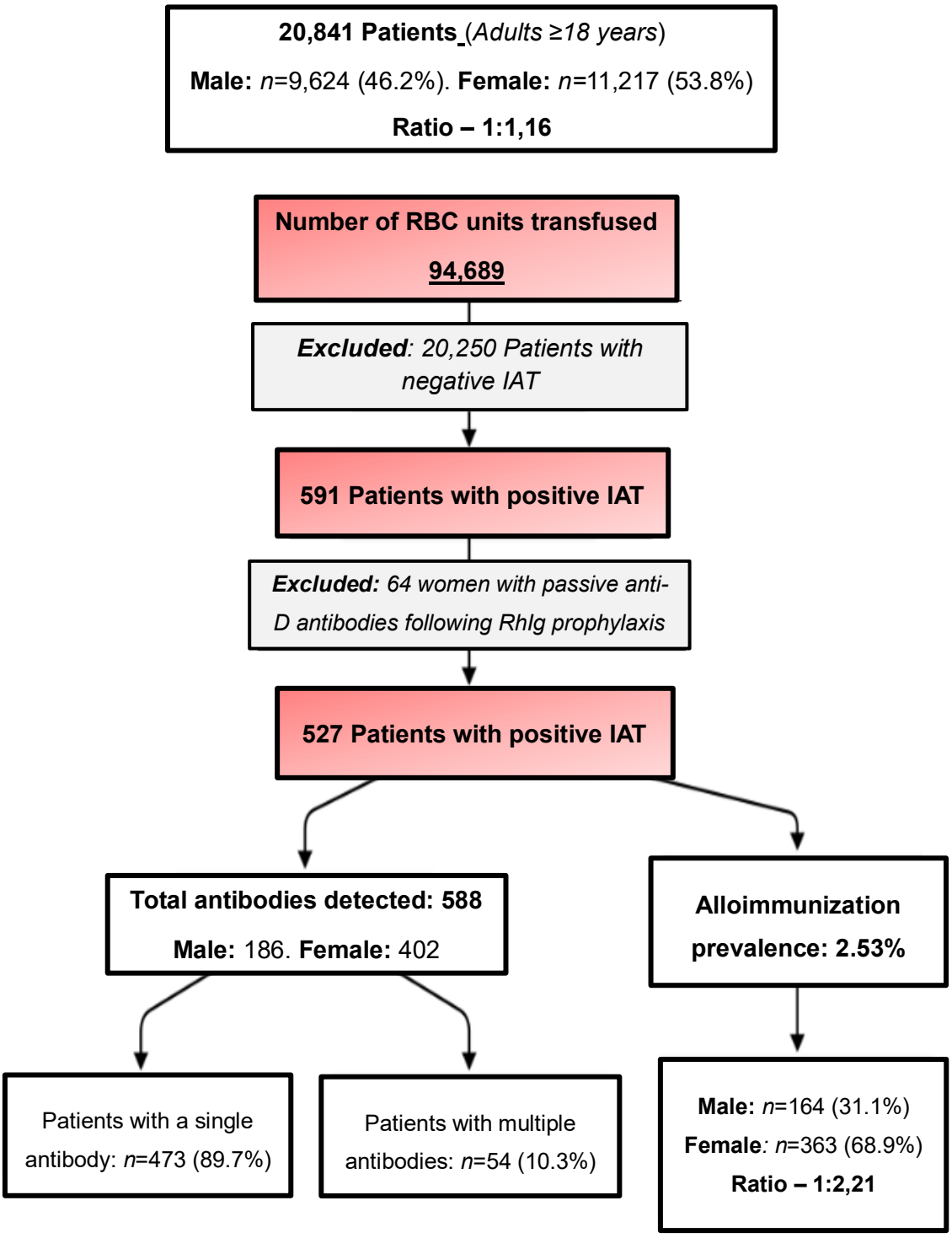


**Figure 4.1.** Patient inclusion flowchart and alloimmunization prevalence overview. The final analysis included 527 patients with positive IAT after exclusions. Women with passive anti-D antibodies following RhIg prophylaxis were excluded from the prevalence analysis. Alloimmunization prevalence, sex distribution, and number of antibodies are summarized.

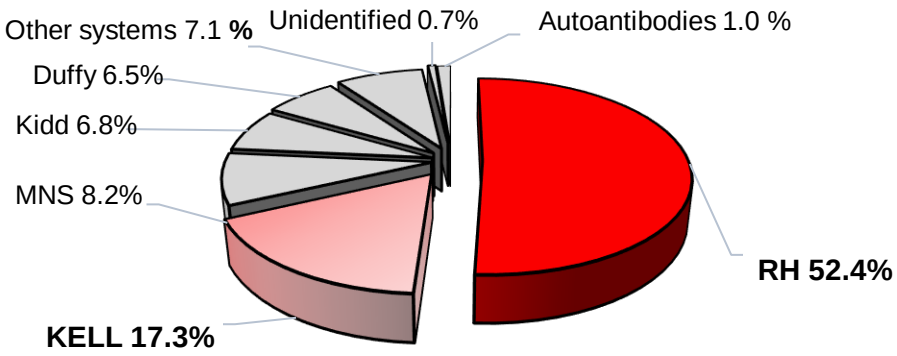


**Figure 4.2.** Antibody distribution by blood group system. Pie chart showing the relative distribution of 588 alloantibodies across major blood group systems. The Rh system predominated, followed by Kell, MNS, Kidd and Duffy.

To complement the distribution of antibodies by system, Table 4.1 presents the phenotypic frequency of Rh and Kell antigens among all blood donors at ULSAR in 2022, alongside the number and percentage of corresponding alloantibodies identified in patients. The close similarity between local antigen frequencies and those described in Caucasian populations reflects a predominantly white donor base[90] – an observation with relevant implications for transfusion safety, explored further in the discussion.

### 4.1.2 Antibody Distribution by Sex and Blood Group System

To enhance clarity, antibodies were aggregated by system and stratified by sex. Female predominance was observed across all systems, particularly within the Rh system, where 212 antibodies were identified in women compared to 96 in men (Figure 4.3). Similar patterns were noted in the Kell system (74 vs. 28), MNS (29 vs. 18), and Duffy (32 vs. 6). This pattern of sex-based disparity in alloantibody formation raises the hypothesis of female sex as a potential independent predictive factor for immunogenicity, which will be further explored in subsequent statistical models.

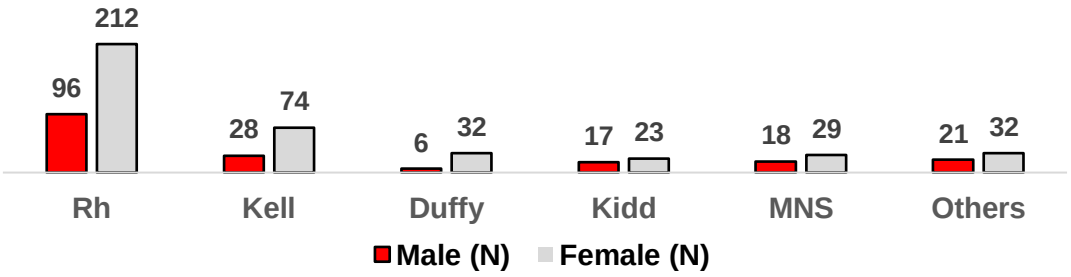


**Figure 4.3.** Distribution of identified alloantibodies by blood group system and sex. Female patients presented with a higher number of antibodies across all systems, particularly within the Rh and Kell systems.

**Table 4.1.** Phenotypic frequencies of red cell antigens among ULSAR blood donors (2022), compared with reported frequencies in Caucasian and Black populations. The number and proportion of corresponding alloantibodies detected in the patient cohort are also presented.

| | Antigen | Antibody | |
|---|---|---|---|
| **Blood Group System** | **Frequency in 2022 ULSAR Blood Donors* /Caucasian**/Blacks*****[90] | **Total sample** | |
| | | **N** | **%** |
| **RH** | | | |
| *D* | 86.3% ≃ 0.86 * / 0.85** / 0.92*** | 147 | 25.0 % |
| *C* | 66.7 % ≃ 0.67 * / 0.70** / 0.27*** | 46 | 7.8 % |
| *c* | 81.9 % ≃ 0.82 * / 0.80** / 0.96*** | 20 | 3.4 % |
| *E* | 27.4 % ≃ 0.27 * / 0.30** / 0.22*** | 78 | 13.3 % |
| *e* | 98.4 % ≃ 0.98 * / 0.98** / 0.98*** | 4 | 0.7 % |
| *Cw* | 0.001** | 13 | 2.2 % |
| **KELL** | | | |
| *K* | 9.2 %≃0.09 * / 0.09** / 0.02*** | 96 | 16.3 % |
| *Kp$^a$* | 0.02** | 6 | 1.0 % |
| **DUFFY** | | | |
| *Fy$^a$* | 0.66**/0.1*** | 35 | 6.0 % |
| *Fy$^b$* | 0.83**/0.23*** | 3 | 0.5 % |
| **KIDD** | | | |
| *JK$^a$* | 0.77**/0.92*** | 30 | 5.1 % |
| *JK$^b$* | 0.73**/0.49*** | 10 | 1.7 % |
| **MNS** | | | |
| *M* | 0.78** / 0.74*** | 22 | 3.7 % |
| *N* | 0.68** / 0.75*** | 1 | 0.2 % |
| *S* | 0.57** / 0.31*** | 24 | 4.1 % |
| *U* | 1** /0.98*** | 1 | 0.2 % |
| **LEWIS** | | | |
| *Le$^a$* | 0.22** | 22 | 3.7 % |
| *Le$^b$* | 0.72** | 9 | 1.5 % |
| **Lutheran** | | | |
| *Lu$^a$* | 0,08** | 6 | 1,0 % |
| *Lu$^b$* | 0,99** | 1 | 0,2 % |
| **Others:** *P1, Vell, Chido* | P1(0,79**/0,94***) Vell (0,99**); Chido (0,97**) | 4 | 0,7 % |
| **Autoantibodies** | | 6 | 1, 0 % |
| **N.i.** | | 4 | 0,7 % |

### 4.1.3 Number of Antibodies per Patient

Most alloimmunized patients (89.7%) developed a single alloantibody. However, 10.3% formed multiple antibodies. The most frequent combinations occurred within the Rh system, particularly anti-D+anti-C, which accounted for 15 of the 54 multi-antibody cases. Other Rh combinations included anti-C+anti-E, anti-c+anti-E, and anti-D+anti-E. These patterns align with the known immunogenicity of Rh antigens and their high prevalence among donor units. Additionally, several combinations involved antigens from multiple systems, especially Rh and Kell (e.g., anti-D+anti-K, anti-C+anti-$JK^b$), indicating broader antigenic exposure. The rare presence of three or more antibodies – observed in seven patients – further reinforces the cumulative effect of complex transfusion histories and underscores the need for phenotype-guided matching in high-risk populations (Figure 4.4).

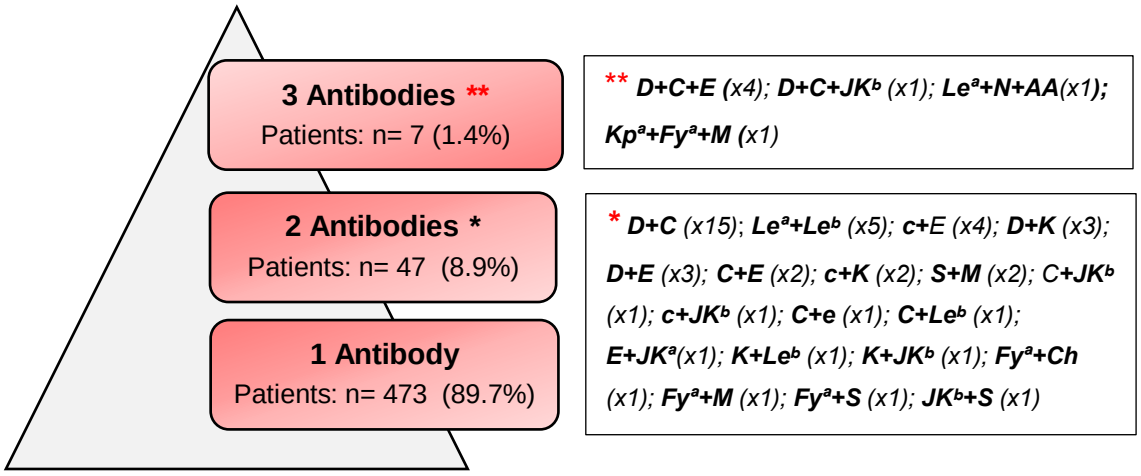


**Figure 4.4.** Distribution of patients according to the number of alloantibodies. Among the 23 unique combinations observed, 8 involved antibodies within a single blood group system – most commonly Rh – while the remaining 15 combined antigens from multiple systems. The Rh system was present in 58% of all combinations, followed by Kidd, MNS, Kell and Duffy. These findings underscore the immunogenic burden of Rh antigens and the increasing antigenic complexity in multi-transfused patients.

## 4.2. Immunogenicity and Post-Transfusion Follow-Up Timing

Building upon the general characterization of alloimmunized patients, this section addresses the core objective of this thesis: evaluating the immunogenic potential of specific red cell antigens and the extent to which suboptimal post-transfusion follow-up may compromise antibody detection. To do so, three structured databases were developed, each representing a different follow-up pattern after antigenic exposure. This segmentation allows for a comparative analysis of immunogenicity and supports a predictive modeling of undiagnosed alloimmunization.

### 4.2.1 Subgroup Characterization by Follow-Up Timing

**Table 4.2.** Subgroup characterization based on post-transfusion follow-up timing

| | Database 1 Ideal Timing (*n*=1131) | | Database 2 Delayed Timing (*n*=831) | | Database 3 No follow-up (*n*=2790) | | Database (1 to 4) Total (*n*=20841) | |
|---|---|---|---|---|---|---|---|---|
| | ***n*** | **%** | ***n*** | **%** | ***n*** | **%** | ***n*** | **%** |
| **Sex** | | | | | | | | |
| *Female* | 515 | 45.5 | 419 | 50.4 | 1418 | 50.8 | 11,217 | 53.8 |
| *Male* | 616 | 54.5 | 412 | 49.6 | 1372 | 49.2 | 9,624 | 46.2 |
| **Age categories** | | | | | | | | |
| *18 to 59 years* | 137 | 12.1 | 116 | 14.0 | 378 | 13.5 | 4,876 | 23.4 |
| *≥ 60 years* | 994 | 87.9 | 715 | 86.0 | 2412 | 86.5 | 15,965 | 76.6 |
| **Number of RBC units** | | | | | | | | |
| *1 to 10* | 520 | 46.0 | 571 | 68.7 | 2676 | 95.9 | 19,102 | 91.7 |
| *11 to 24* | 407 | 36.0 | 229 | 27.6 | 109 | 3.9 | 1,382 | 6.6 |
| *25 to 49* | 38 | 3.3 | 10 | 1.2 | 2 | 0.1 | 140 | 0.7 |
| *≥ 50* | 166 | 14.7 | 21 | 2.5 | 3 | 0.1 | 217 | 1.0 |
| **ABO blood group** | | | | | | | | |
| *A* | 539 | 47.7 | 388 | 46.7 | 1306 | 46,8 | 9,879 | 47.4 |
| *B* | 105 | 9.3 | 99 | 11.9 | 254 | 9.1 | 2,105 | 10.1 |
| *AB* | 37 | 3.3 | 33 | 4.0 | 88 | 3.2 | 646 | 3.1 |
| *O* | 450 | 39.7 | 311 | 37.4 | 1142 | 40.9 | 8,211 | 39.4 |
| **Rh Blood Group** | | | | | | | | |
| *Rh positive* | 1015 | 89.7 | 733 | 88.2 | 2446 | 87.7 | 18,403 | 88.3 |
| *Rh negative* | 116 | 10.3 | 98 | 11.8 | 344 | 12.3 | 2,438 | 11.7 |
| **Age (mean ± SD; min–max)** | 73.87 ± 12.75; 24-102 | | 72.98 ± 12.91; 29-102 | | 74.20 ± 14.46; 24-101 | | 68.87 ± 22.35; 18-102 | |
| **RBC units (mean ± SD; min–max)** | 17.15 ± 23.11; 2-437 | | 8.93 ± 8.74; 2-146 | | 3.72 ± 3.21; 2-56 | | 4.54 ± 7.89; 1-437 | |
| **Alloimmunization prevalence** | 11.1% | | 4.6% | | NA | | 2.53% | |
| ***p*-value (Group 1 vs. Group 2)** | <0.05 | | | | | | | |
| ***p*-value (Group 1 vs. Total cohort)** | <0.05 | | | | | | | |

To contextualize the analysis, patients were divided into three subgroups according to the timing of post-transfusion antibody screening. All had received RBC units with Rh and/or Kell antigens they lacked. Database 1 (Ideal Timing) was tested within 30–180 days, database 2 (delayed Timing) after 180 days, and database 3 had no recorded follow-up. **Table 4.2** summarizes the demographic and transfusional characteristics of these three groups. The ideal timing database (*n*=1131) included a slightly higher proportion of male patients (54.5%) and showed the highest transfusion burden, with a mean of 17.15 RBC units (SD = 23.11; range 2–292). The delayed timing group (*n*= 831) had a comparable sex distribution and a slightly younger average age (72.98 years), but received fewer RBC units on average (mean=8.93; SD=8.74; range 2–146). The no follow-up database (*n*=2790), the largest of the three, was predominantly composed of older patients (86.5% aged ≥60 years), with the lowest transfusion exposure (mean=3.72 units; SD=3.21; range 1–56). In terms of ABO distribution, group A was the most prevalent across all subgroups (ranging from 46.7% to 47.7%), followed by groups O, B, and AB. When stratified by Rh status, Rh-positive patients represented the majority in all subgroups: 89.7% in the ideal timing, 88.2% in the delayed timing, and 87.7% in the no follow-up. A fourth column was added to aggregate all transfused patients included in the study (*n*=20,841), combining the three sensitized groups with the broader group of non-included cases individuals. This total cohort preserved the slight female predominance (53.8%) observed in the overall population and displayed a predominantly older age profile, with 76.6% aged 60 or older. Most patients received between 1 and 10 RBC units (91.7%), and the ABO and Rh distribution remained consistent with subgroup patterns - blood group A being the most common (47.4%) and Rh-positive individuals representing 88.3%. Importantly, the prevalence of alloimmunization was statistically significant between groups: 11.1% in the ideal timing group compared to 4.6% in the delayed group ($p<0.05$). Moreover, when comparing the ideal-timing group with the total cohort (2.53%), the difference was significantly higher ($p<0.05$), emphasizing the relevance of follow-up timing in detecting alloimmunization events.

### 4.2.2 Antigen-Specific Immunogenicity: Ideal Timing vs. Delayed Timing

Can the timing of post-transfusion IAT influence the detection of alloantibodies through their evanescence – and, by extension, hide their immunogenicity? Could we, in fact, be talking about significant differences? Looking at Table 4.3, the answer appears to be yes. Patients who underwent IAT within the 30 to 180-day window – the period considered most appropriate for alloantibody detection – consistently showed higher immunogenicity rates across all antigens studied. In particular, this difference reached statistical

significance for anti-C, anti-E, and anti-K, where the likelihood of antibody detection was clearly greater when follow-up occurred in the ideal timeframe.

Even for anti-D, the detection rate was higher within this window (72.7% vs. 48.4%), although the difference did not meet the threshold for statistical significance. For anti-c and anti-e, event counts were low, which limited the possibility of a possible statistical significance – still, their detection also favoured timely follow-up.

These findings suggest a clear trend: when follow-up is delayed, antibodies may be missed – not necessarily because they were never produced, but possibly because they were no longer detectable at the time of testing.

**Table 4.3.** Immunogenicity of Rh and Kell antigens according to post-transfusion follow-up timing. Number of exposed patients, corresponding antibody detections, calculated immunogenicity rates, and p-values are presented for each antigen.

| | **Ideal Timing** | **Delayed Timing** | ***p*** |
|---|---|---|---|
| **Exposed to D** | 44 | 31 | 0.052 |
| *Anti-D detected* | 32 | 15 | |
| *% Immunogenicity* | 72.7% | 48.4% | |
| **Exposed to C** | 266 | 199 | <0.05 |
| *Anti-C detected* | 14 | 3 | |
| *% Immunogenicity* | 5.3% | 1.5% | |
| **Exposed to c** | 237 | 149 | 0.579 |
| *Anti-c detected* | 10 | 4 | |
| *% Immunogenicity* | 4.2% | 2.7% | |
| **Exposed to E** | 368 | 245 | <0.05 |
| *Anti-E detected* | 29 | 4 | |
| *% Immunogenicity* | 7.9% | 1.6% | |
| **Exposed to e** | 32 | 23 | 0.257 |
| *Anti-e detected* | 3 | 0 | |
| *% Immunogenicity* | 9.4% | 0.0% | |
| **Exposed to K** | 561 | 381 | <0.05 |
| *Anti-K detected* | 40 | 9 | |
| *% Immunogenicity* | 7.1% | 2.4% | |

### 4.2.3. Sex-Stratified Immunogenicity by Timing: Ideal vs. Delayed

After establishing that timing matters overall, male and female patients were separated to understand whether the trends held true across both groups, or whether different patterns emerged. The results are telling (Table 4.4 and Table 4.5). In women, the effect of timing was particularly relevant. Immunogenicity rates were consistently higher when follow-up occurred within the optimal 30 to 180 days. For anti-C, anti-E, and anti-K, this difference was not only visible – it was statistically significant. Even for anti-D, although the *p*-value remained above 0.05, the contrast (76.5% vs. 50.0%) was considerable. Among men, the pattern was still present, but less pronounced. Anti-E was the only antibody to reach statistical significance, with detection falling from 6.4% to just 0.8% when follow-up was delayed. For other antigens – including anti-K and anti-C – the same drop in detection was observed, but without statistical weight. And for anti-D, while immunogenicity was higher with timely testing (70.4% vs. 46.7%), the result did not reach significance.

Taken together, these findings raise an important point: when follow-up is late or absent, we risk missing antibodies that were likely there. And in women, who showed consistently higher immunogenicity across antigens, this risk seems even more pronounced. The question now is not whether timing matters – but how much it matters for different patients, and what that should mean for post-transfusion policy.

**Table 4.4.** Immunogenicity of Rh and Kell antigens in female patients according to post-transfusion follow-up timing. Number of exposed female patients, detected antibodies, and immunogenicity rates are shown for each antigen. Differences between ideal and delayed timing are statistically evaluated where appropriate.

| | Ideal Timing | | | Delayed Timing | | | |
|---|---|---|---|---|---|---|---|
| **Antibody** | **Exposed** | **Detected** | **%** | **Exposed** | **Detected** | **%** | ***p*** |
| ***Anti-D*** | 17 | 13 | 76.5 | 16 | 8 | 50.0 | 0.114 |
| ***Anti-C*** | 130 | 9 | 6.9 | 107 | 1 | 0.9 | <0.05 |
| ***Anti-c*** | 99 | 8 | 8.1 | 81 | 4 | 4.9 | 0.551 |
| ***Anti-E*** | 165 | 14 | 9.1 | 116 | 3 | 2.6 | <0.05 |
| ***Anti-e*** | 16 | 3 | 18.8 | 12 | 0 | 0.0 | -* |
| ***Anti-K*** | 255 | 27 | 10.6 | 186 | 5 | 2.7 | <0.05 |

**Table 4.5.** Immunogenicity of Rh and Kell antigens in male patients according to post-transfusion follow-up timing. Number of exposed male patients, detected antibodies, and immunogenicity rates are shown for each antigen. Differences between ideal and delayed timing are statistically evaluated where appropriate.

| | Ideal Timing | | | Delayed Timing | | | |
|---|---|---|---|---|---|---|---|
| **Antibody** | **Exposed** | **Detected** | **%** | **Exposed** | **Detected** | **%** | ***p*** |
| **Anti-D** | 27 | 19 | 70.4 | 15 | 7 | 46.7 | 0.129 |
| **Anti-C** | 136 | 6 | 4.4 | 92 | 2 | 2.2 | 0.479 |
| **Anti-c** | 138 | 2 | 1.4 | 68 | 0 | 0 | -* |
| **Anti-E** | 203 | 15 | 6.4 | 129 | 1 | 0.8 | <0.05 |
| **Anti-e** | 16 | 0 | 0.0 | 11 | 0 | 0.0 | -* |
| **Anti-K** | 306 | 13 | 4.2 | 195 | 4 | 2.1 | 0.215 |

***** Statistical significance could not be evaluated for some antigens due to the absence of events in one or more groups

## 4.3. Predictors of Immunogenicity in Combined Follow-Up Cohorts (Ideal and Delayed)

After establishing the influence of follow-up timing on alloantibody detection, the next step involved combining patients from the ideal and delayed testing groups into a single cohort. This approach enabled broader exploration of other factors that may contribute to antigen-specific immunogenicity, including sex, age, blood group, Rh, and transfusion burden.

### 4.3.1 Immunogenicity by Sex

As shown in Table 4.6, immunogenicity rates were consistently higher in female patients across all antigens analyzed. While some differences were modest and did not reach statistical significance – such as anti-D, anti-C, anti-E, and anti-e – others stood out more clearly. In particular, the antibodies anti-c and anti-K exhibited significantly higher detection rates in women compared to men (6.7% vs. 1.0% and 7.3% vs. 3.4%, respectively), both reaching statistical significance. These findings suggest that female patients may be more prone to alloantibody formation, possibly due to a combination of immunological, hormonal, and exposure-related factors.

**Table 4.6.** Immunogenicity by sex

| | Female | Male | *p* |
|---|---|---|---|
| **Exposed to D** | 33 | 42 | 1.000 |
| Anti-D detected | 21 | 26 | |
| *% Immunogenicity* | 63.6% | 61.9% | |
| **Exposed to C** | 237 | 228 | 0.341 |
| *Anti-C detected* | 10 | 8 | |
| *% Immunogenicity* | 4.2% | 3.5% | |
| **Exposed to c** | 180 | 206 | <0,05 |
| *Anti-c detected* | 12 | 2 | |
| *% Immunogenicity* | 6.7% | 1.0% | |
| **Exposed to E** | 281 | 332 | 0.591 |
| *Anti-E detected* | 17 | 16 | |
| *% Immunogenicity* | 6.0% | 4.8% | |
| **Exposed to e** | 29 | 26 | 0.238 |
| *Anti-e detected* | 3 | 0 | |
| *% Immunogenicity* | 10.3% | 0.0% | |
| **Exposed to K** | 441 | 501 | <0.05 |
| *Anti-K detected* | 32 | 17 | |
| *% Immunogenicity* | 7.3% | 3.4% | |

### 4.3.2 Immunogenicity according to age group

Age-related differences in alloantibody development were analyzed by comparing patients under 60 years old with those aged 60 and above (Table 4.7). The results did not show any statistically significant associations for any specific antigen. However, certain trends were observable: younger patients showed higher immunogenicity for anti-D and anti-c, whereas older patients had higher rates for anti-C, anti-E, anti-e, and anti-K. It is also important to note that the sample size for older patients was substantially larger, which may influence interpretation. Taken together, these findings suggest that age, in isolation, did not emerge as a major determinant of alloantibody response in this cohort.

**Table 4.7.** Immunogenicity by age group. Number of patients exposed to Rh and Kell antigens, corresponding antibody detections, immunogenicity rates, and p-values comparing individuals aged 18–59 years and those aged ≥60 years.

| | 18 to 59 years | ≥ 60 years | *p* |
|---|---|---|---|
| **Exposed to D** | 6 | 69 | 0.401 |
| Anti-D detected | 5 | 42 | |
| *% Immunogenicity* | 83.3% | 60.9% | |
| **Exposed to C** | 80 | 385 | 0.328 |
| Anti-C detected | 1 | 17 | |
| *% Immunogenicity* | 1.3% | 4.4% | |
| **Exposed to c** | 55 | 331 | 0.123 |
| Anti-c detected | 4 | 10 | |
| *% Immunogenicity* | 7.3% | 3.0% | |
| **Exposed to E** | 68 | 545 | 0.566 |
| Anti-E detected | 2 | 31 | |
| *% Immunogenicity* | 2.9% | 5.7% | |
| **Exposed to e** | 9 | 46 | 0.579 |
| Anti-e detected | 0 | 3 | |
| *% Immunogenicity* | 0.0% | 6.5% | |
| **Exposed to K** | 102 | 840 | 0.351 |
| Anti-K detected | 3 | 46 | |
| *% Immunogenicity* | 2.9% | 5.5% | |

### 4.3.3 Immunogenicity According to Blood Group (ABO and Rh)

To explore the role of blood group in alloantibody formation, immunogenicity was assessed across ABO and Rh. It's worth exploring whether innate differences between ABO and Rh groups could also shape immune responses.

Let's begin with the ABO system (Table 4.8). Despite some variation in immunogenicity rates across blood groups – for example, anti-C appeared slightly more frequent in group AB – no statistically significant associations emerged. Overall, ABO group does not seem to meaningfully affect the likelihood of antibody formation in this cohort.

**Table 4.8.** Immunogenicity rates by ABO blood group. Number of patients exposed to each antigen, number and percentage of detected alloantibodies, and *p*-values comparing across ABO groups.

| | **A** | **AB** | **B** | **O** | ***p*** |
|---|---|---|---|---|---|
| **Exposed to D** | 24 | 0 | 5 | 46 | 0,991 |
| Anti-D detected | 15 | 0 | 3 | 29 | |
| *% Immunogenicity* | 62.5% | NA | 60.0% | 63.0% | |
| **Exposed to C** | 195 | 13 | 50 | 207 | 0,378 |
| Anti-C detected | 4 | 1 | 3 | 9 | |
| *% Immunogenicity* | 2.1% | 7.7% | 6.0% | 4.3% | |
| **Exposed to c** | 194 | 19 | 30 | 143 | 0,549 |
| Anti-c detected | 8 | 0 | 0 | 6 | |
| *% Immunogenicity* | 4.1% | 0.0% | 0.0% | 4.2% | |
| **Exposed to E** | 279 | 28 | 89 | 217 | 0,659 |
| Anti-E detected | 13 | 1 | 4 | 15 | |
| *% Immunogenicity* | 4.7% | 3.6% | 4.5% | 6.9% | |
| **Exposed to e** | 24 | 0 | 3 | 25 | 0,217 |
| Anti-e detected | 0 | 0 | 0 | 3 | |
| *% Immunogenicity* | 0.0% | 0.0% | 0.0% | 12.0% | |
| **Exposed to K** | 461 | 28 | 97 | 356 | 0,504 |
| Anti-K detected | 22 | 1 | 3 | 23 | |
| *% Immunogenicity* | 4.8% | 3.6% | 3.1% | 6.5% | |

However, the Rh system told a different story (Table 4.9). Individuals with Rh-negative blood showed a significantly higher rate of anti-C formation (14.5% vs. 2.2%, $p < 0.05$). It's worth noting that anti-D – the most immunogenic of all – could not be compared across Rh groups, since Rh-positive individuals naturally express the D antigen and are not at risk for sensitization. Additionally, comparing anti-c and anti-e formation, between Rh groups, is complicated by the fact that Rh-negative individuals are typically homozygous for c and e antigens (cc/ee), which limits the validity of such comparisons and introduces methodological bias.

**Table 4.9.** Immunogenicity rates by Rh status (positive vs. negative). Includes number of exposures, antibody detections, and calculated immunogenicity percentages for each antigen. Statistically significant comparisons are highlighted.

| | Rh (-) | Rh (+) | *p* |
|---|---|---|---|
| **Exposed to D** | 74 | 0 | NA |
| Anti-D detected | 47 | 0 | |
| *% Immunogenicity* | 63.5% | 0.0% | |
| **Exposed to C** | 62 | 403 | <0.05 |
| Anti-C detected | 9 | 9 | |
| *% Immunogenicity* | 14.5% | 2.2% | |
| **Exposed to c** | 1 | 385 | 0.964 |
| Anti-c detected | 0 | 14 | |
| *% Immunogenicity* | 0.0% | 3.6% | |
| **Exposed to E** | 34 | 579 | 0.704 |
| Anti-E detected | 2 | 31 | |
| *% Immunogenicity* | 5.9% | 5.4% | |
| **Exposed to e** | 0 | 55 | NA |
| Anti-e detected | 0 | 3 | |
| *% Immunogenicity* | NA | 5.5% | |
| **Exposed to K** | 118 | 824 | 0.075 |
| Anti-K detected | 2 | 47 | |
| *% Immunogenicity* | 1.7% | 5.7% | |

### 4.3.4 Immunogenicity and Number of RBC Units Transfused

The potential link between transfusion volume and alloimmunization risk was examined by analysing immunogenicity in patients sensitized to Rh and/or Kell antigens, categorized by the total number of RBC units they had received (Table 4.10). These categories do not represent the number of units carrying the specific antigen in question, but rather serve as a proxy for cumulative exposure – the more units a patient receives, the greater the chance of being exposed to an unmatched antigen.

Looking across the data, some trends begin to emerge. For both anti-E and anti-K, immunogenicity rates increase with transfusion volume, particularly in patients who received more than 25 units. In the case of anti-K, this increase is statistically significant. For other antigens, this pattern was less evident – a finding that may be partly explained by smaller subgroup sizes, which limit the statistical power to detect such effects. Still, the overall distribution suggests a possible cumulative impact of transfusion burden on

alloimmunization risk. It is also important to consider that patients receiving high numbers of units are often subject to more frequent IAT testing, which may increase the chance of antibody detection.

**Table 4.10.** Immunogenicity according to categories of transfused RBC units.

| | **≤ 10** | **11 to 24** | **25 to 49** | **≥ 50** | ***p*** |
|---|---|---|---|---|---|
| **Exposed to D** | 37 | 27 | 4 | 7 | 0.457 |
| Anti-D detected | 21 | 18 | 2 | 6 | |
| *% Immunogenicity* | 56.8% | 66.7% | 50.0% | 85.7% | |
| **Exposed to C** | 266 | 141 | 12 | 46 | 0.059 |
| Anti-C detected | 8 | 4 | 2 | 3 | |
| *% Immunogenicity* | 3.0% | 2.8% | 16.7% | 6.5% | |
| **Exposed to c** | 206 | 140 | 10 | 30 | 0.233 |
| Anti-c detected | 4 | 7 | 1 | 2 | |
| *% Immunogenicity* | 1.9% | 5.0% | 10.0% | 6.7% | |
| **Exposed to E** | 322 | 206 | 15 | 70 | <0.05 |
| Anti-E detected | 14 | 9 | 2 | 8 | |
| *% Immunogenicity* | 4.3% | 4.4% | 13.3% | 11.4% | |
| **Exposed to e** | 37 | 12 | 1 | 5 | 0.282 |
| Anti-e detected | 1 | 2 | 0 | 0 | |
| *% Immunogenicity* | 2.7% | 16.7% | 0.0% | 0.0% | |
| **Exposed to K** | 476 | 316 | 27 | 123 | <0.001 |
| Anti-K detected | 19 | 12 | 0 | 18 | |
| *% Immunogenicity* | 4.0% | 3.8% | 0.0% | 14.6% | |

## 4.4 Predictive Modeling of Alloimmunization in Patients Without Follow-Up

### 4.4.1 Context and Rationale

A total of 2,790 patients (database 3) exposed to Rh and/or Kell antigens had no post-transfusion follow-up testing, due to the absence of a national post-transfusion surveillance policy. This represents a significant loss of clinical information. What happened to them remains unknown. Based on the previous findings, a logistic regression model was developed to estimate the probability of undetected alloantibody formation in this population.

### 4.4.2 Model Development and Variables

Logistic regression models were built using the Forward Wald method to identify predictors of immunogenicity for each antibody. Several variables were tested. Significant predictors were retained in the final models for anti-C, anti-c, and anti-K. For anti-D, anti-E, and anti-e, no variables were retained. The final models and predictive performance are summarized in Table 4.11.

**Table 4.11.** Logistic regression models (Forward Wald) for predicting alloantibody formation. Only models retaining at least one significant predictor were considered valid for prediction.

| | | Coefficient | Standard Error | Wald | *p* |
|---|---|---|---|---|---|
| ***Anti-D*** | Constant | 0,981 | 0,339 | 8,396 | <0,05 |
| | % correctly classified = 72.7% | | | | |
| ***Anti-C*** | Constant | -1.427 | 0.455 | 9.855 | <0,05 |
| | RH positive | -1.918 | 0.580 | 10.951 | <0,05 |
| | % correctly classified = 94.7% | | | | |
| ***Anti-c*** | Constant | -2.944 | 0.478 | 37.994 | <0,001 |
| | Sex: Male | -1.763 | 0.810 | 4.742 | <0,05 |
| | Number of Units | 0.025 | 0.012 | 4.602 | <0,05 |
| | % correctly classified = 96.2% | | | | |
| ***Anti-E*** | Constant | -2.459 | 0.193 | 161.496 | <0,001 |
| | % correctly classified = 92.1% | | | | |
| ***Anti-e*** | Constant | -2.269 | 0.606 | 13.993 | <0,001 |
| | % correctly classified = 90.6% | | | | |
| ***Anti-K*** | Constant | -2.353 | 0.238 | 97.664 | <0,001 |
| | Sex: male | -0.944 | 0.351 | 7.219 | <0,05 |
| | Number of Units | 0.009 | 0.004 | 4.378 | <0,05 |
| | % correctly classified = 92.7% | | | | |

**Note:** Independent variables entered into the model: age, number of RBC units transfused, male sex, Rh positive, blood group A, B, AB, and O. Models retaining only the constant were not considered valid for prediction, as no explanatory variable contributed significantly to immunogenicity.

Key findings from Table 8 include:

- Rh positivity contributed negatively and significantly to predicting anti-C formation.
- The number of transfused RBC units was a positive and significant predictor for anti-K and anti-c development.
- Male sex was a negative and significant predictor for anti-K and anti-c immunogenicity.
- The classification accuracy for the models predicting anti-c, anti-C, and anti-k was equal to or greater than 92.7%

### 4.4.3 Regression Equations

The resulting equations for predicting immunogenicity in patients without follow-up are shown below, based on the retained predictors:

Anti-C Model:

$$\log\left(\frac{p}{1-p}\right) = -1.427 - 1.918 \times (\text{Rh-positive})$$

Anti-c Model:

$$\log\left(\frac{p}{1-p}\right) = -2.944 - 1.763 \times (\text{Male sex}) + 0.025 \times (\text{Number of units transfused})$$

Anti-K Model:

$$\log\left(\frac{p}{1-p}\right) = -2.353 - 0.944 \times (\text{Male sex}) + 0.009 \times (\text{Number of units transfused})$$

### 4.4.4 Predicted Immunogenicity in the No Follow-Up Group

Based on these predictive parameters, the following estimated results were obtained for patients without follow-up (Table 4.12).

**Table 4.12.** Predicted probabilities of alloantibody formation in patients without follow-up, based on logistic regression models for anti-c and anti-K.

| | **No follow-up** |
|---|---|
| **Exposed to c** | 509 |
| Anti-c detected | 16 |
| *% Immunogenicity* | 3.1% |
| **Exposed to K** | 1096 |
| Anti-K detected | 69 |
| *% Immunogenicity* | 6.3% |

The absence of any significant predictors in the models for anti-D and anti-e immunization is likely related to the small sample sizes, which may not provide sufficient variability to detect statistical significance. The model for anti-C immunization is also not considered robust, as it retained only one significant predictor, which is insufficient for reliably classifying the immunogenic response. It is estimated that 3.1% developed anti-c antibodies and 6.2% developed anti-K antibodies. As a final step, the predicted immunogenicity rates for anti-c and anti-K in patients without follow-up were compared to observed rates from the ideal follow-up cohort. The model estimated a 3.1% immunogenicity rate for anti-c versus 4.2% observed, and 6.3% for anti-K versus 7.1% observed. In both cases, the differences were not statistically significant (*p*=0.5950 and *p*=0.5866, respectively). While this cannot be considered a formal validation, this comparison serves as a basic consistency check. The close alignment between predicted and observed rates suggests that the model is internally coherent and captures relevant patterns in the training data.

# Chapter 5. Discussion

This study retrospectively analysed 20,841 adults who received RBC transfusions at ULSAR between 2002 and 2022, either during hospitalization or in outpatient settings (including the day hospital of immunohematology). Over this 20-year period, 94,689 units of RBCs were transfused, and 527 patients were found to have clinically significant alloantibodies, resulting in an overall alloimmunization prevalence of 2.53%.

This rate is slightly higher than those reported in some well-established cohorts. For example, the multicenter REDS-III study in the USA, which included more than 300,000 patients, reported a prevalence of 2.07%[91]. Evers et al., in a Dutch cohort of 21,512 patients transfused between 2006 and 2013, found a prevalence of 2.2%[92]. Politou et al., in Greece, described a lower rate of 1.16% among 53,800 transfused patients[93]. But context also matters. Unlike the aforementioned studies, this cohort was monitored over two consecutive decades, with systematic records of each transfusion episode and the corresponding antibody screening results. This long-term follow-up makes a difference: it increases the likelihood of detecting late-appearing alloantibodies and recapturing evanescent antibodies that re-emerge after subsequent exposures. In this light, the rate we report here reflects not just the frequency of alloimmunization, but also the hemovigilance system in place. The antigenic profile of our donor population also plays a role. As detailed in Table 4.1, the ULSAR blood donor base is predominantly Caucasian, with antigen frequencies comparable to those described for white European populations[28]. The recipient population, however, includes a substantial proportion of individuals with African ancestry, many of whom have erythrocyte phenotypes that diverge significantly from the donor pool. This antigenic mismatch, especially in chronically transfused patients, increases the risk of alloimmunization[10 94]. In patients with SCD, alloimmunization rates can reach 47% in the absence of extended phenotype matching strategies[95 96]. Patients transfused in their baseline states of health are thought to be less likely to become alloimmunized, this heightened risk in SCD stems from a combination of frequent transfusion exposure, antigenic disparity with predominantly white donors, and a chronic inflammatory state inherent to the disease[97 60]. Of note, patients with SCD are, by necessity, transfused during times of inflammation. Other patient populations with high rates of RBC alloimmunization include those with myelodysplastic syndrome (MDS)[98], and thalassemia major[99].

The sex imbalance among alloimmunized patients deserves emphasis. While the overall transfused population had a male-to-female ratio of 1:1.16, the alloimmunized group showed a significantly different distribution of 1:2.21. This trend – also observed by Winters et al. (1:2.7)[100], - suggests a greater susceptibility to alloimmunization in women.

In our cohort, a total of 588 alloantibodies were identified in 527 patients, yielding a mean of 1.12 antibodies per alloimmunized patient. The vast majority (89.7%) developed only one alloantibody, while the remaining 10.3% had two or more. These figures closely mirror the findings of Politou et al., who reported that 88.8% of alloimmunized patients had only one antibody. Among patients with multiple alloantibodies, frequent combinations included anti-D+anti-C and anti-C+anti-E within the Rh system, as well as anti-D+anti-K across systems. The most common combination was anti-D+anti-C, consistent with findings from other studies[101 102].

The higher occurrence of alloantibodies against antigens of Rh and Kell systems (69,7%) is in agreement with the literature; both systems have highly immunogenic antigens[55 103 104]. The Rh system alone comprised 52.4%, with anti-D (25.0%), anti-E (13.3%), and anti-C (7.8%) being the most prevalent. Particularly noteworthy is anti-D, which remained the most common specificity, despite the exclusion of 64 cases of passive anti-D following RhIg prophylaxis. Its persistence underscores the strong immunogenicity of the RhD antigen, well-documented in the literature[7 42]. The Kell system accounted for 17.3% of the antibodies, primarily anti-K. For instance, the Kell antigen is present in approximately 9% of European donors but is responsible for a disproportionately large share of alloantibodies due to its capacity to elicit strong immune responses[44]. This distribution aligns with other European and North American studies[91 92 93]. Antibodies from the Kidd ($JK^a$, $JK^b$) and Duffy ($Fy^a$, $Fy^b$) systems remain clinically significant, accounting for approximately 6–7% of the identified alloantibodies. Kidd antibodies are known for their rapid evanescence, which can compromise detection and increase the risk of DHTR[18 27]. As previously said, anti-$Fy^a$ antibodies are especially relevant in patients of African descent with the Fy(a–b–) phenotype, which is often incompatible with $Fy^a$+ units from Caucasian donors[47 48].

Taken together, these findings reinforce the need for effective communication between clinicians and IHS. When transfusion requests contain only general diagnoses like "anemia," they may fail to trigger extended phenotyping protocols. By explicitly noting diagnoses such as "SCD", "Thalassemia Major" or "MDS," clinicians help ensure more precise matching, improving transfusion safety and minimizing alloimmunization risk.

The results of this study clearly demonstrate that the interval between transfusion and IAT has a decisive impact on the ability to detect alloimmunization. Among patients who underwent follow-up within the ideal timing window (30 to 180 days), the prevalence of alloimmunization was 11.1%, in contrast to 4.6% observed in the delayed timing group (>180 days), a statistically significant difference ($p$ <0.05). This disparity becomes even more pronounced when compared to the overall prevalence of 2.53%, further emphasizing that the timing of post-transfusion monitoring is a critical variable in identifying the immune response. Failure to conduct screening within this sensitive timeframe compromises not only the diagnosis itself, but also our ability to assess the true extent of alloimmunization in the clinical setting. The higher prevalence observed in the ideal timing group suggests that many alloantibodies may simply go undetected when screening is delayed. This issue is particularly relevant in the case of evanescent alloantibodies. The importance of this sensitive timeframe is also supported by the findings of Reverberi et al., who reported an overall non-persistence rate of 37%, with marked variation according to antibody specificity – anti-D being the most persistent and anti-JK$^a$ among the least durable[52]. Stack et al. demonstrated that approximately two- thirds of alloantibodies disappear from the serum over a five-year period, with nearly half becoming undetectable within the first six months after identification[21]. This concern is further reinforced in his editorial “Timing is everything”[105], where he warns that up to 70– 80% of alloantibodies may eventually evanesce if no follow-up testing is performed. Without such screening, transfusion services remain unaware of prior sensitization, increasing the risk of anamnestic responses and DHTR. In a study involving 18,750 veterans, Tormey and Stack confirmed high evanescence rates and warned of the increased risk of inadvertent re-exposure and subsequent HTR[106]. These findings strongly support the importance of timely detection and long-term antibody tracking.

The decision to focus on the immunogenicity of Rh and Kell system antigens was based on both scientific and methodological grounds. These antigens are not only consistently cited in the literature as the most immunogenic and frequently implicated in alloimmunization events, but they also accounted for the majority of clinically significant alloantibodies identified in our cohort (approximately 70%). Moreover, from a practical standpoint, antigenic information on the ABO, Rh, and Kell systems is routinely available for all RBC units. This availability enabled matching each patient’s antigen profile with the transfused units, allowing assessment of antigenic mismatches and subsequent alloantibody formation. Extended phenotyping for other erythrocyte systems is not routinely performed for donors or recipients, making such analyses inconsistent and methodologically unsound in this context.

Comparison of the detection rates for anti-C, anti-E, anti-K, and anti-D alloantibodies between the optimal and late follow-up groups revealed a consistent pattern: the 30 to 180-day window maximizes detection. The differences were statistically significant for anti-C, anti-E, and anti-K. Although anti-D did not reach statistical significance, the observed discrepancy remains clinically relevant[4]. When stratified by sex, female patients exhibited higher detection rates for all major antibodies within the optimal follow-up window, with statistically significant differences observed for anti-C, anti-E, and anti-K. Several factors may contribute to this trend, including immunological and hormonal differences, as well as prior exposures, such as pregnancies, that may prime the immune system to respond more robustly and rapidly[107]. The detection patterns observed in this study reinforce the appropriateness of the 30–180-day follow-up window. This interval, supported by prior empirical and kinetic studies, captures the majority of primary alloantibody responses while minimizing the risk of evanescence-related under detection [54 55 56 66 88]. Beyond the timing of post-transfusion antibody screening, other factors may influence the antigen-specific immunogenicity of RBC antigens. By combining patients from both the ideal and delayed follow-up cohorts, the analysis was broadened to explore the potential impact of variables such as sex, age, ABO and Rh blood groups, and the total number of transfused units. The aim here was to identify consistent patterns that could help shape future preventive strategies and guide a more personalized approach to transfusion therapy. Among all the variables considered, sex stood out as the most relevant. Women showed higher immunogenicity rates for all antibodies studied, with statistically significant differences observed for anti-c, and anti-K. These results lend further support to the idea that female sex, in itself, represents a risk factor for alloimmunization – a trend consistently observed in other studies as well[107 108]. In addition to underlying immunological and hormonal differences, prior exposure during pregnancy may act as an additional sensitizing trigger[109]. Studies such as that by Verduin et al. also highlight sex as a meaningful predictive variable in this context[110].

The role of age in transfusion-related alloimmunization is complex. In this study, patients aged ≥ 60 had higher immunogenicity rates for anti-C, anti-E, anti-e, and anti-K, while anti-D and anti-c had higher immunogenicity rates among those under 60. None of these differences reached statistical significance. Still, the overall distribution was clear: 90.8% of alloimmunized patients were 60 or older, and only 9.2% were younger. This trend aligns with previous studies by Politou et al,.[93]

While one might expect immune senescence to reduce responses in the very elderly, Moncharmont et al., showed that older patients remain immunologically competent and capable of producing significant alloantibody responses[111]. From a practical, laboratory standpoint, age may act as a confounding variable. Younger adults often belong to high-risk groups in whom extended matching (Rh and Kell) is either mandated or strongly recommended. In these populations, the selection of antigen-compatible units aims to avoid early alloimmunization that could compromise future transfusion management. In contrast, such phenotypic precision is not always applied in older patients, especially when the transfusional need is urgent or the life expectancy is shorter. This discrepancy in clinical practice may further skew age-related alloimmunization patterns. So, age appears to reflect a balance between two competing factors: on one hand, cumulative exposure, on the other hand, immune response may decline gradually with age. Nonetheless, age alone in this study is not an independent predictor of alloimmunization risk.

Similar to other authors, no correlation was observed between alloimmunization and the different blood types of the ABO system[103]. The Rh system proved to be more relevant, primarily due to the high immunogenicity of the D antigen[112]. Among Rh-negative individuals, the rate of anti-C alloimmunization was higher compared to Rh-positive patients. This finding suggests an increased susceptibility of RhD-negative individuals to other Rh antigens, such as C and E, likely due to both the absence of prior exposure and the structural homology among Rh proteins. Indeed, given the high degree of similarity between Rh antigens, especially between D and C/E, it has been proposed that individuals who completely lack RhD expression may be more prone to mounting immune responses against these related antigens[113 114].

In this study, the number of RBC units transfused emerged as a relevant variable. The development of anti-E and anti-K antibodies showed statistically significant associations with transfusion burden, while anti-C demonstrated a strong upward trend that did not reach statistical significance. This association is well documented in the literature. Studies conducted by Santos et al., and Alves et al., observed that alloimmunized patients had received, on average, significantly more units than their non-alloimmunized counterparts[56 103]. Also, Zalpuri et al., who conducted a prospective study in a cohort of previously non-transfused, non-alloimmunized patients observed a clear, incremental increase in alloimmunization risk with the number of transfused units[115]. This aligns well with the results found in this study and reinforces the idea that transfusion burden plays a central role in alloantibody development. While transfusion volume alone is not sufficient to predict alloimmunization, it serves as a meaningful proxy for antigen exposure

The predictive modelling approach added a new layer to this study. It provided a way to estimate what may be happening among patients who were exposed but never tested again. These individuals represent a blind spot created by the absence of a national post-transfusion follow-up policy. By applying patterns learned from patients with proper monitoring, it was possible to generate plausible estimates of missed alloimmunization. More than a statistical exercise, this model underscores the consequences of system-level gaps. Of course, this was an internal model, and its predictions need to be interpreted with that limitation in mind. Still, the alignment with observed data from the best- monitored group suggests that these estimates are not far from reality. In conclusion, predictive modelling here served a dual role: as a practical workaround for missing data, and as a reminder of what's at stake when long-term transfusion outcomes are not systematically tracked.

# Chapter 6. Study Limitations

Despite its comprehensive approach, this study has several limitations that should be acknowledged, as summarized in Table 6.1.

**Table 6.1** Study Limitations

| Limitation | Description |
|---|---|
| **□ Lack of Direct Measurement of Antibody Evanescence** | The study evaluates alloantibody detection over time but does not directly measure evanescence, potentially underestimating low-titer antibodies. |
| **📊 Limited Clinical Data** | Patient comorbidities and immunosuppressive therapies were not included, restricting a full risk assessment of alloimmunization. |
| **🔬 Complexity of Alloimmunization Factors** | Genetic, clinical, and transfusion-related factors interact dynamically, making causality difficult to establish in a retrospective study. |
| **🚫 Selection Bias** | The exclusion of pediatric patients limits the generalizability of the findings. |
| **📈 External Influences** | Shifts in patient demographics, such as an aging population and changing disease prevalence, may have impacted the study`s results. |
| **📄 Publication Bias** | The literature reviewed may overrepresent studies with significant findings while underreporting negative or non-significant results. |

By acknowledging these limitations, this study provides a transparent framework for interpreting its results and identifying opportunities for future research. In particular, prospective studies that directly assess antibody evanescence and incorporate broader clinical variables may further clarify the dynamics of RBC alloimmunization.

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# Chapter 7. Recommendations and Future Perspectives

This chapter outlines the key recommendations emerging from the findings of this study, as well as future directions that may contribute to improved transfusion safety and a deeper understanding of RBC alloimmunization. The order of recommendations reflects a deliberate progression: from immediate clinical implications and direct study findings, through structural and technical solutions, and culminating in broader strategies for long-term prevention.

## 7.1 Strengthen Post-Transfusion Monitoring Policies

The results of this study suggest that the timing of post-transfusion IAT can significantly influence detection rates. Given the risk of underdiagnosing alloimmunization due to antibody evanescence, especially when testing is delayed, it may be advisable to implement a structured follow-up protocol. A follow-up window between 30- and 180-days post-transfusion appears to offer greater reliability for detecting clinically relevant antibodies. Introducing such a measure could enhance transfusion safety and support timely identification of at-risk patients.

## 7.2 Establish a Centralized National Immunohematologic Database

In Portugal, as in most countries, transfusion records are maintained locally, with no comprehensive national database. Implementing such a centralized platform could significantly enhance transfusion safety by integrating patient antibody histories and transfusion requirements across hospitals[116]. This would ensure that prior alloantibodies are known at any IHS, saving time and resources, avoiding redundant antibody investigations, and alerting to antibodies that have evanesced but remain clinically relevant[57]. Successful international models support this approach[117 118]. The Netherlands' Transfusion Register of Irregular Antibodies (TRIX) registry has documented over 80,000 alloantibodies and reduced DHTR by ~50%. The registry has also optimized pretransfusion testing and antigen-negative unit allocation, improving donor-recipient matching strategies[119 120]. France's *Établissement Français du Sang Alpes-Méditerranée* connects 14 blood banks to 149 hospitals, ensuring real-time access to transfusion data[117].

A unified system could also reduce serious errors. For example, the Pittsburgh Central Transfusion network reported 94 WBIT (wrong-blood-in-tube) errors over 17 years, 57% of which were caught through discrepancies in historical ABO records, resulting in a 38% improvement in error detection[58].

### 7.3 Expand Recruitment of Ethnically Diverse Blood Donors

An additional challenge in alloimmunization prevention is the increasing genetic diversity of transfusion recipients, particularly in countries like Portugal, where immigration and multiculturalism are reshaping the population's antigenic profile. The findings of this study suggest a predominantly Caucasian donor base, as evidenced by the similarity between local antigen frequencies and those reported in European populations. Many clinically significant blood group antigens are ethnically dependent, and IHS must adapt to ensure that the available donor pool reflects the diversity of patients in need. Transfusion services in Portugal should prioritize targeted blood donation campaigns aimed at recruiting donors from underrepresented ethnic backgrounds, ensuring greater antigenic compatibility between donors and recipients and reducing the risk of alloimmunization. Without a diversified donor base, the effectiveness of extended antigen matching will remain limited[121] [122].

### 7.4 Promote Molecular Genotyping for At-Risk Patients

Serologic phenotyping has long been considered the gold standard for determining blood group antigen expression. Nonetheless, it faces notable limitations in specific clinical scenarios, such as recently transfused patients (where circulating donor RBC confound accurate typing), individuals with autoantibodies, and those receiving monoclonal antibody therapies (e.g., anti-CD38, anti-CD47)[94] [123]. Additionally, variant alleles may yield false-positive or false-negative results, compromising extended phenotyping in urgent settings and heightening the risk of alloimmunization[124]. At the Hospital Fernando da Fonseca (Lisbon), for instance, genotyping of 21 multitransfused SCD patients revealed that ~90% harbored the FY*null01 mutation in the FYB promoter, which enabled the safe use of Fyb+ blood units in individuals with an apparent Fy(a–b–) phenotype. This strategy drastically expanded the donor pool, mitigating alloimmunization rates in a population often reliant on rare phenotype matches[62]. It also allows refined management of weak and partial D variants in pregnant women, avoiding unnecessary RhIg prophylaxis, and preserving limited RhD-negative inventories for those who really requires them[125] [126] (Appendix F: Serologic Weak D phenotype).

### 7.5 Align Transfusion Practices with PBM

PBM is a multidisciplinary, evidence-based strategy designed to minimize unnecessary transfusions and reduce exposure to donor RBC antigens, thereby lowering the risk of alloimmunization[127]. Studies have established a clear correlation between transfusion volume and alloantibody formation[115]. PBM strategies – such as preoperative anemia correction, restrictive transfusion triggers, and perioperative blood conservation – have been shown to reduce transfusion rates by up to 40% while maintaining patient safety. By reducing transfusion dependency and optimizing blood usage, PBM emerges as a critical approach to mitigating alloimmunization risks in transfusion-dependent patients[128].

### 7.6 The Future of Transfusion Medicine

Reducing alloimmunization rates will require close collaboration between transfusion specialists, geneticists, and computational scientists. Donor genetic phenotypes must be made available on a broader scale to enable extended matching for patients with rare antigen profiles[129]. Regional and national antigen databases have proven feasible, but their implementation must ensure data security and equitable donor recruitment[130]. Personalized transfusion strategies, supported by predictive algorithms and automated alloantibody monitoring, will be essential[131]. Emerging technologies like artificial intelligence may also support the future of transfusion medicine. Preliminary studies suggest potential in predicting transfusion needs, managing inventories, and identifying complications. However, these tools are still maturing and should be seen as complementary to clinical expertise and robust practices.

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# Chapter 8. Conclusion

Evidence from this study suggests that RBC alloimmunization remains a relevant clinical challenge, particularly involving Rh and Kell antigens. Antibody detection proved highly dependent on timing: screening performed between 30 and 180 days after transfusion markedly increased identification rates, revealing the impact of antibody evanescence and limitations of current monitoring practices.

By combining clinical data with predictive modelling, the analysis inferred a non-negligible number of undetected cases in patients without follow-up. This gap highlights that analytical precision alone is insufficient if not complemented by structured post-transfusion surveillance capable of capturing clinically relevant alloimmunization events.

Ultimately, the findings support the implementation of standardized follow-up protocols and centralized antibody registries to ensure continuity of immunohematologic information. Beyond its immediate scope, this work contributes to a more integrated vision of transfusion safety – where data systems and clinical decision-making converge to minimize preventable risks and improve long-term patient care.

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# Appendices

## Appendix A: Clinical Significance of Blood Groups

**Table A1:** Clinical Significance of Blood Groups. This original table summarizes the key antigens, immunogenicity, and clinical implications of various blood group systems, highlighting their relevance in transfusion medicine and alloimmunization. High immunogenicity systems, such as Rh and Kell, are major contributors to HDFN and DHTR, while others, such as Lutheran and Lewis, typically result in less severe outcomes

| System | Key Antigens | Immunogenicity | Clinical Implications | References |
|---|---|---|---|---|
| **Rh** | D, C, c, E, e | High (D) | HDFN, DHTR, alloimmunization risk in multi-transfused patients. | Arthur & Stowell, 2022; Hendrickson et al., 2016 |
| **Kell** | K, k | High (K) | Fetal anemia, hemolysis in DHTR. | Reid et al., 2012; Zimring & Hudson, 2016 |
| **Kidd** | $Jk^a$, $Jk^b$ | Moderate | Evanescence, DHTR, hyperhemolysis. | Stack et al., 2016; Schonewille et al., 2000 |
| **Duffy** | $Fy^a$, $Fy^b$ | Moderate | Malaria resistance (Fy[a-b-]), compatibility challenges. | Arthur & Stowell, 2022 |
| **MNS** | M, N, S, s | Variable | Hemolysis (S, s), rarely significant (M, N). | Reid & Lomas-Francis, 2003; Garratty, 2004 |
| **Lewis** | $Le^a$, $Le^b$ | Low | Mild hemolysis in enzymatic tests. | Daniels, 2013; Garratty, 2004 |
| **Diego** | $Di^a$, $Di^b$ | Moderate | Hemolysis in specific populations (Asian, Native American). | Reid et al., 2012; Westhoff, 2007 |
| **Lutheran** | $Lu^a$, $Lu^b$ | Low | Anti-$Lu^b$ may cause severe hemolysis; Anti-$Lu^a$ typically mild. | Reid & Lomas-Francis, 2003; Daniels, 2013 |

## Appendix B: Transmembrane structure of RhD, RhCE, and RhAG proteins

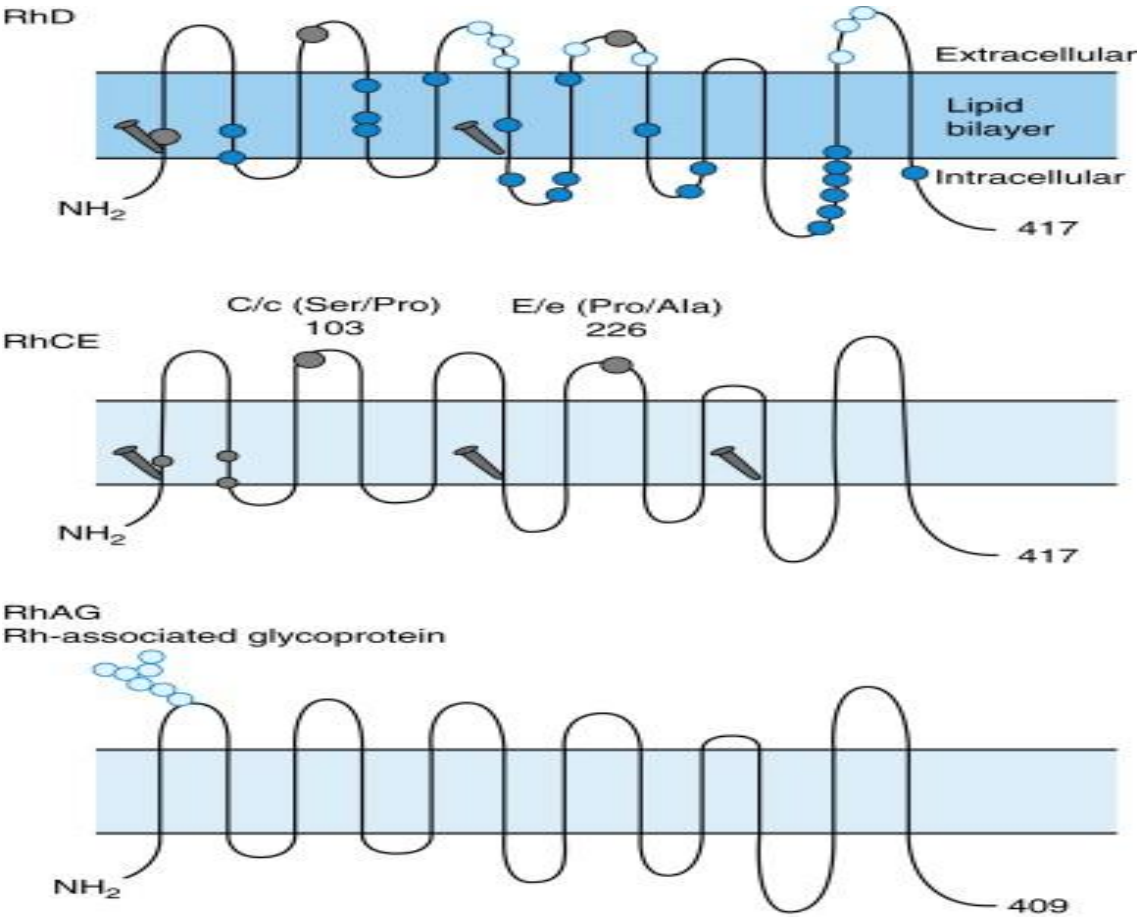



**Figure B1 –** Main structural features of Rh blood group system proteins. The RhD protein shows a greater number of extrextracellular epitopes, contributing to its high immunogenicity. The amino acid residues C/c (Ser/Pro at residue 103) and E/e (Pro/Ala at residue 226) are highlighted in RhCE. The Rh-associated glycoprotein (RhAG), on the other hand, serves as a functional support for the Rh complex. *Adapted from* Arthur, C., & Stowell, S. (2022).

## Appendix C: Summary of Molecular and Host Factors Influencing Alloimmunization.

**Table C1.** Summary of Molecular and Host Factors Influencing Alloimmunization. This table is an original work synthesized with supporting references.

| Factor | Description | Clinical implications | References |
|---|---|---|---|
| **Molecular Characteristics** | | | |
| ***Polymorphism*** | Highly polymorphic antigens, such as RhD, present multiple Epitopes, increasing imune activation potential. | Higher risk of alloimmunization with RhD or Kell antigens. | Arthur & Stowell (2023) |
| ***Amino Acid Substitutions*** | Changes in amino acid sequences create novel epitopes, boosting antigen recognition and immunogenicity. | Enhanced detection and avoidance strategies for high-risk antigens. | Avent & Reid (2000) |
| ***Antigen Density*** | Dense expression of antigens on RBC surfaces, like RhD, significantly increases immunogenic response. | Challenges in identifying compatible RBC units for high-density antigens. | Castleman & Kilby (2020) |
| **Host Factors** | | | |
| ***Genetic Predispositions*** | HLA haplotypes, such as HLA-DRB1*15, predispose individuals to heightened alloimmune reactions. | Tailored transfusion approaches based on HLA profiling can improve outcomes. | Schonewille et al. (2000) |
| ***Chronic Inflammation*** | Conditions such as SCD heighten immune activation through pro-inflammatory cytokines. | Careful monitoring in patients with chronic inflammatory diseases is essential. | Schonewille et al. (2000) |
| ***Prior Antigenic Exposure*** | Sensitization from previous transfusions or pregnancies increases the likelihood of alloimmunization. | Frequent antibody screening for sensitized individuals after exposures. | Hendrickson & Tormey (2016) |
| ***Immunosuppressive States*** | Advanced age, malignancies, or other immunosuppressive conditions alter immune responses. | Risk of missed alloimmunization in immunocompromised patients requires follow-up. | Arthur & Stowell (2023) |

## Appendix D: RhIg Prophylaxis

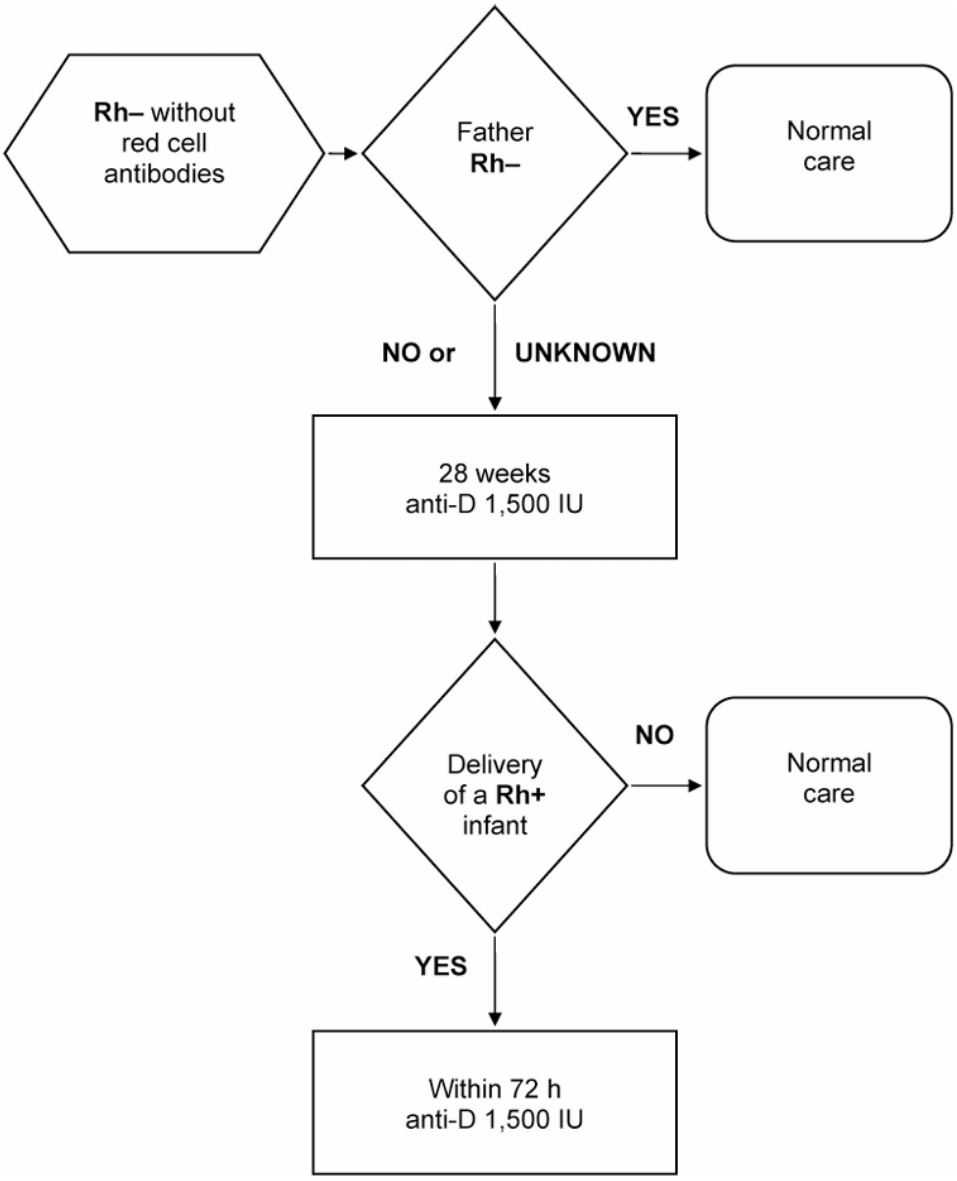



**Figure D1 –** RhIg Prophylaxis Flowchart: Flow diagram outlining the recommended prophylactic administration of anti-D immunoglobulin for Rh-negative pregnant women. Key decision points include paternal Rh typing (if known), gestational age (28 weeks), and neonatal Rh status, with a 1,500 IU dose administered within 72 hours postpartum if the infant is confirmed to be Rh-positive.

*Adapted from* Hirose TG, Mays DA. The safety of RhIG in the prevention of haemolytic disease of the newborn. J Obstet Gynaecol. 2007

# Appendix E: Other Antibodies in HDFN

**Table E1.** Other Antibodies in HDFN. This table is an original work, synthesized from Bowman et al., 2007; Ohto et al., 2020; and Reid & Lomas-Francis, 2015

| Antibody | System | Mechanism of Action | Clinical Impact | References |
|---|---|---|---|---|
| **Anti-Fy$^a$, Anti-Fy$^b$** | Duffy System | Extravascular hemolysis mediated by macrophages | Mild to moderate anemia | Bowman et al., 2007 [132] |
| **Anti-S, Anti-s** | MNS System | Extravascular hemolysis similar to Anti-D | Mild to severe Anemia | Reid & Lomas-Francis, 2015 [133] |
| **Anti-U** | MNS System | Severe cases documented in U-Negative individuals; extravascular hemolysis. | Severe anemia; Hydrops fetalis | Reid & Lomas-Francis, 2015 [133] |
| **Anti-Ge3** | Gerbich System | Induces apoptosis of erythroid progenitors, impairing erythropoiesis | Severe anemia | Ohto et al., 2020 [73] |
| **Anti-M** | MNS System | Disruption of erythropoeiesis vi agglutination or partial phagocytosis of progenitors. | Mild to moderate Anemia | Moise, 2008 [74] Ohto et al., 2020 [73] |
| **Anti-Vel** | Vel System | Intravascular hemolysis; rare but severe cases documented | Severe anemia | Reid & Lomas-Francis, 2015 [133] |

## Appendix F: Serologic Weak D phenotype

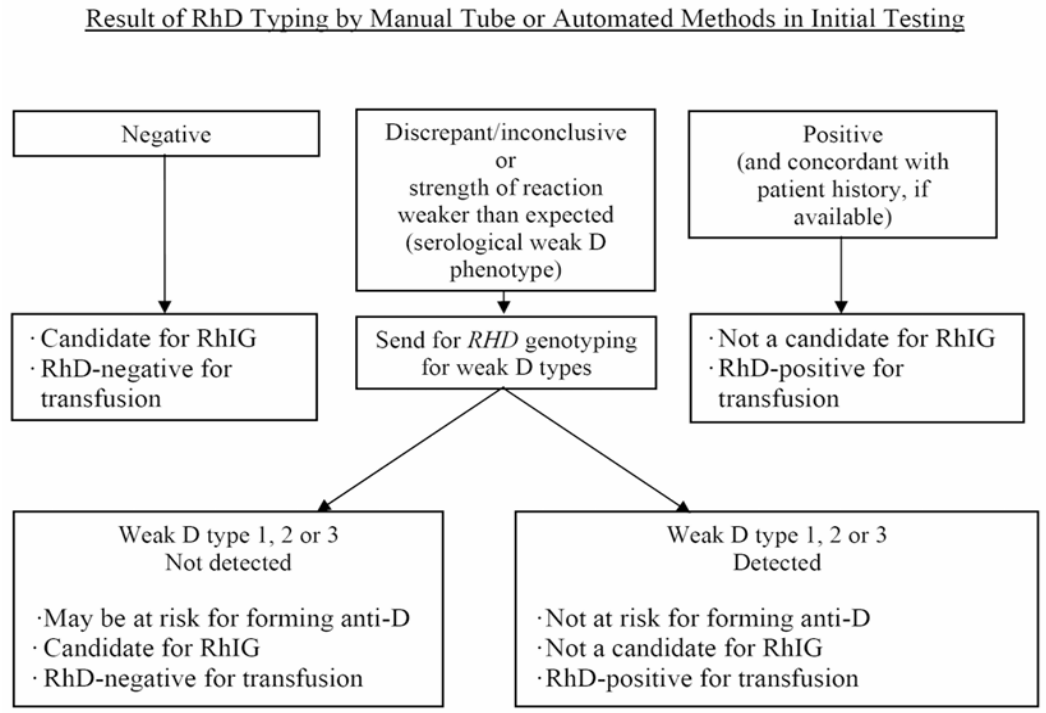



**Figure F1:** Algorithm for resolving serological weak D phenotype test results by *RHD* genotyping to determine candidacy for Rh immune globulin (RhIG) and RhD type for transfusions.

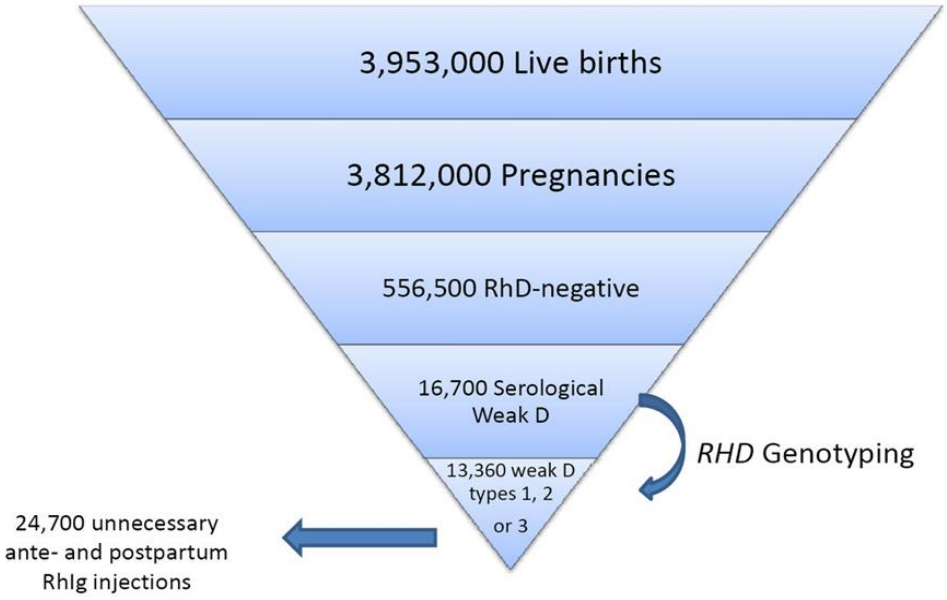



**Figure F2:** Unnecessary RhIG injections. Of 3,953,000 live births in the United States each year, an estimated 13,360 mothers have a serologic weak D phenotype which, if confirmed by *RHD* genotyping, could be managed as RhD-positive. Adjusting for antepartum and postpartum dosing, *RHD* genotyping of pregnant women with a serological weak D phenotype could prevent 24,700 unnecessary injections of RhIG.

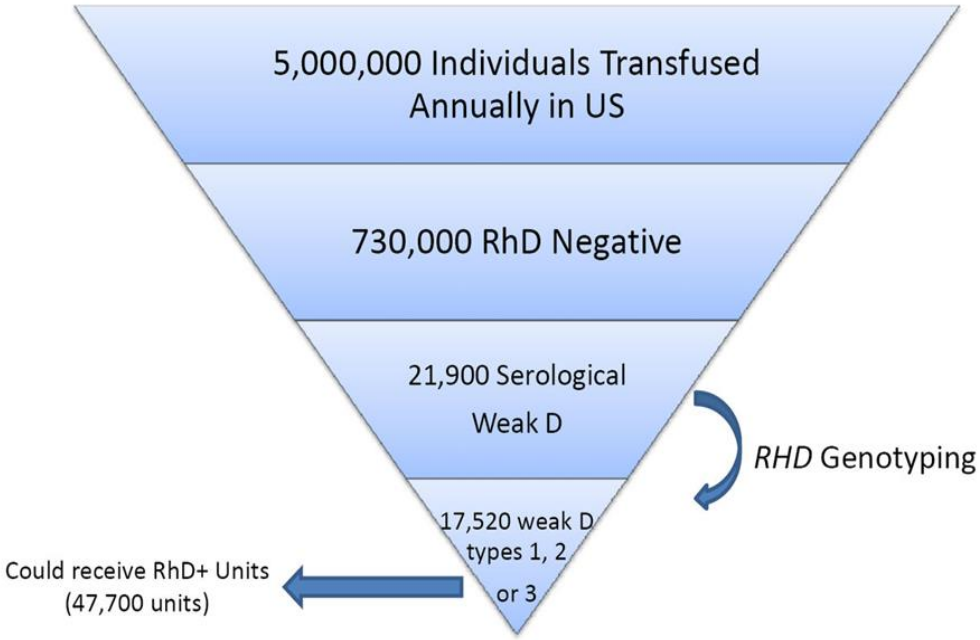



**Figure F3:** Unnecessary Transfusion of RhD-negative RBC Units. Of 5,000,000 individuals transfused annually in the USA, an estimated 17,520 with a serological weak D phenotype will be typed as RhD-negative. If their *RHD* genotype were determined, they could be managed safely as RhD-positive. Since the average number of RBC units is 2.7 per recipient, *RHD* genotyping could make 47,700 units of RhD-negative RBCs available to patients who require them.

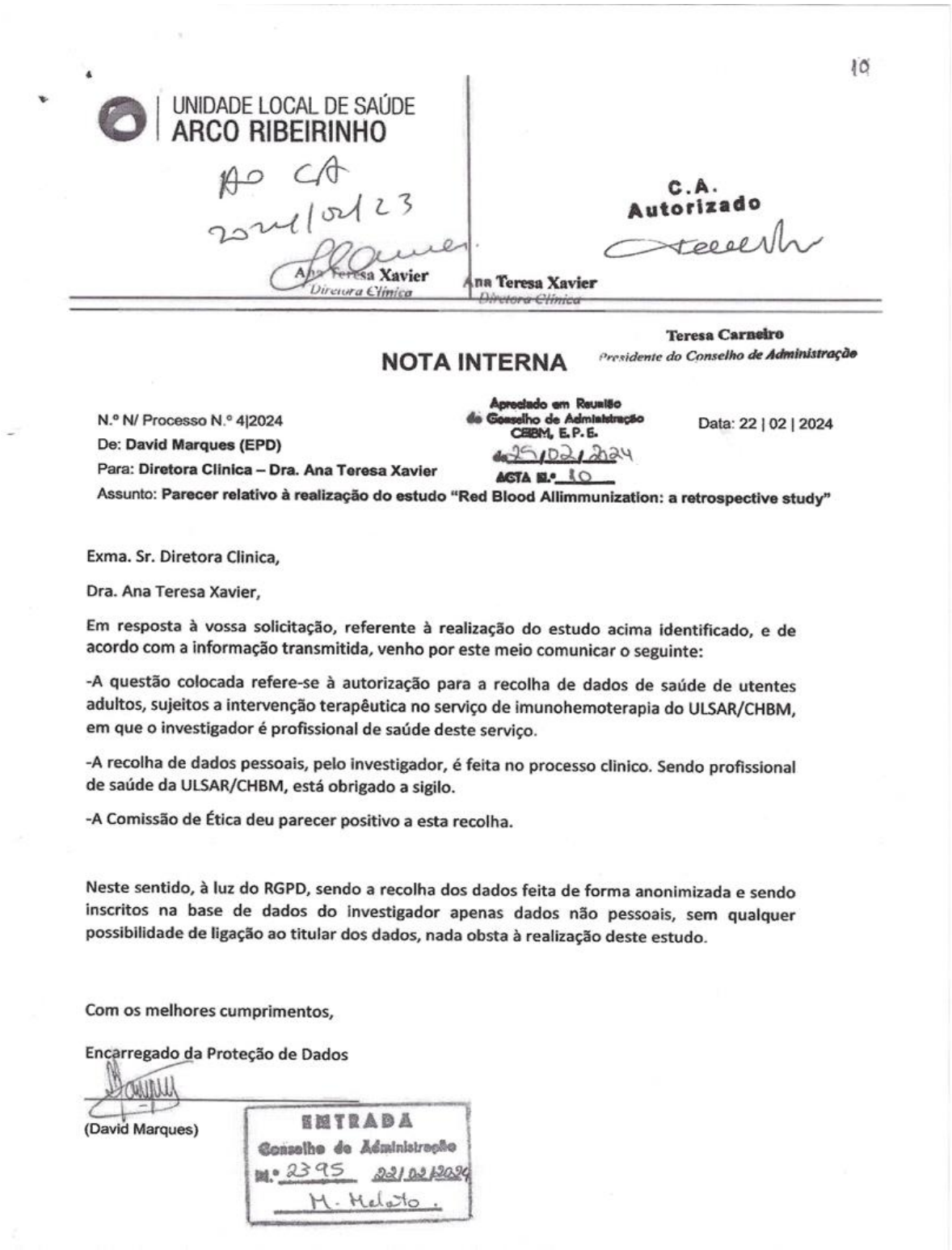
# Annexes

## Annex A. Resident Population

**Table AA1 –** Resident Population (2011 and 2021 Censuses), by Municipality of the ULSAR, by Sex and Respective Percentage Variation. Source: INE, 20

| | Men | | | Women | | | Total | | |
|---|---|---|---|---|---|---|---|---|---|
| | **2011** | **2021** | **Var. (%)** | **2011** | **2021** | **Var. (%)** | **2011** | **2021** | **Var. (%)** |
| **Alcochete** | 8,494 | 9,204 | +8,4% | 9,075 | 9,941 | +9,5% | 17,569 | 19,145 | +9,0% |
| **Barreiro** | 37,347 | 36,708 | -1,7% | 41,417 | 41,651 | +0,6% | 78,764 | 78,359 | -0,5% |
| **Moita** | 31,708 | 31,353 | -1,1% | 34,321 | 34,909 | +1,7% | 66,029 | 66,262 | +0,4% |
| **Montijo** | 24,723 | 27,057 | +9,4% | 26,499 | 28,632 | +8,0% | 51,222 | 55,689 | +8,7% |
| **Total** | **102,272** | **104,322** | **+2,0%** | **111,312** | **115,133** | **+3,4%** | **213,584** | **219,455** | **+2,7%** |

## Annex B. Authorization to Conduct the Study by the ULSAR Board of Directors,



10

UNIDADE LOCAL DE SAÚDE
ARCO RIBEIRINHO

Ao CA
22/02/23
Ana Teresa Xavier
Diretora Clínica

C.A.
Autorizado
Ana Teresa Xavier
Diretora Clínica

Teresa Carneiro
Presidente do Conselho de Administração

**NOTA INTERNA**

Apreciado em Reunião do Conselho de Administração CHBM, E.P.E. de 25/02/2024 ACTA N.º 10

N.º N/ Processo N.º 4|2024
De: **David Marques (EPD)**
Para: **Diretora Clínica – Dra. Ana Teresa Xavier**
Data: 22 | 02 | 2024
Assunto: **Parecer relativo à realização do estudo "Red Blood Allimmunization: a retrospective study"**

Exma. Sr. Diretora Clinica,

Dra. Ana Teresa Xavier,

Em resposta à vossa solicitação, referente à realização do estudo acima identificado, e de acordo com a informação transmitida, venho por este meio comunicar o seguinte:

-A questão colocada refere-se à autorização para a recolha de dados de saúde de utentes adultos, sujeitos a intervenção terapêutica no serviço de imunohemoterapia do ULSAR/CHBM, em que o investigador é profissional de saúde deste serviço.

-A recolha de dados pessoais, pelo investigador, é feita no processo clinico. Sendo profissional de saúde da ULSAR/CHBM, está obrigado a sigilo.

-A Comissão de Ética deu parecer positivo a esta recolha.

Neste sentido, à luz do RGPD, sendo a recolha dos dados feita de forma anonimizada e sendo inscritos na base de dados do investigador apenas dados não pessoais, sem qualquer possibilidade de ligação ao titular dos dados, nada obsta à realização deste estudo.

Com os melhores cumprimentos,

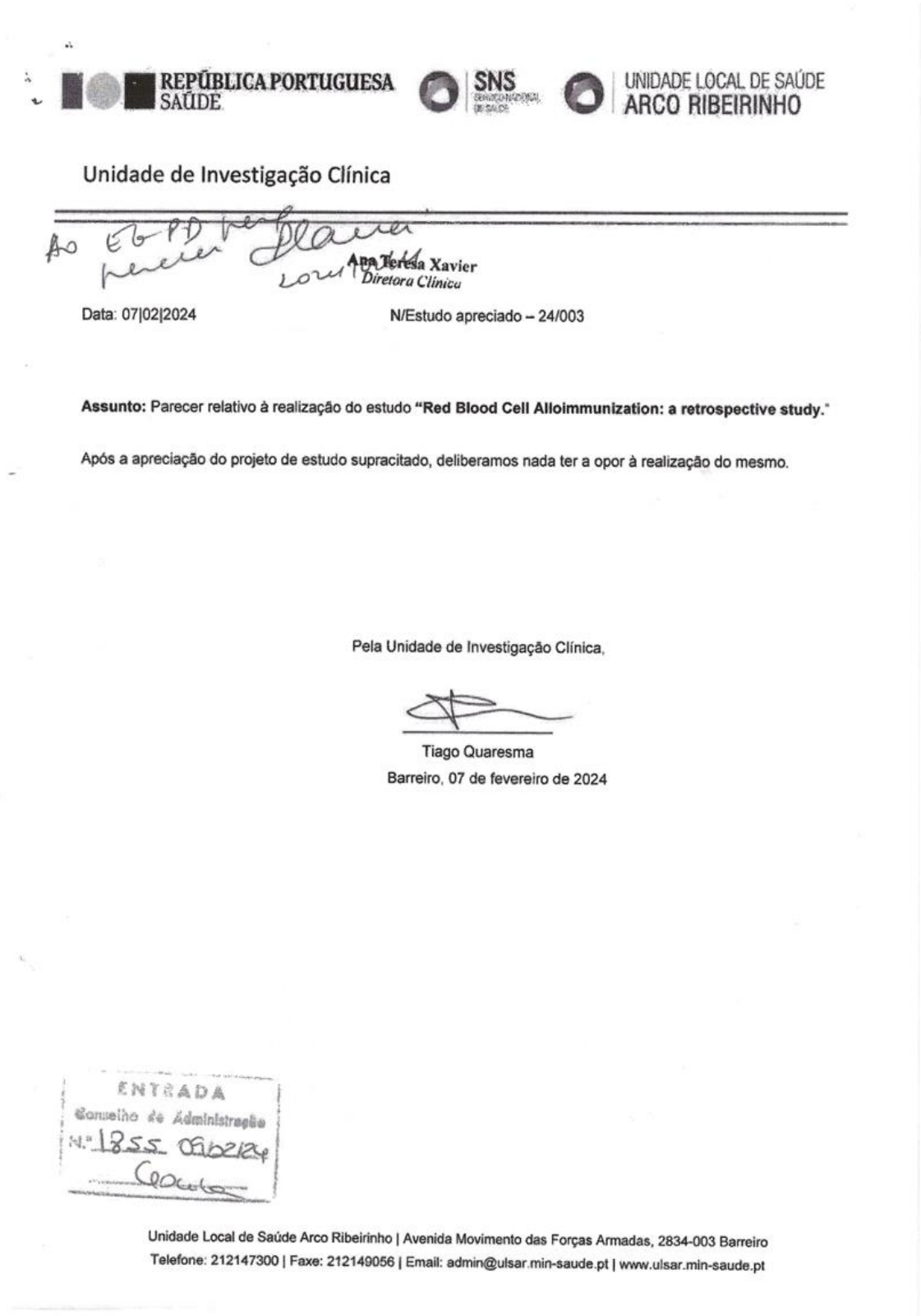
Encarregado da Proteção de Dados

(David Marques)

ENTRADA
Conselho de Administração
N.º 2395 22/02/2024
M. Meloto

**Figure BB1 -** Authorization to Conduct the Study by the ULSAR Board of Directors, following a favorable opinion from the ULSAR Ethics Committee.

## Annex C. Authorization to Conduct the Study Granted by the ULSAR Clinical Research Unit



REPÚBLICA PORTUGUESA
SAÚDE

SNS

UNIDADE LOCAL DE SAÚDE
ARCO RIBEIRINHO

Unidade de Investigação Clínica

Ao EC PD para parecer

Ana Teresa Xavier
Diretora Clínica

Data: 07|02|2024

N/Estudo apreciado – 24/003

**Assunto:** Parecer relativo à realização do estudo **"Red Blood Cell Alloimmunization: a retrospective study."**

Após a apreciação do projeto de estudo supracitado, deliberamos nada ter a opor à realização do mesmo.

Pela Unidade de Investigação Clínica,

Tiago Quaresma

Barreiro, 07 de fevereiro de 2024

ENTRADA
Conselho de Administração
N.º 1855 07/02/24

Unidade Local de Saúde Arco Ribeirinho | Avenida Movimento das Forças Armadas, 2834-003 Barreiro
Telefone: 212147300 | Faxe: 212149056 | Email: admin@ulsar.min-saude.pt | www.ulsar.min-saude.pt

**Figure CC1 –** Authorization to Conduct the Study Granted by the ULSAR Clinical Research Unit

## Annex D. Authorization from the ESSL Ethics Committee.

**REFERÊNCIA INTERNA DO PROJETO: CE-ESTeSL-Nº.85-2023** – Filipe Candeias
**TÍTULO DO PROJETO**: Red Blood Cell Alloimmunization: a retrospective study
**TIPO de Projeto/Estudo:** Dissertação de Mestrado (Tecnologias Clinico-Laboratoriais)
**ORIENTADOR:** Professora Doutora Ana Ramos
**EQUIPA:** Orientador Prof.ª Dra. Edna Ribeiro; Coorientador Dr. António Robalo Nunes;
**INSTITUIÇÃO PROMOTORA:** Escola Superior de Tecnologia da Saúde de Lisboa, do Instituto Politécnico de Lisboa
**INSTITUIÇÃO(ÕES) ENVOLVIDAS**: Centro Hospitalar Barreiro-Montijo
**SUBMISSÃO do PROJETO:** 12 de março de 2024

Exmo. Sr. Professor Doutor António Robalo Antunes
Exma. Senhora Professora Doutora Edna Ribeiro
Exmo. Sr. Dr. Filipe Candeias, estudante de mestrado

A Comissão de Ética da ESTeSL (CE-ESTeSL) aprovou por unanimidade a emissão de parecer favorável suportado no parecer favorável da CES e da Administração do Hospital onde se realizará o estudo. No entanto, a CE-ESTeSL em conformidade com as boas práticas, recomenda que o responsável pela anonimização dos dados seja um elemento externo à equipa de investigação.

O presente parecer tem em consideração a versão submetida do projeto e demais documentação enviada. Eventuais alterações nestes documentos determinam a necessidade de revisão do presente parecer.

Lembramos que todos os estudos que envolvem a autorização dos participantes e a recolha de amostras e dados anonimizados e/ou codificados têm de cumprir com o estabelecido no Regulamento Geral sobre a Proteção de Dados de 27 de abril de 2016.

Por último, solicita-se que, ao abrigo do artº 19 da Lei 21/2014 de 16 de abril e do disposto no nº 23 da atual versão da Declaração de Helsínquia, seja dado conhecimento à CE-ESTeSL do relatório final, com as conclusões do estudo, bem como de eventuais alterações ao protocolo de investigação e demais informações tidas por relevantes.

Aproveitamos ainda para desejar o maior sucesso no desenvolvimento deste trabalho.

Com os melhores cumprimentos,

Rute Borrego

Rute Borrego | Professora Adjunta
Presidente da Comissão de Ética

Av. D. João II, lote 4.69.01- Parque das Nações
1990-096 Lisboa | Portugal
conselhodeetica@estesl.ipl.pt
+351 218980488 (ext. 674)

**Figure DD1 –** Authorization from the ESTeSL Ethics Committee.