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# Red Blood Cells

## Functions and Significance

*Edited by Vani Rajashekaraiah*

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# Meet the editor

Dr. Vani Rajashekaraiah is a Professor at the Department of Biotechnology & Genetics at JAIN (Deemed-to-be University) in Bengaluru, India. She has 18 years of teaching proficiency in Molecular Biology, Genetics and Genetic Engineering and 22 years of research experience in Hematology and Oxidative Stress Biology. She has a doctoral degree in Zoology from Bangalore University, Bengaluru. Her current research focuses on blood banking solutions and drug-induced thrombocytopenia. Her research achievements include 5 funded research projects, 48 publications in reputed journals with 634 citations, 37 conference presentations and one patent grant in India in 2023. She received a CSIR research fellowship in 2002, Seed Money to Young Scientist for Research in 2013 from the Government of Karnataka, and the Jain University Achiever Award in 2015 and 2020 for Quality Research.

# Contents

# Preface

Red blood cells have evolved progressively towards specific functions with their unique structural components. These cells are very efficient, and each stage, i.e. differentiation, maturation and senescence, is constantly regulated.

This book delves into the aspects of the differentiation of red blood cells, their structure, and their functions, which can be used as reliable biomarkers in different clinical conditions.

The first section gives an overview of the blood systems, the development of the red cells, their different stages, and their unique membrane structure with specialized proteins, making them highly specialized effective cells.

The second section gives insights into various red cell indices with reference to landmark studies and reticulocytes and their clinical significance as diagnostic markers in various situations. The final chapter introduces the influential factors of alloimmunization and its significance.

Thus, this book attempts to introduce the readers to the less explored facets of red blood cells in terms of their functions and clinical significance.

I am grateful to IntechOpen for the invitation and the opportunity to be the academic editor of this book on red blood cells. I thank Publishing Process Managers Dominik Samardzija, Dorian Salatic, and their entire team for their complete support of this publication. I also acknowledge the contributions of all the authors of the book chapters and the reviewer of my book chapter.

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Section 1

# Functional Aspects

Chapter 1

# Introductory Chapter: Red Blood Cell – A Highly Specialized Cell

*Vani Rajashekaraiah*

## 1. History

Jan Swammerdam, a Dutch biologist, first described red blood cells (RBCs) in 1668, and later, Antonie van Leeuwenhoek published the unique features of human red blood cells in 1675. George Gulliver published the primary features of red cell membranes in 1862, and Gorter and Grendel provided the first insights into the structure of the membrane in 1925 [1].

## 2. Differentiated cell

Red blood cells/erythrocytes are unique among mammalian cells. They have evolved as a highly differentiated cell to deliver oxygen throughout the circulation. Mammals are the only vertebrates with enucleated erythrocytes under homeostatic conditions. Mature RBCs lack mitochondria and, therefore, are dependent on simple glycolysis through the Embden–Meyerhof pathway for energy production. The deletion of organelles in mature red blood cells has led to increased surface-area-to-volume ratio, facilitating efficient gas exchange, as well as cellular hemoglobin (Hb) capacity and the ability to traverse fine capillaries [2].

The erythrocyte membrane and hemoglobin are pivotal components of RBCs in terms of structure and functions. Hb is made of four subunits with a heme group, where oxygen binds reversibly to the iron atom of each heme group so as to be transported to all tissues.

The specialized cytoskeleton of the red cell membrane enables it to undergo large reversible deformations while maintaining its structural integrity. The extensive protein–protein interactions in the cytoskeleton with transmembrane channels link the cytoskeleton and the membrane, maintaining the biconcave shape and reversibly deformability. The membrane is composed of cholesterol and phospholipids anchored to a skeletal protein network made of spectrin, actin, protein 4.1R, adducin, dematin, tropomyosin, and tropomodulin. It accounts for all of its diverse antigenic, transport, and mechanical characteristics. The primary regulators of this high elasticity are (i) the geometry of the cell, specifically cell surface-area-to-volume ratio; (ii) the cytoplasmic viscosity determined by hemoglobin; and (iii) membrane deformability [3].

## 3. Life cycle

Human red blood cells have a lifespan of 120 days, later they are cleared by macrophages in the spleen and liver [4]. Normally 1% of RBCs are synthesized each day but their production can increase during acute or chronic stress, such as trauma or hemolysis. Erythropoiesis starts with the pluripotent stem cells of the bone marrow. These stem cells proliferate and differentiate into progenitor-committed cells and further to precursors and finally mature RBCs. During terminal erythropoiesis, the nucleus and other organelles are extruded and the enucleated reticulocytes enter the bloodstream and complete the maturation process through many stages, such as chromatin condensation, budding, and mitophagy [5]. Loss of the erythroid nucleus facilitated the evolution of mammalian endothermy towards further specialization for efficient gas exchange. Erythropoiesis is regulated at each stage through cytokines, transcription factors, and modifications of histones and microRNAs [6].

The normoxic and hypoxic cycling of RBC exposes it to oxidative insults that result in continuous biochemical, physical, and immunological changes. These include lipid and protein oxidation, with hemoglobin denaturation arising from oxidative damage leading to inactive hemoglobin aggregates.

The age-related changes include dehydration with increased density and membrane Immunoglobulin G, cell shrinkage, loss of membrane phospholipid asymmetry with phosphatidylserine exposure reduction in sialic acid, cholesterol, phospholipids, and microvesiculation. This eventually triggers removal from the circulation system through the mononuclear phagocytic cells primarily in the spleen, and also in the liver and lymph nodes [7, 8].

## 4. Functions

Red cells influence blood flow through blood viscosity and hemostasis. Although plasma exhibits Newtonian fluid mechanics, whole blood is considered a non-Newtonian fluid due, to RBCs, as they are responsible for nearly 50% of blood viscosity. The ability to deform rapidly in response to fluid shear stresses is governed by cytoplasmic viscosity, determined by hemoglobin concentration [8].

The membrane proteins exhibit diverse functions as transport proteins, adhesion proteins, and signaling receptors. Membrane proteins with transport function include band 3 (anion transporter), aquaporin 1 (water transporter), Glut1 (glucose transporter), sodium, calcium and potassium, and chloride ion channels [9].

Assumed to be inert oxygen carriers for ages, red blood cells are emerging as important modulators of the innate immune response. Evidence suggests that these cells have a direct role in the innate immune system [5–7] and inflammation. Erythrocytes bind and scavenge chemokines, nucleic acids, and pathogens in circulation [10, 11].

They play a significant role in systemic redox regulation as they contain complex redox systems for the preservation of cellular integrity, cellular metabolism, and cellular shape and flexibility.

RBCs possess robust antioxidant systems, both enzymatic and nonenzymatic, which can neutralize these reactive species. The key function of the antioxidant system is to keep hemoglobin in a reduced form, thereby preserving its ability to bind oxygen. These systems also reduce Reactive oxygen species (ROS).

generation and protect the cellular membrane lipids, proteins, channels, and metabolic enzymes from oxidative stress [12].

RBCs can reflect the homeostasis of the organism due to their constant movement through circulatory networks and interactions with all tissues. Therefore, they can be employed as biomarkers under various disease conditions and act as targets for therapeutic studies.

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Chapter 2

# Band 3 Protein: A Critical Component of Erythrocyte

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## Abstract

The erythrocyte membrane plays an important role in maintaining the structure, biological transport, and homeostasis of erythrocytes. The membrane consists of various unique proteins that serve specific functions. Band 3, an integral membrane protein of erythrocytes, constitutes about one-third of the membrane proteins. The amino-terminal region, positioned on the cytoplasmic side, comprises binding sites for hemoglobin, glycolytic enzymes, and ankyrin. Band 3 plays a crucial role in maintaining the structural integrity by connecting the lipid bilayer to the underlying cytoskeletal network. It has a versatile role in cellular dynamics, intracellular trafficking, cellular aging, gas exchange, cellular adhesion, and erythropoiesis. Oxidative modifications in band 3 can be detrimental to membrane structure, compromising its integrity, functionality, and cellular interactions. The intricate chemistry between band 3 and various cellular components unravels its significance in erythrocyte physiology and aging. Therefore, it can be employed as a potential molecular target for therapeutic interventions.

**Keywords:** erythrocytes, band 3, cell membrane, oxidative stress, anion exchange, AE1

## 1. Introduction

Erythrocytes constitute a significant portion of blood and have a distinctive role due to oxygen transport. These cells also facilitate the transport of carbon dioxide and thereby regulate blood pH [1]. Erythrocytes contain hemoglobin, which efficiently carries oxygen due to the affinity of heme group for oxygen. Hemoglobin undergoes breakdown on aging, resulting in the separation of iron and globin, which can be later recycled for the synthesis of new hemoglobin. This ensures continual regeneration and maintenance of oxygen-carrying function [2].

## 2. Erythrocyte membrane structure and function

Erythrocyte membrane is responsible for the antigenic transport and mechanical properties. It plays an important role in the intricate dynamics of erythrocyte biology. The membrane consists of two domains: lipid bilayer and cytoskeleton. The lipid bilayer consists of hydrophilic peripheral proteins internally, hydrophobic integral proteins in the middle (mainly band 3 and glycophorins), and external hydrophilic proteins. The cytoskeleton comprises specific peripheral proteins, including spectrin, ankyrin, actin, and Band 4.1R, 4.2 [1, 3].

Spectrin serves as the primary membrane protein in erythrocytes and exhibits self-associative properties. It forms a lattice structure in conjunction with other membrane proteins and actin, creating a supportive network on the inner aspect of the lipid bilayer. This lattice imparts unique properties of strength and elasticity to erythrocytes [4].

Ankyrin plays a crucial role in connecting the spectrin-actin cortical cytoskeleton with the cytoplasmic domains of integral membrane proteins. It forms a bridge between adhesion molecules and ion channels and integrates the structural and functional components of the erythrocyte membrane.

Glycophorins, also known as sialoglycoproteins, constitute approximately 2% of the total membrane protein in erythrocytes. These proteins are situated at the actin junctional complexes, acting as anchors that connect the cytoskeleton to the lipid bilayer. The amino terminals of glycophorins serve as the binding sites of antigens of ABO and MN blood groups. The inner carboxyl terminal faces the cytoplasm and interacts with the cytoskeleton [5].

### 2.1 Band 3 structure

Band 3 is an erythrocyte membrane protein with a molecular mass of 95 kDa, constituting about one-third of the membrane proteins. Band 3 is exposed on both membrane faces with a relatively low carbohydrate content (8% by weight). The amino-terminal region (41 kDa), positioned on the cytoplasmic side, contains a highly extended structure with binding sites for hemoglobin, glycolytic enzymes, and ankyrin [6].

Each unit of band 3 contains a single site for numerous stilbene disulfonate derivatives, which are potent inhibitors of anion transport. The membrane and cytoplasmic domains of adjacent subunits interact, forming band 3 dimers when the cytoplasmic domains are oxidatively cross-linked with cuprous ion and o-phenanthroline [7]. The cytoplasmic domain of band 3 differs among different species [8].

The carboxy terminus (52 kDa) is primarily composed of alpha-helical structures and arranged cylindrically, with multiple positively charged amino acids. These charges influence ion distribution across the membrane, repelling cations and attracting anions, thereby enhancing anion transport in both directions.

Various types of physical and biochemical evidence strongly suggest that band 3 exists as either a dimer or a tetramer within the membrane. Band 3 undergoes a monomer-dimer-tetramer equilibrium when isolated in non-ionic detergents. The predominant form of the protein is a dimer under normal conditions, with the possibility of tetramer formation [9].

### 2.2 Band 3 functions

Band 3 protein regulates various functions and plays a crucial role in maintaining erythrocyte stability and functional regulation. The band 3 region of the

electrophoretic profile is recognized as anion exchanger 1 (AE1). Its functions include anion exchange-assisted oxygen transport, maintenance of structural integrity, regulating erythrocyte properties, formation of Wright (Wr) blood group antigen, and senescence. These functions may be influenced by its interactions with lipids or lipid domains in the plasma membrane [10].

### *2.2.1 Anion exchange*

Erythrocytes are indispensable players in the exchange of gases within our bodies. The metabolically active cells can be distinguished from inactive ones by a combination of three mechanisms: the oxy/deoxy conversion of hemoglobin, carbonic anhydrase reaction, and the chloride shift.

Chloride shift, an anion exchange mechanism, crucial for delivering oxygen efficiently, is a result of synergy between hemoglobin, carbonic anhydrase, and the band 3 protein [11]. Carbon dioxide ($CO_2$) produced in peripheral cells diffuses into the erythrocytes as they traverse through the capillaries. Carbonic anhydrase converts the diffused $CO_2$ into bicarbonate ($HCO_3$), triggering the chloride-bicarbonate exchange by the band 3 protein [12].

Anion exchange activity results in the conversion of weaker carbonic acid to stronger hydrochloric acid, inducing intracellular acidification. This transient effect facilitates the dissociation of oxygen from oxyhemoglobin ($HbO_2$) in metabolically active cells. The concentration of carbon dioxide and acidity (Bohr effect) ensures that deoxyhemoglobin (HbH+) accepts protons, preventing further dissociation of oxygen from $HbO_2$ [12].

Amino acid residues, including lysine, arginine, and glutamic acid, are known to be crucial for the anion exchange activity of band 3 protein [13]. Intracellular histidine residue of band 3 protein also participates in anion exchange [14].

### *2.2.2 Cytoskeletal interaction and structural integrity during erythropoiesis*

Band 3 plays a crucial role in maintaining the structural integrity by connecting the lipid bilayer to the underlying cytoskeletal network in the early stages of erythroid differentiation. One of its primary functions is to form essential linkages with ankyrin, a cytoskeletal protein, thereby tethering the membrane to the spectrin-based skeletal network. Protein 4.2 modulates the interaction between band 3 and ankyrin during erythropoiesis [15].

The formation of a ternary junctional complex involving band 3, Rhesus-associated glycoprotein (RhAG), and other membrane proteins with protein 4.1R contributes significantly to membrane cohesion [15]. This complex, comprising β-spectrin and actin in the cytoskeleton, plays a vital role in maintaining the structural stability of the membrane throughout erythropoiesis. Interactions with additional cytoskeletal proteins, such as adducin and dematin, enhance the role of band 3 in connecting the bilayer to the membrane skeleton [16]. This is fundamental in preventing vesiculation and preserving the optimal surface area of the membrane.

### *2.2.3 Regulating glycolysis*

Band 3 is also involved in the regulation of glycolysis. Glycolytic enzymes can bind to the cytoplasmic domain leading to inhibition. This can be reversed by phosphorylation of tyrosine 8 and/or 21 on band 3. It is a unique method of controlling glycolysis as it relies on reversible covalent inhibition. The inhibitory membrane binding site

and the phosphorylation of specific tyrosine residues act as the regulators, influencing the activity of glycolytic enzymes [17].

### 2.2.4 Oxygen transport

Deoxyhemoglobin (deoxyHb) strongly and reversibly binds to band 3. This interaction acts like a molecular switch and can impact various erythrocyte functions based on oxygen levels. As oxygen increases, deoxyHb strengthens its association with band 3 and stabilizes the cell membrane [18, 19]. The flexible and unstructured nature of the amino terminus of band 3 provides an eightfold higher affinity to deoxyHb than to oxyhemoglobin (oxyHb) [20–22].

The association of glycolytic enzymes (GEs) on band 3 overlaps with the deoxyHb-binding site of the protein. DeoxyHb displaces GEs, thereby enhancing glucose consumption by glycolysis [23]. The central cavity being inaccessible upon Hb oxygenation allows the GEs to bind to the protein. This leads to a shift in glucose consumption from the pentose phosphate pathway at higher $O_2$ levels to glycolysis at low $O_2$ levels [23, 24].

### 2.2.5 Wright (Wr) blood group antigen formation

Band 3 plays a crucial role in the formation of the Wright (Wr) blood group antigen. The interaction between band 3 and glycophorin A (GPA) is vital to this process. The negatively charged phospholipids and cholesterol in erythrocyte membrane interact with band 3, creating an annulus around it. GPA interacts with band 3 outside of the Ankyrin complex to form the Wright blood group antigen [25]. This interaction is specific and involves the notable interaction of Glu658 in band 3 with Arg61 in GPA.

The GPA/band 3 complex, constituting the Wright blood group antigen, promotes the clustering of band 3 within the erythrocyte membranes. This complex formation is integral to the expression of the Wr blood group antigen in mature erythrocytes. Specific mutations, such as the Glu658Lys mutation, have been associated with the creation of the Wrb antigen [26].

### 2.2.6 Senescence

Senescence in erythrocytes includes biochemical, physical, conformational, and structural alterations mediated by oxidative and glycation events. Band 3 is critical to this phenomenon.

Tyrosine phosphorylation of band 3 by tyrosine kinase $p72^{syk}$ leads to cell dehydration and $K^+$ efflux through the Gardos channel [27]. This is induced by $Ca^{2+}$ (the Gardos effect) causing cell shrinkage leading to erythrocyte senescence [28].

In senescent erythrocytes, the breakdown of hemoglobin molecules results in the formation of hemichromes. This induces the clustering of nearby band 3 molecules on the cell surface and exposes concealed antigenic peptides (neo-antigens) on its surface [29–31]. The exposed antigen facilitates the recognition and targeting of senescent erythrocytes for their reticuloendothelial removal while hindering their endothelial adhesion. Fc receptor-dependent phagocytosis is a mechanism responsible for removing erythrocytes. These autoantibodies target a 62 kDa band 3 fragment found on senescent cells, emphasizing the importance of band 3 in the removal of senescent erythrocytes [32].

## 3. Oxidative stress

Oxidative stress (OS) is a physiological condition that occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the body's ability to detoxify or repair the damage caused by these highly reactive molecules. ROS, which includes free radicals like superoxide anion, hydroxyl radical, and non-radical species such as hydrogen peroxide, are natural byproducts of cellular metabolism. These are highly reactive and capable of causing damage to cellular structures [33].

Erythrocytes possess an efficient endogenous antioxidant system, consisting of superoxide dismutase, catalase, glutathione, peroxidase, peroxiredoxin-2, and glutathione [34]. Oxidative damage has been demonstrated to decrease erythrocyte survival and their rheological properties affecting their homeostasis [35, 36].

Band 3 protein can act as a marker of OS for the identification of specific oxidative modifications, such as cysteine oxidation. This is closely linked to band 3 protein, rendering it vulnerable to redox reactions [14, 37].

Band 3 modifications can impact the structure and function of erythrocytes.

Altered functionality: Oxidative modifications on band 3 can affect the anion exchange and transport capabilities of the membrane [37]. Elevated levels of ROS have been associated with OS and alterations in antioxidant defenses [38]. Oxidative reactions also induce Caspase-3 activation leading to the partial degradation of band 3 [39, 40].

Membrane integrity: Oxidative stress-induced modifications in band 3 may compromise the stability of the membrane. Erythrocytes activate tyrosine kinases during OS, causing tyrosine phosphorylation in the cytoplasmic domain of the band 3 protein. This phosphorylation mediates interactions with ankyrin, leading to membrane destabilization [41, 42].

Cell signaling: Band 3 is involved in cell signaling and interactions with other proteins. Oxidative stress-induced changes in band 3 could influence cell signaling pathways, leading to cellular responses and adaptations [37].

Cytoskeleton interaction: Band 3 interacts with the cytoskeleton, contributing to membrane stability. Oxidative modifications might interfere with these interactions, affecting the overall structure and shape of the cell [37]. Caspase-3 activation cleaves the cytoplasmic end of band 3, which disrupts its interactions with cytosolic proteins and interferes with its linkage to ankyrin [39]. These disruptions contribute to phosphatidylserine (PS) exposure, emphasizing the impact of OS, caspase-3 activation, band 3 alterations in erythrocyte deformability [39]. The intricate interplay of these molecular events underscores the multifaceted role of OS in shaping the biomechanical properties and physiological fate of erythrocytes.

Transport disruption: Band 3 plays a role in transporting ions across the membrane. Oxidant molecules circulating in the bloodstream exert their effects on the plasma membrane. They potentially impact the integrity of band 3 protein and, consequently, the transport systems [35, 36].

Cellular aging: Oxidation of band 3 has implications in the aging of erythrocytes. It contributes to the exposure of senescent-specific neo-antigens, which subsequently bind autologous immunoglobulin G (IgG), triggering the removal of erythrocytes from circulation. Moreover, the binding of IgG has been associated with the formation of band 3 clusters, initiated by the interaction of denatured oxidized hemoglobin (hemichromes) with band 3 [43–45].

## 4. Studies on band 3 protein

Studies focusing on the band 3 membrane protein in erythrocytes have been conducted, revealing valuable insights into its essential functions.

### 4.1 Enzymatic degradation of band 3

Enzymatic modifications of band 3 have provided interesting insights into its susceptibility to degradation. One specific enzyme, neutral proteases from erythrocyte membranes influenced by calcium ions, has a role in cleaving band 3 [46].

### 4.2 Role in gas exchange

Membrane proteins of band 3 anion exchanger (AE1) in erythrocytes in both mice and humans demonstrated the association of Rh-associated glycoprotein (RhAG) and Rh (a key part of gas channel) with band 3. Band 3 serves as the core of a macro complex comprising integral and peripheral membrane proteins. This macro complex plays a coordinated role as an integrated $CO_2/O_2$ gas exchange unit within the erythrocyte [47].

### 4.3 Implications in diseases

Band 3 has been identified as a major player in enhancing adhesion to endothelial cells in malaria-infected and sickle erythrocytes. Synthetic peptides derived from distinct regions of band 3, particularly from its outer parts, have demonstrated the ability to prevent abnormal adherence of sickle cells to endothelial cells. They show the significance of band 3 in mediating cellular interactions and provide potential therapeutic targets for conditions characterized by altered cell adhesion [48].

Band 3 protein plays a crucial role in erythrocyte homeostasis, particularly under hyperglycemic conditions associated with diabetes. The higher anion exchange capability in erythrocytes under hyperglycemia emphasizes band 3 protein's sensitivity to glycated Hb levels and its impact on erythrocyte function [49].

### 4.4 Intracellular trafficking

The interaction between band 3 and Glycophorin A (GPA) was studied using transgenic mice producing human GPA. These form a close network in membrane stability and play a significant role in intracellular trafficking [50].

### 4.5 Pulmonary gas exchange

Contributions of band 3 to pulmonary gas exchange were studied in the canine models. Inhibition of Carbonic Anhydrase (CA) leads to a significant decrease in both $CO_2$ and $O_2$ showing a significant role of band 3 and carbonic anhydrase in pulmonary gas exchange. Erythrocyte membrane band 3 protein contributes to CA-catalyzed processes in pulmonary gas exchange [51].

The functions of band 3 are depicted in **Figure 1**.

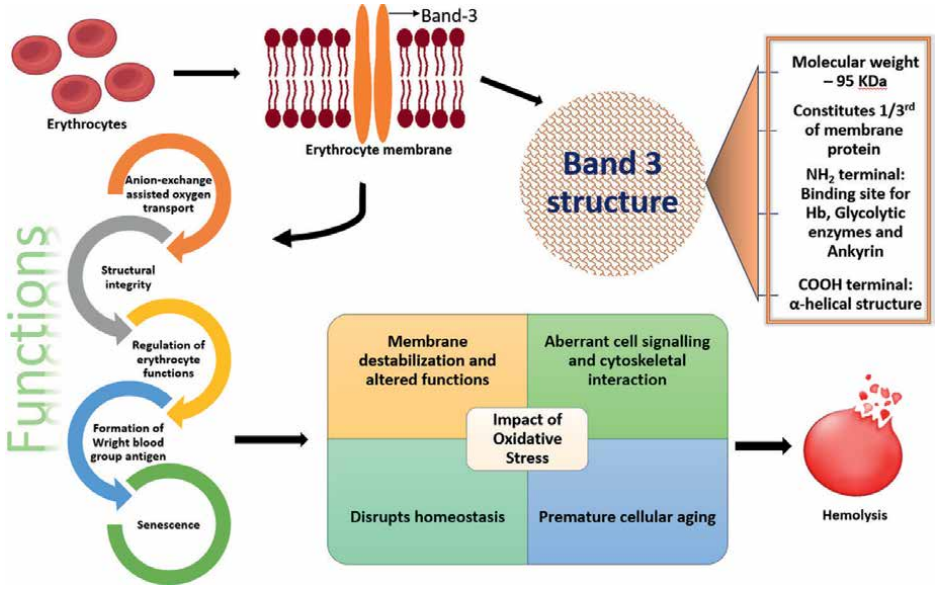


**Figure 1.**
*Comprehensive view of band 3 structure and functions.*

## 5. Conclusion

Band 3 plays a versatile role in cellular dynamics, intracellular trafficking, and cellular aging, maintaining structural integrity, gas exchange, and cellular adhesion. Oxidative modifications to band 3 can have profound effects on membrane structure, compromising its integrity, functionality, and interactions with other cellular components. It serves a vital role in erythropoiesis. The intricate interplay between band 3 and various cellular components unravels its significance in erythrocyte physiology and aging. Band 3 has an integral role in maintaining erythrocyte homeostasis and cell integrity. Therefore, it can be employed as a potential molecular target for therapeutic interventions.

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## Conflict of interest

The authors declare no conflict of interest.

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Chapter 3

# Erythrocyte Blood Systems

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## Abstract

Red blood cells are made up of many structures of carbohydrate, protein, or lipid origin that give rise to erythrocyte antigens that define blood systems. The importance of these antigens lies in the fact that there are pathophysiological mechanisms in blood transfusions, pregnancies, and transplants that generate incompatibilities between donor and recipient due to the erythrocyte antibodies resulting from these responses. The above has been the basis for the formation of the 45 blood systems that we currently know. Although the best known are the ABO and Rh blood systems, responsible for immediate hemolytic transfusion reactions and hemolytic disease of the fetus and newborn, respectively. Other blood systems, such as Kell, Duffy, Kidd, MNs, and Diego, are also clinically significant and important in transfusion medicine. Therefore, all the genetic and biochemical complexity that defines the characteristics of antigens and antibodies, their frequencies in certain populations, and the association with infectious and non-infectious pathologies, are lines of research of interest that try to relate the disease with the belonging of some blood phenotype.

**Keywords:** blood systems, red blood cells, antigens, antibodies, transfusion medicine

## 1. Introduction

Blood group systems are groups of one or more antigens (proteins and oligosaccharides) expressed on the surface of red blood cells and other tissues. They are under the control of a single gene locus or two or more homologous genes very closely linked and with little or no recombination between them [1–3]. Antigenic configurations give way to phenotypes and the generation of antibodies that react to antigenic stimuli that do not exist in an individual [1].

Among the 45 blood systems described [2], there are those that historically have the greatest clinical importance, due to being associated with mild to severe hemolytic transfusion reactions (HTR), mild to severe hemolytic disease of the fetus and newborn (HDFN), and transplant-associated reactions. The ABO and Rh blood systems are the most important, followed by the Kell, Duffy, Kidd, and MNS systems, while the rest of the blood systems to a lesser extent are also associated with some of these clinical conditions [3]. **Table 1** shows the most representative blood systems and their association with clinical conditions.

This chapter, apart from addressing the essential characteristics of the main blood systems, seeks to establish the role played by the molecules and antigens responsible for designating the antigens of blood groups, associating their biological function and

| Blood system | Major antigens | HTR (Mi-Mo-S) | HDFN (Mi-Mo-S) | Transplant | Relationship with clinical conditions |
|---|---|---|---|---|---|
| ABO | A | Yes (Mi/S) | Yes (Mi/Mo) | Yes (solid organ, HSC) | Clearance of von Willebrand factor (venous thromboembolism). SARS CoV2 infection. |
| | B | Yes (Mi/S) | Yes (Mi/Mo) | | |
| MNS | M | Yes. Rare | Yes. Rare | No | Receptor for complement, bacteria, and viruses. Resistance to *Plasmodium falciparum*. Autoanti-N in dialyzed patients. |
| | N | Yes. Rare | Yes. Rare | | |
| | S | Yes (Mi/Mo) | Yes (Mi/S) | | |
| | S | Yes (Mi) | Yes (Mi/S) | | |
| | U | Yes (Mi/S) | Yes (Mi/S) | | |
| P1Pk | P1 | Yes (Mi/Mo) | No | No | Shiga toxin adhesion. Ovarian cancer, HIV infection. Early miscarriages (Anti-PP1Pk). |
| | P | Yes | Yes. Rare | | |
| | $P^k$ | Yes (Mi/S) | Yes (Mi/S) | | |
| Rh | D | Yes (Mi/S) | Yes (Mi/S) | Yes (HSC) | Integrity of the erythrocyte membrane. Transport of ammonium and $CO_2$. |
| | C | Yes (Mi/S) | Yes (Mi) | | |
| | E | Yes (Mi/Mo) | Yes (Mi) | | |
| | c | Yes (Mi/S) | Yes (Mi/S) | | |
| | e | Yes (Mi/Mo) | Yes (Mi) | | |
| Kell | K | Yes (Mi/S) | Yes (Mi/S) | No | Bacterial infections. McLeod phenotype and chronic granulomatous disease. |
| | k | Yes (Mi/Mo) | Yes (Mi/S) | | |
| | $Kp^a$ | Yes (Mi/Mo) | Yes (Mi/S) | | |
| | $Kp^b$ | Yes (Mi/Mo) | Yes (Mi/Mo) | | |
| | $Js^a$ | Yes (Mi/Mo) | Yes (Mi/S) | | |
| | $Js^b$ | Yes (Mi/Mo) | Yes (Mi/S) | | |
| Lewis | $Le^a$ | Yes. Rare | No | No | Receptor for *Helicobacter pylori*. Susceptibility to infectious mononucleosis, severe alcoholic cirrhosis, alcoholic pancreatitis, and fucosidosis. |
| | $Le^b$ | No | No | | |
| Duffy | $Fy^a$ | Yes (Mi/S) | Yes (Mi/S) | Yes (Renal, HSC) | Resistance to *Plasmodium vivax* and *Plasmodium knowlesi*. Innate and acquired immunity. Susceptibility to HIV infection. Prostate cancer. |
| | $Fy^b$ | Yes (Mi/S) | Yes (Mi) | | |
| | Fy3 | Yes (Mi/Mo) | Yes (Mi) | | |
| | Fy5 | Yes (Mi) | No | | |
| | Fy6 | | | | |
| Kidd | $Jk^a$ | Yes (Mi/S) | Yes (Mi/Mo) | Yes (Renal, HSC) | Urea transporter. Bladder cancer. Acute rejection in kidney transplantation. |
| | $Jk^b$ | Yes (Mi/S) | Yes (Mi) | | |
| | Jk3 | Yes (Mi/S) | Yes (Mi) | | |
| Diego | $Di^a$ | Yes (Mi/S) | Yes (Mi/S) | No | Integrity of the erythrocyte membrane. Population marker. |
| | $Di^b$ | Yes (Mi/Mo) | Yes (Mi) | | |
| | $Wr^a$ | Yes (Mi/S) | Yes (Mi/S) | | |
| | $Wr^b$ | No | No | | |

*HTR: hemolytic transfusion reaction. HDFN: hemolytic disease of the fetus and newborn. HSC: hematopoietic stem cells. Mi: mild. Mo: moderate. S: severe.*

**Table 1.**
*Blood systems and association with clinical conditions.*

the probable relationship with some pathologies, which are current lines of research to understand something more about the importance of erythrocyte blood systems.

In this sense, the ABO system plays an important role in compatibility between donor-recipient, both in transfusions and organ transplants. The Rh system is crucial in the management and prevention of HDFN. Several blood group systems are associated with the susceptibility of infections of the following pathogens: *Plasmodium vivax*, *Helicobacter pylori*, norovirus, SARS CoV2, and HIV. Other blood systems have been related to immunity and inflammation processes, in clinical conditions such as venous thromboembolism, myocardial infarction, atherosclerosis, and cancers of the breast, pancreas, lung, gastrointestinal, ovarian, bladder, and prostate [1].

## 2. Erythrocyte blood systems

### 2.1 ABO system

The ABO system was discovered by Karl Landsteiner in 1900, it is the most important system in transfusion medicine and transplantation, due to the presence of natural antibodies reactive at 37°C and complement binders. These antibodies can produce very serious intravascular hemolytic reactions when incompatible ABO red blood cells are transfused. ABO antigens are widely distributed in the body, in red blood cells, lymphocytes, platelets, epithelial and endothelial tissues [3–5].

The presence of these natural hemolytic antibodies and the location of the antigens in blood and tissues makes it feasible that the transfusion of ABO-incompatible blood can produce acute intravascular hemolysis with renal failure and potentially be fatal. For its part, the transplantation of ABO-incompatible solid organs can trigger a severe hyperacute reaction of the graft, and the transplantation of ABO-incompatible hematopoietic stem cells can produce acute hemolysis. Due to the clinical consequences associated with ABO incompatibilities, blood classification and ABO compatibility testing remain the basis of pre-transfusion and transplant evaluation [3–5].

By the presence or absence of antigens A and B on the red blood cell membrane, phenotypes A, B, AB, and O are defined. The ABO system is also characterized by the presence or absence of natural antibodies (isohemagglutinins), directed toward absent A and/or B antigens. There is a reciprocal and inverse relationship between the presence of ABO antigens (A and/or B) in red blood cells and the presence of antibodies (anti-A, anti-B, or both) in the serum. For example, the O phenotype does not have A and B antigens on their red blood cells but naturally possesses both anti-A and anti-B. It is hypothesized that the presence of these natural antibodies is due to the stimulation of the intestinal bacterial flora, which possess structures similar to ABO antigens in their lipopolysaccharide coats [3–5].

ABO antigens are not direct gene products, so the ABO gene and its 7 exons encode glycosyltransferases. Glycosyltransferase A catalyzes the addition of UDP-N-acetylgalactosamine to the H antigen, forming the A antigen, while glycosyltransferase B catalyzes the addition of UDP-galactose to the H antigen, forming the B antigen. Therefore, any genetic alteration that modifies the activity or specificity of glycosyltransferases can alter the ABO phenotype. Mutations that completely annul the enzymatic activity give rise to the O phenotype, without forming antigens A or B and preserving the H antigen. If the genetic alteration decreases the activity of the enzyme, it decreases the conversion of H antigen, favoring the formation of weak A or B phenotypes [3–5].

The ABO blood classification is the most commonly performed test in blood banks, where direct (antigens) and reverse (antibodies) testing is performed on all donors and patients [4].

ABO antibody production begins at birth, peaks between 5 and 10 years of age, and declines thereafter. Titers are usually too low to detect until 3–6 months of age. Therefore, most of the antibodies found in the umbilical cord come from maternal serum. For this reason, the serum test should be performed after 6 months of age [3, 4].

On the other hand, older people also have lower levels of anti-A and anti-B; therefore, antibodies may be undetectable in the reverse group [3, 4].

Anti-A (phenotype B) and anti-B (phenotype A) antibodies are predominantly made up of IgM-type antibodies and may contain small amounts of IgG. In contrast, the serum of people with phenotype O contains anti-A, anti-B, and anti-A,B antibodies, predominantly of the IgG type. The anti-A,B antibody reacts with both A and B cells and cannot be separated into anti-A and anti-B [3, 4].

In the context of HDFN, that caused by ABO incompatibility is the most common, but it is also the mildest to moderate form. It frequently occurs in mothers of phenotype O, who have anti-A, anti-B, or anti-A,B type IgG antibodies in their serum, which cross the placenta and attack the red blood cells of their children with phenotypes A or B, favoring complement-mediated hemolysis [3, 4].

In Caucasians, phenotypes O and A are the most common (45 and 40%, respectively), followed by phenotype B (11%) and phenotype AB (4%). The frequency of phenotype B in blacks and Asians is higher (20 and 27%, respectively) than in Caucasians (11%) [6].

Finally, it is known that people with phenotypes A and B have a lower clearance of von Willebrand factor, which leads to a higher risk of venous thromboembolism, in relation to people with phenotype O. On the other hand, people with phenotype O would have a lower risk for SARS CoV2 infection and its associated complications relative to people with phenotype A [1].

### 2.2 Rh system

It is the most important blood system after ABO, being highly immunogenic and complex, with numerous polymorphisms and clinically significant alleles. The discovery of the Rh system is attributed to several groups of researchers whose observations contributed to understanding the pathogenesis of HDFN [3].

Currently, the Rh system is made up of 56 antigens carried in two proteins of 417 amino acids (RhD and RhCE), which are encoded by the RHD and RHCE genes, respectively. The RhD protein expresses the D antigen, while the RhCE protein expresses the C, c, E, and e antigens, which are responsible for most clinically significant antibodies [2, 3].

The terms "RhD positive" and "RhD negative" refer to the presence or absence of D antigen in red blood cells [3, 4].

The D antigen is highly immunogenic, which makes it the most clinically important antigen after the ABO system antigens. It is estimated that 30–85% of RhD-negative people who receive an RhD-positive transfusion produce anti-D antibodies that cause HDFN (in the case of pregnant women), autoimmune hemolytic anemias (AIHA), and transfusion reactions [3–5].

The antigenic diversity and complexity of the Rh system are generated by the arrangement and proximity of the RHD and RHCE genes on the short arm of

chromosome 1, which facilitates the appearance of multiple genetic exchange events that give rise to weak and partial variants of the D antigen. From a clinical point of view, the partial DVI variant is postulated to be the most important in the white population [4].

In 2015, the American Association of Blood Banks (AABB) and the College of American Pathologists (CAP) gathered enough evidence recommending RHD genotyping in pregnant women when a weak D phenotype is detected by routine methods. This measure would optimize the rational use of preventive anti-D immunoglobulin in pregnant women and, on the other hand, would improve the use of RhD-negative blood products in patients who are really at risk of developing anti-D [7, 8].

The Rh complex interacts with band 3, GPA, GPB, LW, and CD47, and is associated with the membrane skeleton of red blood cells *via* ankyrin and protein 4.2. This complex maintains the integrity of the erythrocyte membrane. Rh proteins can transport ammonia and $CO_2$.

RhD incompatibility remains the leading cause of HDFN. In some individuals with Rh-nulled phenotype, compensated hemolytic anemia may occur. In conditions such as leukemia, myeloid metaplasia, myelofibrosis, and polycythemia, reduced expression of Rh antigens may occur [6].

### 2.3 Kell system

The Kell system consists of 38 antigens carried on a 732-amino acid type II transmembrane glycoprotein (also known as CD238). Kell glycoprotein is a zinc-dependent metalloproteinase that has endothelin-converting enzyme-3 activity. A single disulfide bond binds the KEL protein to the membrane of the XK protein that carries the Kx antigen. The absence of XK leads to reduced expression of Kell antigens in the membrane of red blood cells [2].

The most important antigens are K, k, $Kp^a$, $Kp^b$, $Js^a$, and $Js^b$ [3].

Antigen K has a prevalence of approximately 9% in Caucasians, 1.5% in Africans, and rare in Asians. K antigen is highly prevalent in all populations.

Kell system antibodies are responsible for the pathogenesis of HDFN. Anti-K antibodies have the potential to cause severe HDFN, with lower amniotic fluid bilirubin concentrations, hyperbilirubinemia, reticulocytosis, and erythroblastosis, when compared to anti-D-mediated HDFN of similar severity. The lower degree of hemolysis and fetal anemia observed suggests that anti-K acts predominantly by a mechanism of suppression of erythropoiesis. The Kell glycoprotein is expressed in very early phases of the erythroid maturation process, which allows anti-K antibodies to inhibit erythropoiesis and cause aplastic anemia that can overcome the hemolytic component of fetal anemia [3, 4].

Antibodies from the Kell system have been responsible for severe AIHA. The presence of an antibody is often associated with an apparent depression of Kell antigens. Although most anti-K antibodies originate from pregnancy or transfusions, a few cases of apparent non-immune anti-K originating from erythrocyte antigens have been described. In some cases, antibodies were found in healthy men who donated blood and had no history of transfusions [3, 4].

### 2.4 Duffy system

The Duffy system was discovered by Cutbush and Mollison in 1950 and comprises five antigens ($Fy^a$, $Fy^b$, Fy3, Fy5, and Fy6) located on the Duffy antigen receptor for

chemokines (DARC) glycoprotein encoded by the ACKR1 gene (CD234). The ACKR1 gene is present on chromosome 1 [9].

The polymorphism of the Duffy system is due to the presence of the antigens, $Fy^a$ and $Fy^b$, which are the products of the codominant alleles FYA and FYB, respectively. Four phenotypes are known from these antigens, Fy(a + b−), Fy(a + b+), Fy(a−b+), and Fy(a−b−) [3, 4].

The $Fy^a$ and $Fy^b$ antigens are very sensitive to most proteolytic enzymes (papain, ficin, and bromelain), but are not destroyed by trypsin [3, 4].

In Africans, there is the Fy allele, which is more abundant than the $Fy^a$ *and* $Fy^b$ alleles. Individuals homozygous for the Fy allele are of erythrocyte Fy(a−b−) phenotype. The coding region of the Fy allele in Africans is identical to that of the *Fyb* allele, therefore, the Duffy glycoprotein is not produced and the $Fy^b$ antigen is not expressed in red blood cells, due to the presence of a mutation in the promoter region of DARC, which prevents binding to the transcription factor GATA-1, The expression of the gene in red blood cells does not result [4].

Duffy glycoprotein is present in red blood cells, vascular endothelial cells, alveolar epithelial cells, renal collecting tube cells, and Purkinje cells in the brain [9].

In the African population, the Fy(a−b−) phenotype denotes the absence of the Duffy glycoprotein in red blood cells but not in other tissues. This helps explain why these individuals do not develop anti-$Fy^b$ and can develop anti-Fy3 or anti-Fy5 in a very exceptional way. On the other hand, in the Caucasian population, the Fy(a−b−) phenotype is very rare, being associated with inactive mutations in the DARC genes. These individuals do not have Duffy glycoproteins in their red blood cells and are not expected to express them in other tissues. All of them were characterized by the presence of anti-Fy3 in their serum, an antibody that reacts with all red blood cells, except those of the Fy(a−b−) phenotype [3, 4].

Anti-$Fy^a$ is an antibody 20 times more common than anti-$Fy^b$. The predominant IgG subtype is IgG1 and these antibodies are usually detected through a human antiglobulin test. Anti-$Fy^a$ and anti-$Fy^b$ can cause acute or delayed HRT. Although they are generally mild, some have been found to be fatal. Anti-Fy3 has been documented in acute or delayed type HTR, while anti-Fy5 has been involved in late HTR. Both Fya and Fyb are associated with mild to severe HDFN generation [9].

The DARC glycoprotein is a receptor for chemokines and immune system cells related to innate and chronic immunity. It is also the recipient of the malaria parasites *Plasmodium vivax and Plasmodium knowlesi*. Therefore, people with Fy(a−b−) phenotype or point mutations do not bind to chemokines and resist invasion of these parasites [6].

Mutations in the DARC gene have been associated with an increased risk of susceptibility to HIV infection, regulating prostate cancer growth. FY antigens may act as minor histocompatibility antigens in renal allograft rejection [6].

### 2.5 Kidd system

It was discovered in 1951 in the context of the HDFN [6]. The Kidd system comprises three antigens ($Jk^a$, $Jk^b$, Jk3) located on a type 3 membrane glycoprotein, with 10 membrane domains, which functions as primary transporter of urea on red blood cells [2]. The Kidd gene (SLC14A1) contains 11 exons, of which 4–11 encode the mature protein, which is located on chromosome 18q12.3 [6].

The $Jk^a$ and $Jk^b$ antigens are the allele products, represented in the Kidd glycoprotein. They have a similar prevalence in Caucasian and Asian populations, but

$Jk^a$ is more abundant than $Jk^b$ in Africans. Kidd antigens are resistant to proteolytic enzymes such as papain and ficin [3, 4].

The null phenotype, Jk(a−b−), is usually the product of a homozygous silent gene present at the *JK* locus. Although the null phenotype is very rare in most of the population, it is relatively common in Polynesia, with a prevalence of about 1 in 400 individuals, but as high as 1.4% in the inhabitants of the Island of Niue in the South Pacific. Immunized individuals carrying the Jk(a−b−) phenotype can produce an antibody, the anti-Jk3 [3, 4].

Anti-Jka and anti-Jkb antibodies are not common and are generally found in patients who present other erythrocyte antibodies simultaneously. A particular characteristic of these antibodies is that they are usually anamnestic, that is, they may not be detected by conventional serological methods and reappear after a new antigenic stimulus. Due to the above, its detection turns out to be a challenge for laboratory workers, added to the fact that its expression depends on cells that express a double dose of antigen, Jk(a+b−) or Jk(a−b+). Clinically they are associated with acute or delayed HTR. Only in very rare cases can they cause mild HDFN [3, 4].

The urea transporter in red blood cells plays a role in urea concentration by speeding up the transport of urea across the red blood cell membrane as it pass through the descending and ascending straight vessels. Urea crosses the membrane of Jk(a−b−) red blood cells 1000 times slower than normal red blood cells. Although Jk(a−b−) individuals do not exhibit clinical symptoms, their ability to concentrate urine is reduced by approximately one-third [6].

It has been observed that genetic variations of SLC14A1 may be involved in the incidence of bladder cancer [1] Antibodies of the Kidd system are involved in acute rejection of kidney transplantation, suggesting that antigens of the Kidd system may behave as histocompatibility antigens [3, 4].

### 2.6 MNS system

The MNS system is highly complex as it comprises 48 antigens [2]. It was discovered in 1927 by Landsteiner and Levine. As in the Rh system, the complexity is due to recombination between homologous genes. Antigens are localized to glycophorin A (GPA, CD235A), glycophorin B (GPB, CD235B), or hybrids of both glycophorins. GPA forms an association with Band 3 on the erythrocyte membrane, and both GPA and GPB possess the highest amount of sialic acid in the erythrocyte membrane. *GYPA* and *GYPB*: the genes encoding GPA and GPB span seven and five exons, respectively. GPA is limited to the erythrocyte series and is frequently used as an erythroid marker. Both GPA and GPB are considered receptors for complement, bacteria, viruses, and the malaria parasite, *Plasmodium falciparum*. A third gene, GYPE, does not normally encode proteins in the membrane of red blood cells, but the gene is thought to be involved in the cell membrane composition of hybrid proteins [3, 4].

M and N antigens are antithetic and polymorphic antigens present in GPA, while S and S antigens are antithetic and polymorphic antigens present in GPB. The U antigen is also important and is located in the GPB. Another important characteristic to mention is that the M and N antigens of GPA are sensitive to trypsin, while the S, s and U antigens are resistant. In contrast, with treatment of erythrocytes with alphachymotrypsin, the activity of M, N and U is resistant, while the expression of S and s is completely destroyed. M, N, S, and s antigens are altered by treatment of erythrocytes with papain, ficin, bromelain, or pronase. All of these properties are used in the laboratory to establish the specificity of clinically significant antibodies [3–6].

Anti-M is a fairly common antibody that can be produced naturally, while anti-N is quite rare. Most anti-M and anti-N antibodies are not clinically relevant, except when they are active at 37°C. When active anti-M or anti-N antibodies are found at 37°C, the indirect antiglobulin test (IAT) is used in the laboratory. Very occasionally, these antibodies have been associated with HTR or HDFN. Anti-S, anti-s, and anti-U are generally IgG antibodies reactive at 37°C, which have been associated with cases of HTR and are responsible for severe and fatal HDFN [3, 4].

### 2.7 Lewis system

Mourant discovered the Lewis system in 1946. It consists of two main antigens, $Le^a$ and $Le^b$, and three common phenotypes: Le(a+b−), Le(a−b+), and Le(a−b−). Antigens are expressed in red blood cells, platelets, endothelium, kidney, and genitourinary and gastrointestinal epithelia. Lewis antigens are not synthesized by red blood cells but are passively adsorbed into erythrocyte membranes, and can be eluted from red blood cells after transfusion or in response to increased plasma volume of circulating lipoproteins. For example, the Lewis antigen is often decreased in red blood cells during pregnancy, and women are transiently typed as having the Le(a−b−) phenotype [3, 4].

Antibodies against Lewis antigens are generally IgM and natural. They are usually found in the serum of people with the Le(a−b−) phenotype, containing a mixture of anti-$Le^a$, anti-$Le^b$ and anti-$Le^{a+b}$. It is rare to find anti-$Le^a$ in individuals with the Le(a−b+) phenotype and anti-$Le^b$ in the Le(a+b−) phenotype. These antibodies are found quite frequently during pregnancy with the temporary Le(a−b−) phenotype [3, 4].

Most Lewis antibodies are reactive agglutinins in saline medium and at room temperature. Sometimes, Lewis antibodies can be detected in the antiglobulin phase. Very rarely, antibodies can cause hemolysis in vitro. Hemolysis is most commonly seen when fresh serum containing anti-$Le^a$ or anti-$Le^{a+b}$ is studied, and particularly when tested with enzyme-treated red blood cells [3, 4].

Lewis antibodies are not associated with HDFN, as they are predominantly IgM and do not cross the placenta. In addition, Lewis antigens are poorly expressed in the red blood cells of a neonate; most newborns type as Le(a−b−) with human Lewis antibodies [3, 4].

There are several studies that try to explain the association between Lewis antigens and susceptibility to *Helicobacter pylori* infection in patients with alterations to the gastric mucosa [6, 10].

Lewis antigens decrease in expression from red blood cells in the course of infectious mononucleosis, severe alcoholic cirrhosis, alcoholic pancreatitis, and pregnancy. On the other hand, patients with fucosidosis may increase the expression of Lewis antigens in their saliva and red blood cells [6].

### 2.8 P1PK system

Landsteiner and Levine discovered the P1PK system in 1927, via the P-antigen, which is now known as P1. The antigens that are currently part of the system are P1, $P^k$, and NOR, which are not primary gene products and are located on glycolipids. The A4GALT gene encodes 4-α-galactosyltransferase, the enzyme that synthesizes the P1 and $P^k$ antigens. The P1 antigen is found in both glycolipids and glycoproteins, while the $P^k$ antigen is found only in glycolipids. The immediate precursors for system

antigens are paragloboside for P1 antigen, lactosylceramide for $P^k$ antigen, and globoside for NOR antigen [2, 6].

Transcriptional regulation determines the two most prevalent phenotypes (P1 and P2) in the P1PK blood system. The $P^1$ alleles encode the P1 and $P^k$ antigens, while the $P^2$ alleles encode only $P^k$. The NOR allele also encodes P1 and $P^k$. Null alleles encode a non-functional galactosyltransferase, resulting in the p-phenotype (P1-$P^k$-NOR) if inherited on both chromosomes [2, 6].

The presence of the P1 antigen in red blood cells denotes the P1 phenotype, while the absence of the P1 antigen gives rise to the P2 phenotype [3, 4].

Anti-P1 is an antibody commonly found in the serum of P2 individuals (25–66%). Anti-P1 is a naturally occurring IgM antibody and is often detected as a weak agglutinin at room temperature. It is rare to find anti-P1 reagents at 37°C or showing hemolysis in vitro. Since anti-P1 is usually IgM, anti-P1 does not cross the placenta and therefore does not cause HDFN. Anti-P1 titers are often high in patients with hydatidosis, fascioliasis, and poultry breeders [6].

In the laboratory, it can be seen that the expression of anti-P1 varies between individuals and that the reaction intensity decreases during in vitro storage. Thus, anti-P1 may not react with all P1 red blood cells studied. To do this, it is suggested to incubate the sample at low temperatures (4°C) or perform tests with erythrocytes treated with enzymes. Another alternative is to use inhibition tests with hydatid cyst fluid or substance P1 from pigeon eggs, which would be more useful when analyzing samples that contain multiple antibodies [3].

Anti-PP1Pk (historically known as anti-Tja) is a mixture that can be separated into anti-P, anti-P1 and anti-Pk in the serum of individuals of phenotype p. Antibodies are potent hemolysins and are associated with HTR and occasionally with HDFN. There is a link between anti-PP1Pk and early miscarriage [3, 4].

P1 and $P^k$ antigens have been associated with increased susceptibility to disease. For example, the adhesion of Shiga toxin and several pathogens to these antigens has been reported. A possible role of these antigens in patients with ovarian cancer has been studied. The presence of the $P^k$ antigen may play a role in HIV infection [6].

### 2.9 Diego system

The Diego system was discovered by Layrisse in 1955 in the context of HDFN and is made up of 23 antigens located on the Band 3 glycoprotein, an erythrocyte anion exchanger or solute transporter of the 4Al family (SLC4A1). The transmembrane domains of band 3 function as an anion transporter in red blood cells, while the long amino-terminal region of the protein is critical for the maintenance of the shape integrity of red blood cells through their interaction with the cytoskeleton [2]. The Band 3 gene (SLC4A1) consists of 20 sequence-encoding exons located on chromosome 17 [2].

The most relevant antigens are $Di^a$, $Di^b$, $Wr^a$, and $Wr^b$ [4], which are considered population genetic markers [11].

The $Di^a$ antigen has a high prevalence in natives of North and South America, ranging from 2% in Caracas Indians (Venezuela) to 54% in Kainganges Indians (Brazil). It has a prevalence of 5% in the Chinese population and of 12% in Japanese population, and is very rare in European and African populations [6]. The $Di^b$ antigen is highly prevalent in almost all populations [3, 4].

Anti-$Di^a$ and anti-$Di^b$ antibodies are generally of the IgG type (lgG1 and IgG3) and usually require the human antiglobulin test for detection, although some examples

can be detected by direct agglutination. Anti-$Di^a$ has been found in 3.6% of poly-transfused patients in Brazil and there are cases associated with severe HDFN. In rare cases, anti-$Di^b$ has been found to be responsible for severe HDFN. Apart from an example of anti-$Di^a$ as the cause of a late post-transfusion reaction, no anti-$Di^a$ or anti-$Di^b$ has been responsible for an HTR [3, 4].

Given the frequencies observed for the $Di^a$ antigen in different populations, it is necessary to have antibody detection and identification methodologies in blood banks that include anti-$Di^a$ in the study panels. This measure allows controlling alloimmunization and reducing the incidence of HTR and HDFN associated with the $Di^a$ antigen [11, 12].

## 3. Conclusions

Knowledge of erythrocyte blood systems since 1900 accounts for the genetic diversity of populations that are classified into certain phenotypes of clinical importance, where the hemolytic impact of antibodies in post-transfusion and post-transplant reactions is known, in addition to being responsible for the pathophysiology of HDFN. The main systems continue to be ABO and Rh, associated with hemolytic conditions that range from mild, moderate, and severe, to the death of patients.

The antigens that define phenotypes and blood systems are located on glycoprotein molecules and oligosaccharides present in the membrane of red blood cells as well as in other tissues. These molecules have certain functions such as maintaining the integrity of red blood cells, receptors for pro-inflammatory cytokines, ion and solute transporters, receptors for pathogens, etc.

In addition to these already known characteristics, there are other lines of evidence about the role that these molecules and antigens may have in the different pathophysiological conditions in the field of oncohematological, cardiovascular, oncogenic, infectious disorders, etc. Undoubtedly, advances in research will be able to establish certainties about the participation of erythrocyte antigens in the different clinical conditions under study.

## Conflict of interest

The authors declare no conflict of interest.

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Section 2

# Clinical Aspects

Chapter 4

# Clinical Significance of Red Blood Cell Indices

*Minseon Park and Yoon Hwan Chang*

## Abstract

Red blood cell (RBC) indices not only assess anemia but also play a crucial role in predicting and evaluating significant health issues, such as cardiovascular morbidity and mortality from cancer, cardiovascular disease, and infection. Ongoing research is expected to further elucidate these associations. Factors associated with RBC indices and red blood cell distribution width (RDW) beyond the normal range include nutritional imbalances, underlying chronic disease conditions, oxidative stress, chronic inflammation, and insulin resistance. Compared to studies related to outcomes, research on factors that lead to changes in RBC indices is limited, indicating the need for further investigation. Some RBC parameters, such as hemoglobin/RDW and mean corpuscular volume, are highlighted as promising, cost-effective, and readily accessible prognostic and diagnostic tools for cardiovasulcar disease, cancer, and all-cause mortality. Their versatility, along with insights into the underlying physiological changes, suggests they warrant further exploration and validation through prospective studies. This could represent a significant advancement in prognosis and patient management for some diseases.

**Keywords:** hemoglobin, hematocrit, mean corpuscular volume, red cell distribution width, mean corpuscular hemoglobin

## 1. Introduction

### 1.1 Red blood cell (RBC) indices

In peripheral blood, the RBC values include RBC count, hemoglobin concentration (Hb), and packed cell volume, along with RBC indices such as mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) [1]. Modern automated hematology analyzers also calculate RBC distribution width (RDW), a coefficient of variation (CV) of RBC volume distribution [1]. RDW is an index of the degree of anisocytosis in the RBC population (variation in MCV).

MCV, MCH, and MCHC values are determined based on the levels of hemoglobin (Hb), hematocrit (Hct), and RBC count [2].

$$\mathrm{MCV} = \mathrm{Hct} \times 1000 / \mathrm{RBC}\left(\text{in millions}/\mu\mathrm{L}\right), \quad \text{expressed in femtoliters}\left(\mathrm{fL}\right) \tag{1}$$

$$\mathrm{MCH} = \mathrm{Hb}\left(\text{in g/L}\right) / \mathrm{RBC}\left(\text{in millions}/\mu\mathrm{L}\right), \quad \text{expressed in picograms}\left(\mathrm{pg}\right) \tag{2}$$

$$\mathrm{MCHC} = \mathrm{Hb}\left(\text{in g/dL}\right) / \mathrm{Hct}, \text{expressed in g/dL} \tag{3}$$

RBC indices are measurements of the size and hemoglobin content of red blood cells. They are useful in diagnosing various types of anemia and other blood disorders. While they are not yet direct markers of the risk of cardiovascular disease (CVD), cancer, and mortality. Abnormalities in RBC indices can sometimes be associated with certain cardiovascular conditions and may serve as potential predictors of mortality in some situations.

This chapter will review well-established causes and conditions related to changes in RBC indices, along with emerging evidence for the clinical significance of changing RBC indices such as MCV and RDW, as well as the potential pathophysiologic mechanisms associated with these changes. Among them, MCV and RDW will be mainly discussed.

### 1.2 The cause of abnormal RBC indices

In erythropoiesis, the development of red blood cells includes the compaction of nuclear chromatin, referred to as nuclear maturation, the production of Hb in the cytoplasm known as cytoplasmic maturation, and a decrease in cell size as a result of division and loss of water [3].

Abnormal RBC indices may suggest various underlying conditions, such as nutritional deficiencies, genetic disorders, chronic illnesses, and certain medications. Elevated MCV can indicate several conditions, including vitamin B12 deficiency, folate deficiency, alcoholism, liver disease, hypothyroidism, myelodysplastic syndromes (MDS), and certain drugs [4].

Nuclear maturation abnormalities in megaloblastic anemias caused by folate or vitamin B12 deficiency lead to the presence of large oval red blood cells with a normal amount of Hb. In these cases, MCV and MCH levels are elevated, but the MCHC level remains within the normal range. Anisocytosis is present, and RDW is often elevated.

In macrocytosis associated with liver disease, the cells are enlarged because of an excess red cell membrane, without any issue in nuclear maturation. The cells are round instead of oval, and the RDW is within the normal range [3].

Conditions such as iron deficiency anemia, anemia of chronic disease, thalassemia, and sideroblastic anemia can be indicated by a low MCV [5].

Defective Hb synthesis results in the presence of microcytes with or without anisocytosis. Individuals with heterozygous β-thalassemia typically have small cells (low MCV) with a normal RDW, while those with iron deficiency may show elevated RDW (anisocytosis), even before the onset of anemia and microcytosis [3].

The MCH typically follows the changes in MCV and generally offers similar diagnostic information, and it is at least as sensitive as the MCV in detecting iron deficiency conditions [6]. The MCHC is rarely used for diagnosis but is mainly valuable for quality control reasons, such as detecting sample turbidity [7].

Automated instruments generate volume distribution histograms that reflect the degree of variation in cell size and help to identify the presence of more than one

population of cells. Instruments may also assess the percentage of cells falling above and below given MCV thresholds and alert ('flag' in technical terms) when there is an elevated number of microcytes or macrocytes. These measurements could suggest a slight but significant increase in the percentage of either microcytes or macrocytes before any change in the MCV occurs.

The RDW offers information and measurement of the variation in RBC size, known as anisocytosis.

The RDW is calculated using pulse height analysis and can be shown as either the standard deviation (SD) in fL or as the CV (as a percentage) of the measurement of red cell volume. The RDW SD is determined by calculating the width in fL at the 20% height level of the red cell size distribution histogram, and the RDW CV is calculated as the CV: RDW (CV) % = (1SD/MCV) × 100 [5]. The CV of RDW can help differentiate between iron deficiency (usually elevated RDW) and thalassemia trait (usually normal RDW), as well as between megaloblastic anemia (usually increased RDW) and other reasons for macrocytosis (more often normal RDW) [8].

In addition to its traditional application in the differential diagnosis of anemias, RDW values also indicate abnormalities in RBC production and metabolism, which can be influenced by factors such as age, gender, ethnicity, systemic inflammatory state, and oxidative stress [9].

Extensive research has been conducted on the role of RDW as a biomarker that can predict morbidity and mortality in various clinical conditions, including angina/myocardial infarction, heart failure, trauma, pneumonia, sepsis, intensive care treatment, renal and liver disease, and in the general population [4]. RDW may be a surrogate for systemic inflammation or oxidative stress, but the predictive value of RDW is independent of other inflammatory markers, suggesting that this biomarker is also tracking other mechanistic processes [7].

### 1.3 The cause of spurious results of RBC indices

Although automated cell counters are fast, easy to use, and accurate, some factors can disrupt the machine calculations and lead to spurious results [3].

Double erythrocytes are counted as one in red cell agglutination, while larger clumps are not counted as RBCs at all. This results in a reduction in RBC count and a falsely high MCV. The value of Hb remains unaffected. Prewarming the sample helps to remove these spurious values [3].

In hyperglycemia, RBCs temporarily become hypertonic compared to the isotonic diluting fluid, leading to enlarged cells and an increased MCV. This can be prevented by allowing some time for equilibration following dilution [3].

Hb is measured based on its absorption characteristics. Hyperlipidemia, hyperbilirubinemia, very high white blood cell count, and high serum protein levels can interfere with this measurement, leading to falsely high Hb values [3].

Low temperatures can cause immunoglobulins or fibrinogen to precipitate in the blood sample, leading to interference with cell counts. This can result in falsely high white blood cell counts, as well as slight increases in Hb, Hct, and RBC count, and a slight decrease in MCV. Prewarming the sample to 37°C will correct the inaccurate values [3].

Occasionally, a group of false values could be the first clue to an otherwise unsuspected clinical condition. For example, having a low hematocrit, normal Hb, and high MCV and MCHC is a common sign of cold agglutinins. Because the MCV is an average value, it can appear normal even when two different cell populations are

present, such as in cases of dimorphic anemias or red cell fragmentation with reticulocyte response. Therefore, it is essential to examine the peripheral blood smear when evaluating anemias [3].

## 2. The change of RBC indices with aging

In healthy elderly individuals, the MCV tended to increase with age, while the MCH and MCHC did not show a similar tendency [10].

Given that higher RDW is linked to older age and an increased disease burden, it has the potential to serve as a novel biomarker for various physiological impairments associated with aging [11].

## 3. RBC parameters and physical fitness

The association between RBC parameters and physical fitness is an important area of study in sports science and medicine. Epidemiological studies have demonstrated strong correlations between physical fitness and survival rates from both cardiovascular and non-cardiovascular causes [12, 13]. Fitness measures the body's capacity to transport and utilize oxygen, primarily influenced by training and partially by genetics [14]. Physical activity (PA), on the other hand, is a behavioral trait encompassing the energy expended on both voluntary and involuntary activities throughout the day [14]. Regular exercise induces adaptive changes in the characteristics of erythrocytes.

There is very little evidence regarding the relationship between physical fitness and RBC indices. The relationship of RBC size with physical fitness was evaluated with 2933 non-anemic military males in Taiwan. In this study, microcytosis was associated with lower anaerobic fitness, but not with aerobic fitness. Microcytosis, possibly early iron deficiency, accelerated the accumulation of lactate and led to lower anaerobic fitness in non-anemic military males [15].

There are few studies examining the relationship between the PA level of the general population and RBC indices. Endurance athletes, in contrast to untrained individuals, have been reported to exhibit hematological abnormalities like reduced Hb, Hct, and RBC count as indicative of sports anemia [16].

Recent studies show that both aerobic exercise (13.1 miles) and resistance training reduce RDW levels [17, 18]. Two additional muscle-strengthening sessions per week were associated with an 11% lower risk of elevated RDW. Researchers suggest that resistance training has anti-inflammatory effects and promotes red blood cell maturation [18].

This negative correlation between MSA and RDW may explain the link between RDW and cardiovascular disease. Regular endurance training can stimulate erythropoiesis to meet the increased oxygen demands, reduce oxidative stress, and help control inflammation and iron imbalance [19].

Many studies examining the relationship between physical fitness, exercise training, and RBC indices show an association, but causality remains unclear. Since RBC indices are linked to various health conditions, further research is needed to explore the longitudinal relationship between physical activity, fitness, and the RBC parameters.

## 4. RBC indices and nutrition

### 4.1 RBC indices, eating frequency, and dietary patterns

Eating frequency affects red blood cell (RBC) indices by influencing nutrient absorption and overall health. Regular, balanced meals that supply iron and essential vitamins (B12, folate, B6) are vital for healthy RBC production. Skipping meals or eating irregularly can lead to nutrient deficiencies and anemia.

Research on the relationship between eating frequency and RBC indices is scarce. A recent study examined the relationship between breakfast consumption patterns and their effects on hematological and body composition indices in 500 Indian adolescent girls [20]. Regular breakfast eaters (>60%) were significantly more likely to be non-anemic compared to those who ate breakfast occasionally (13–60%) or never (<3%), highlighting a notable link between breakfast consumption and anemia status.

A randomized cross-over trial investigated the short-term metabolic and cardiovascular effects of one-day water-only fasting [21]. The fasting intervention significantly increased RBC count and hematocrit (Hct) compared to a day on a usual diet, though most factors returned to baseline 48 hours later. Fasting caused immediate changes in biomarkers related to RBC count and Hct.

In a separate randomized controlled trial, the impact of repeated intermittent fasting (a 26-week period of weekly one-day water-only fasting) on RDW-standard deviation (SD) and RDW-coefficient of variation (CV) was examined [22]. RDW serves as an indicator of overall health, potentially highlighting undiagnosed issues in healthy individuals and reflecting illness severity in those with chronic diseases. In this study, RDW showed no significant changes over 6 months. Further research is needed to assess whether intensive fasting, longer interventions, or larger sample sizes could affect RDW levels.

Diet quality is linked to oxidative stress and inflammation, which are believed to contribute to various chronic diseases [23]. Increasing evidence suggests that systemic inflammatory responses can be assessed using hematological parameters readily available in clinical settings, such as WBC, RDW, mean platelet volume (MPV), neutrophil/lymphocyte ratio (NLR), platelet/lymphocyte ratio (PLR), and RDW/platelet ratio [24, 25]. Therefore, diet quality may be associated with these hematological parameters. High diet quality—characterized by abundant fruits, vegetables, nuts, and whole grains—has been associated with lower levels of inflammatory biomarkers, such as WBC count, RDW, NLR, and PLR. However, no significant association between daily dietary patterns and RDW was found in a representative US sample [26], and no meaningful correlation between healthy eating index/alternative healthy eating index and complete blood count (CBC) parameters was observed [27].

Energy metabolism is vital for red blood cell production, and metabolic disturbances can affect hematological parameters. However, research on the dietary impact is limited. To explore this, we analyzed data from the Health Examinees Study (KOGES-HEXA) [28]. **Table A1** provides hematologic parameter data for men and women.

The data show that MCV and MCH decrease with increasing energy intake in males and females.

### 4.2 Impact of nutrients on specific RBC indices

Iron deficiency leads to low Hb, Hct, and MCV, causing iron deficiency anemia. Insufficient protein, iron, vitamin B12, and folate intake can reduce Hb and Hct [29]. Deficiencies in vitamin B12 and folate can raise MCV, leading to macrocytosis. A balanced intake of B vitamins and iron is key to normal MCV. MCH and MCHC reflect Hb in RBCs, and low levels of iron, vitamin B6, and other nutrients can lower these values. A balanced diet with sufficient iron, vitamins (A, B6, B12, folate), and calories is crucial for healthy red blood cell production and function [30].

## 5. RBC indices and metabolic syndrome

Many RBC parameters have been linked to metabolic syndrome (MetS). MetS involves factors like central obesity, high blood pressure, and abnormal lipid levels, leading to increased risks of type 2 diabetes and cardiovascular disease, and it is often linked to insulin resistance and low-grade inflammation [31].

Several studies suggest that higher RBC counts, hemoglobin (Hb), hematocrit (Hct), and red cell distribution width (RDW) are linked to an increased risk of metabolic syndrome (MetS) and may indicate insulin resistance [32]. Although the exact mechanisms are unclear, insulin is thought to stimulate erythroid progenitor cell growth, potentially promoting RBC proliferation and affecting RBC parameters [32]. Additionally, the association between RBC indices and MetS may be related to iron overload caused by insulin resistance [33].

Red cell distribution width (RDW) has been identified as a predictive marker for metabolic syndrome (MetS), with RDW ≥14% being independently associated with an increased risk of MetS [34]. The connection between RDW and MetS may be due to chronic inflammation and proinflammatory cytokines, which inhibit erythropoiesis and lead to elevated RDW levels.

## 6. Clinical significance of MCV

### 6.1 MCV and the risk of CVD incidence and mortality

Cardiovascular disease (CVD) is a major global health concern. Recent studies suggest that mean corpuscular volume (MCV) can predict morbidity and mortality in diseases like CVD. Low MCV and mean corpuscular hemoglobin concentration (MCHC) values indicate microcytic anemia, often due to iron deficiency or chronic conditions like kidney disease [35]. Severe anemia can lead to tissue hypoxia and cardiovascular complications.

A few studies on the relationship between MCV and coronary heart disease (CHD) incidence or restenosis show inconsistent results. One study found that low MCV (<87.5 fl) predicted restenosis risk in stable coronary artery disease patients after percutaneous coronary intervention (PCI) [36]. Other studies linked higher MCV (≥85 fl) with increased cardiovascular morbidity and mortality, especially in chronic kidney disease (CKD) patients. Low MCV may be related to inflammation, while higher MCV could be linked to lower statin use, increasing oxidative stress and inflammation [37, 38].

| Author (year) (reference) | Country | Study design | No. of endpoints/ total subjects | Age (mean ± SD) | RR/HR (95% CI) | FU (month) |
|---|---|---|---|---|---|---|
| Ueda et al. (2013) [39] | Japan | Retrospective | 458 patients with ADHF | 72.4 ± 12.2 | MCV > Total deaths: 173(37.9%), HR 2.288; (95% CI 1.390–3.643) | 20.8 |
| Tennankore et al. (2011) [40] | Canada | Prospective | 23/150 in chronic HD patients | 65.0 ± 17.0 | MCV >102 fl, HR for total death 2.47, (95% CI, 1.07–5.71) | 9 |
| Wu et al. (2018) [41] | Taiwan | Retrospective | 66,294 | 20≤ | MCV ≥ 99 fL, mortality for ischemic heart disease (aHR = 1.44, P for trend = 0.0992), for cerebral ischemic stroke (aHR = 1.66, P for trend = 0.0667), for cerebral hemorrhage stroke (aHR = 1.23, P for trend = 0.6278) | 108 (median f/u: 74.52) |
| Myojo et al. [38] | Japan | Retrospective | 941 | 60≤ | MCV ≥ 99 fL, cardiac mortality after PCI, aHR 3.45, (95%CI: 1.22–9.80) risk for MACCE after PCI aHR 3.21, (95%CI: 1.54–6.70) | 24 |
| Hsieh et al. [42] | Taiwan, Republic of China | Retrospective | 234/1439 patients with CKD | 64.2 ± 12.2 | MCV > total mortality HR, 2.19; (95% CI, 1.62–2.96); CV mortality HR, 3.57 (95% CI, 1.80–7.06) | 22.8 |

*Abbreviation: ADHF, acute decompensated heart failure; PCI, percutaneous coronary intervention; HD, hemodialysis; MACCE, major adverse cardiovascular and cerebrovascular event; CAD, coronary artery disease; RR, relative risk; HR, hazard ratio; FU, follow-up.*

**Table 1.**
*The association between MCV and CV mortality and all-cause mortality.*

**Table 1** summarizes studies on the relationship between MCV and mortality. High MCV or macrocytosis generally predicts poor outcomes in diseases like CHD, CKD, and heart failure [38–42]. While the exact mechanisms are unclear, macrocytosis may indicate a nutritional imbalance, dysfunctional RBC antioxidant capacity, or abnormal hematopoiesis due to multiple comorbidities [43]. Elevated MCV likely serves as a marker for underlying health risks rather than a direct cause of CVD/CHD-related death.

### 6.2 MCV and the risk of cancer and cancer mortality

High MCV or macrocytosis can indicate cancers such as leukemia, myelodysplastic syndromes, and multiple myeloma [44]. It has also been documented as a survival predictor in cancers like lung, gastric, esophageal, and colon cancers [45–50]. One study found that elevated MCV predicted higher all-cause and liver cancer mortality in healthy, non-anemic men [51]. Potential mechanisms include RBC membrane dysfunction, oxidative stress, and nutritional imbalances leading to inflammation. Elevated MCV may reflect underlying health issues, warranting further research into its diagnostic value.

## 7. Clinical significance of high red cell distribution width (RDW)

RDW indicates variability in erythrocyte size and is commonly elevated in anemias like iron deficiency and vitamin B12 deficiency. Beyond diagnosing anemia, RDW is gaining recognition as a predictor of autoimmune diseases and poor prognosis in coronary heart disease (CHD) and heart failure [52]. It also independently predicts cardiac outcomes, such as restenosis and mortality, in patients undergoing PCI [53].

### 7.1 RDW and the morbidity and mortality from cardiovascular disease

A systematic review of 21 studies involving 56,425 patients found that those undergoing PCI with high RDW had significantly increased risks for in-hospital mortality (OR 2.41), long-term mortality (OR 2.44), cardiac mortality (OR 2.65), and major adverse cardiovascular events (MACE) (OR 2.16) [54]. The association between high RDW and adverse cardiac outcomes may be due to chronic inflammation, which contributes to atherogenesis and platelet activation. RDW has been linked to inflammatory markers, impaired hematopoiesis, disrupted iron metabolism, and reduced red blood cell flexibility through the formation of the oxidized low-density lipoproteins [7, 9].

Additionally, elevated RDW is associated with inadequate tissue perfusion, a predictor of post-PCI mortality [3].

### 7.2 RDW and atrial fibrillation

Atrial fibrillation (AF) is the most common arrhythmia, affecting over 33 million people globally and leading to significant health risks [55]. Studies show that 36–82% of AF patients have coronary artery disease (CAD), and about 74% have subclinical coronary atherosclerosis.

RDW is a prognostic marker for atrial fibrillation (AF) and its complications, including stroke, thromboembolism, and mortality. A systematic review of five studies found higher RDW levels in recurrent AF cases [56]. Elevated RDW is linked

to worse outcomes in AF, likely due to inflammation and oxidative stress. It is being considered for inclusion in AF risk scores, though specific thresholds are still under study. More large-scale research is needed to assess its clinical usefulness.

### 7.3 RDW (RDW/Hb) and all-cause mortality and cancer mortality

Elevated RDW is important for diagnosing and monitoring various conditions. Historically, Hb levels have been used to assess cancer treatment tolerance, with low Hb suggesting malnutrition and poor tolerance [2]. The RDW/Hb ratio has emerged as a prognostic marker in cancers like lung, breast, stomach, and lymphoma, reflecting cancer-related inflammation, nutritional deficiencies, and bone marrow disorders [57]. Deficiencies in iron, vitamin B12, and folate, as well as tumors affecting the bone marrow, can alter the RDW/Hb ratio [57].

## 8. Conclusions

RBC parameters like the RDW/Hb ratio and MCV are emerging as cost-effective, accessible tools for diagnosing and predicting cardiovascular disease, cancer, and mortality. Identifying reversible causes of abnormal RBC indices can help in prevention and treatment. Their potential warrants further research and validation in prospective studies.

## Conflict of interest

None.

# Appendix



| Energy intake | Male | | | | | Female | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1st quartile | 2nd quartile | 3rd quartile | 4th quartile | p | 1st quartile | 2nd quartile | 3rd quartile | 4th quartile | p |
| | (N = 5125) | (N = 5125) | (N = 5125) | (N = 5124) | | (N = 9736) | (N = 9736) | (N = 9736) | (N = 9736) | |
| Age, median, IQR | 55.0 [48.0;62.0] | 54.0 [48.0;61.0] | 54.0 [47.0;60.0] | 52.0 [45.0;59.0] | 0.000 | 53.0 [48.0;60.0] | 53.0 [47.0;59.0] | 52.0 [46.0;58.0] | 51.0 [44.0;56.0] | 0.000 |
| BMI, kg/m$^2$ | 24.1 [22.4;26.0] | 24.2 [22.6;26.0] | 24.3 [22.6;26.0] | 24.6 [22.9;26.4] | 0.000 | 23.1 [21.4;25.1] | 23.2 [21.5;25.3] | 23.3 [21.5;25.3] | 23.3 [21.5;25.3] | 0.000 |
| Smoking habit | | | | | 0.000 | | | | | 0.000 |
| Never | 1418 (27.7%) | 1334 (26.0%) | 1272 (24.8%) | 1251 (24.4%) | | 9261 (95.1%) | 9395 (96.5%) | 9407 (96.6%) | 9369 (96.2%) | |
| Ex-smoker | 2125 (41.5%) | 2280 (44.5%) | 2291 (44.7%) | 2119 (41.4%) | | 155 (1.6%) | 122 (1.3%) | 115 (1.2%) | 147 (1.5%) | |
| Current | 1582 (30.9%) | 1511 (29.5%) | 1562 (30.5%) | 1754 (34.2%) | | 320 (3.3%) | 219 (2.2%) | 214 (2.2%) | 220 (2.3%) | |
| Alcohol intake | | | | | 0.000 | | | | | 0.000 |
| Never | 1097 (21.4%) | 966 (18.8%) | 957 (18.7%) | 953 (18.6%) | | 6521 (67.0%) | 6641 (68.2%) | 6399 (65.7%) | 6132 (63.0%) | |
| Ex | 409 (8.0%) | 372 (7.3%) | 331 (6.5%) | 328 (6.4%) | | 203 (2.1%) | 162 (1.7%) | 163 (1.7%) | 189 (1.9%) | |
| Current | 3619 (70.6%) | 3787 (73.9%) | 3837 (74.9%) | 3843 (75.0%) | | 3012 (30.9%) | 2933 (30.1%) | 3174 (32.6%) | 3415 (35.1%) | |
| Daily energy intake | 1316.1 [1154.2;1421.0] | 1630.2 [1562.1;1693.1] | 1906.2 [1830.5;1997.0] | 2395.6 [2219.0;2676.6] | 0.000 | 1115.2 [950.2;1236.3] | 1493.8 [1419.6;1561.9] | 1771.9 [1696.8;1856.3] | 2232.1 [2070.6;2509.7] | 0.000 |
| Hb | 15.3 [14.5;16.0] | 15.3 [14.6;16.0] | 15.4 [14.7;16.0] | 15.4 [14.7;16.1] | 0.000 | 13.3 [12.7;13.9] | 13.3 [12.7;13.9] | 13.3 [12.7;13.9] | 13.3 [12.7;13.9] | 0.473 |
| HCT | 45.0 [43.1;47.0] | 45.1 [43.2;46.9] | 45.3 [43.4;47.1] | 45.3 [43.5;47.3] | 0.000 | 40.0 [38.3;41.8] | 40.1 [38.3;41.8] | 40.1 [38.4;41.8] | 40.1 [38.4;41.7] | 0.501 |
| MCV | 92.6 [90.1;95.3] | 92.4 [89.9;95.0] | 92.2 [89.7;94.8] | 92.0 [89.5;94.7] | 0.000 | 91.9 [89.3;94.4] | 91.7 [89.1;94.2] | 91.6 [88.9;94.1] | 91.4 [88.6;93.8] | 0.000 |

| Energy intake | Male | | | | | Female | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1st quartile | 2nd quartile | 3rd quartile | 4th quartile | p | 1st quartile | 2nd quartile | 3rd quartile | 4th quartile | p |
| | (N = 5125) | (N = 5125) | (N = 5125) | (N = 5124) | | (N = 9736) | (N = 9736) | (N = 9736) | (N = 9736) | |
| MCHC | 31.4 [30.5;32.3] | 31.3 [30.5;32.3] | 31.3 [30.4;32.2] | 31.3 [30.4;32.1] | 0.000 | 30.6 [29.7;31.5] | 30.5 [29.6;31.4] | 30.5 [29.5;31.3] | 30.4 [29.4;31.3] | 0.000 |
| MCHC | 33.9 [33.3;34.4] | 33.9 [33.3;34.5] | 33.9 [33.3;34.5] | 33.9 [33.3;34.5] | 0.110 | 33.2 [32.6;33.8] | 33.2 [32.6;33.8] | 33.2 [32.6;33.7] | 33.2 [32.6;33.7] | 0.001 |
| Platelet | 236.0 [204.0;271.0] | 236.0 [205.0;272.0] | 237.0 [204.0;271.0] | 237.0 [206.0;273.0] | 0.350 | 257.0 [223.0;297.0] | 258.0 [224.0;296.0] | 260.0 [225.0;298.0] | 262.0 [227.0;300.0] | 0.000 |

**Table A1.**
*The association between hematologic parameters and the quartile level of energy intake.*

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Chapter 5

# Clinical Significance of Reticulocytes

*Ashok Kumar Sah and Darla Srinivasa Rao*

## Abstract

Reticulocytes, immature red blood cells, are crucial for assessing erythropoiesis and bone marrow function, offering insights into various hematological conditions. This abstract highlights their clinical significance in evaluating red blood cell production, diagnosing, prognosis, and monitoring treatments. Reticulocyte counts reflect the rate of erythropoiesis in response to physiological or pathological changes, aiding in the assessment of bone marrow function and oxygen delivery capacity. Elevated reticulocyte counts indicate a compensatory response to anemia, while decreased counts suggest impaired erythropoiesis or bone marrow issues. Reticulocyte indices, such as the reticulocyte production index (RPI) and corrected reticulocyte count, provide further insights into erythropoietic dynamics, helping distinguish between hypo- and hyperproliferative conditions. These parameters are also valuable in diagnosing and managing disorders such as hemolytic anemias, bone marrow failures, and myelodysplastic syndromes, as well as in monitoring treatment efficacy. Changes in reticulocyte counts post-treatment, such as with erythropoietin therapy or blood transfusions, offer feedback on therapeutic effectiveness and guide patient care. Overall, reticulocytes are essential for diagnosing, classifying, and managing hematological conditions, improving patient outcomes and care quality.

**Keywords:** retics, immature RBCs, anemia, red cell indices, bone marrow, PCV

## 1. Introduction

RBCs, or immature red blood cells, are referred to as reticulocytes. Organelle remnants and ribosomal RNA (rRNA) identify young, nucleated erythrocytes from adult erythrocytes. Their name originates from their reticular, or network-like, appearance when stained with supravital dyes such as fresh methylene blue or brilliant cresyl blue. It originates from the orthochromatic normoblast in the bone marrow through nuclear exclusion. They enter the peripheral blood after developing in the bone marrow and continuing to differentiate into mature red blood cells there. The reticulocyte count is commonly performed to obtain information about the functional integrity of the bone marrow because it indicates the erythropoietic activity of the bone marrow, the rate of reticulocyte delivery from the bone marrow

into the peripheral blood, and the rate of reticulocyte maturation. Reticulocytosis, or an increase in peripheral blood reticulocytes, is seen in anemic individuals with functional bone marrow, whereas reticulocytopenia, or a reduction in peripheral blood reticulocytes, is seen in anemic patients with dysfunctional bone marrow. Reticulocyte enumeration is helpful for monitoring bone marrow regeneration after chemotherapy or bone marrow transplantation, in addition to being used for the assessment of anemic patients. In a lab context, the reticulocyte may be identified from the mature RBC due to the presence of components like as RNA that are lost during development into the mature RBC. The traditional method of enumerating reticulocytes was created in the 1940s and included manually counting reticulocytes using supravital dyes for RNA and light microscopy. However, since automated reticulocyte enumeration techniques are significantly more precise, accurate, and cost-effective than manual counting, they are becoming more and more frequent in clinical laboratories. These techniques have been around for 10 years. Moreover, the most current techniques provide a variety of reticulocyte-related properties, such as the reticulocyte maturation index and immature reticulocyte fraction, that are not available with light microscopy. These new measures are being assessed in the clinical diagnosis and follow-up of anemia and other diseases [1–3].

### 1.1 Development and maturation

New erythrocytes, leucocytes, and thrombocytes are continuously produced by the bone marrow from hematopoietic stem cells. Myeloid, lymphoid, erythroid, and megakaryocytic cell lines are the main marrow cell lines that arise from populations of progenitor cells that stem cells self-renew and self-differentiate to create. Despite being multipotent, the offspring of early progenitor cells are committed to producing a limited quantity of cell lines. For hematopoietic stem cells to live, multiply, and divide, they need the "microenvironment" that the stromal matrix of the bone marrow supplies.

Hematopoietic growth factors are necessary for the self-renewal of stem cells and for the proliferation and differentiation of lineage-committed progenitor cells. Hormone-like substances called colony-stimulating factors (CSFs) encourage the growth of progenitor cell colonies in vitro. One of the hematopoietic growth hormones, erythropoietin, is the main chemical regulator of erythropoiesis. In response to a drop in renal oxygen tension, the kidney produces erythropoietin, a glycoprotein with a molecular weight of 34,000–39,000 kDa. Erythropoietin promotes the cell cycle entry of resting (G0) committed erythroid stem cells (CFU-E) while also accelerating erythroblast division at all embryonic stages. Since a hematocrit of 40–45% is optimal for delivering oxygen to the tissues, the generation of red blood cells is strictly regulated. When mature red blood cell precursors differentiate into mature red blood cells, they go through a series of increasingly dramatic structural and metabolic changes [4]. The biggest modifications are as follows:

1. Hemoglobin synthesis and cytoplasmic buildup.

2. Loss of mitochondria and the machinery that makes proteins.

3. The nucleus's chromatin contraction, extrusion, and condensation.

4. Exosome formation-induced loss of expression of cell-surface membrane receptors.

5. Modifications in phospholipid and cholesterol levels in the membrane.

6. Variations in the amounts of different intracellular enzymes, such as glucose-6-phosphate dehydrogenase.

These events are reflected in the morphological features of red blood cell progenitors. The pronormoblast, the first morphologically distinct red blood cell, is produced when the CFU-E separates. The mature RBC, reticulocyte, polychromatic normoblast, orthochromatic normoblast, and basophilic normoblast are among the subsequent cellular phases of erythropoiesis [5–7]. The various physically different RBC precursors and RBCs themselves make up the erythron. During this period, complex alterations in cellular biochemistry also take place [8]. A pronormoblast matures into a nonnucleated RBC in 3–5 days. Anucleate red blood cells, also known as reticulocytes, have a somewhat larger size (10–15 μm vs. 6–8 μm) than mature red blood cells. In addition to mitochondria and a small number of ribosomes, the early reticulocyte serves as a Golgi body and a centriole. About twenty to thirty percent of the RBC's total hemoglobin at this stage of development comes from early reticulocytes, which are still producing hemoglobin. However, when the reticulocyte matures into a red blood cell and individual cellular organelles are removed, hemoglobin production gradually declines. During the process of differentiating, the transferrin receptor is removed from the reticulocyte surface membrane. Reticulocytes typically undergo their ultimate maturation and are discharged into the peripheral circulation after spending about 2 days in the bone marrow. When these cells were labeled with tricyclic, heterochromatic, cationic dyes that bind and cross-link RNA and aggregate other organelles, a deep blue precipitate was observed. This precipitate is the source of the term "reticulocyte." Relative RNA concentration can be used to calculate the "age" of reticulocytes. Heilmeyer and Westhäuser divided reticulocytes into four groups (Groups I–IV) using blood samples that had been supravitally colored (**Table 1**) [1]. Very young reticulocytes called stress reticulocytes are released into the bloodstream in reaction to a severe case of anemia. Stress reticulocytes are multilobular and motile, as revealed by phase contrast microscopy, whereas mature reticulocytes are nonmotile and cup-shaped. Stress reticulocytes are only visible in the bloodstream under erythropoietic stress and are not present in people who are not anemic, according to Coulombel et al. [10]. In hematopoietic persons, stress reticulocytes are rapidly eliminated from the bloodstream; in anemic individuals, their lifespan is prolonged, most likely due to splenic adaptation [10, 11]. The separation of stress- and normal-type reticulocytes is accomplished by density-gradient fractionation.

## 2. Clinical significance of reticulocytes

### 2.1 Reticulocytes as a hemolytic marker

Since they have ribosomal-RNA traces, reticulocytes which are nonnucleated direct antecedents of red blood cells have a basophilic cytoplasm and a larger mean

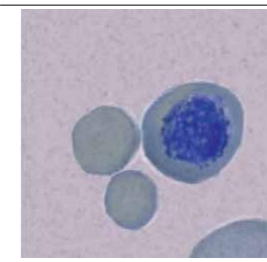
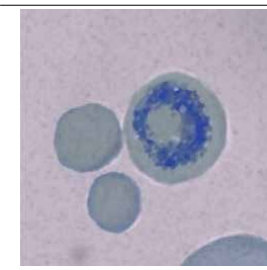
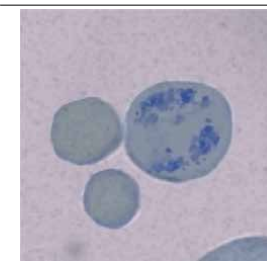
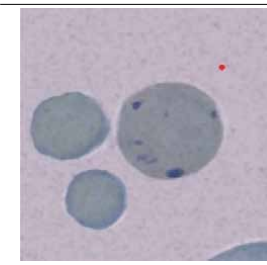
| Groups | Characters of reticulocytes | Structure |
|---|---|---|
| Group I | Very young reticulocytes have a thick, cohesive, dense and clumped mass of RNA and other organelles after the nucleus has been expelled. |  |
| Group II | RNA matures and loses density, a reticular network forms in the original mass's vicinity. Expanded loose reticulum network. |  |
| Group III | RNA then diffuses and becomes less concentrated. Fragmented granules with a network of residual reticulum. |  |
| Group IV | Only a few scattered RNA residues remain in the highest developed reticulocytes before they eventually develop into mature RBCs. Fragmented grains. |  |

**Table 1.**
*Maturation stages of reticulocytes according to Heilmeier classification [9].*

corpuscular volume. They make up a little portion of peripheral red blood cells (typical levels range from 1 to 2%, depending on the laboratory). As a measure of the hemopoietic activity of the bone marrow, reticulocytes are typically elevated in hemolysis and other pathological and physiological situations (e.g., hemorrhage pregnancy, delivery, and acclimatization). However, in hemolytic conditions, concurrent marrow involvement (oncohematologic conditions, dyserythropoietic or bone marrow failure syndromes), iron and vitamin deficiency, infections, or an autoimmune reaction against bone marrow precursors may cause the compensatory

reticulocytosis to be insufficient or nonexistent. The second is particularly noteworthy in autoimmune hemolytic anemia (AIHA), as 39% of children and roughly 20% of adults are known to have reticulocytopenia [12–14]. Reticulocytopenia can often be a clinical emergency with a high transfusion requirement and a poor prognosis, as observed in a small number of emergency cases, refractory, and fatal AIHA cases in recent times [15]. Consequently, the evaluation of reticulocytes ought to be conducted using the recently suggested bone marrow responsiveness index (BMRI) [16]. Reticulocytosis is a crucial measure to track hemolysis recovery or a treatment's effectiveness. When individuals with a deficiency are supplemented with folate, vitamin B12, or iron, the reticulocyte response usually takes 3 to 5 days to occur (a condition known as reticulocyte crisis). Erythropoietin has been shown to improve anemia and reduce/avoid hemolysis related to overtransfusion in patients with inadequate reticulocytosis; this has been observed with thrombopoietin agonists in primary immune thrombocytopenia [17, 18]. Reticulocytes in AIHA typically remain elevated for several days until hemoglobin levels are restored. Reticulocytes are often slightly raised in chronic/congenital hemolytic disorders, but they can rise substantially in an acute hemolytic crisis. Absolute reticulocyte numbers in hereditary spherocytosis drop markedly following splenectomy, which is consistent with the situation of impaired hemolysis [19]. Pyruvate-kinase deficiency does not cause reticulocytopenia, as evidenced by the persistently elevated reticulocyte count that follows splenectomy [20]. Similarly, in Paroxysmal nocturnal hemoglobinuria (PNH) patients, reticulocyte counts frequently stay high even after starting eculizumab therapy because of the ongoing extravascular hemolysis brought on by C3 fragment deposition on PNH red blood cells [21]. Finally, while prosthetic valve replacement typically connotes subclinical hemolysis with normal or slightly lowered hemoglobin levels, reticulocyte count does not alter considerably in these individuals [12, 22].

### 2.2 Diagnosis of anemia

The reticulocyte count is clinically significant for the pathophysiological classification of anemia. It is also clinically significant for the early detection of the marrow's return to normal erythropoiesis following therapeutic intervention (iron, cobalamin, folic acid, ESAs, etc.), after spontaneous or pharmacologically induced marrow aplasia, or after bone marrow transplantation. However, the manual microscopic approach is nearly worthless in cases of severe reticulocytopenia due to its imprecision [23]. It does not clearly distinguish between low and normal reticulocyte levels, nor does it permit the study of subtle but significant fluctuations during the early recovery of erythropoietic bone marrow activity. Flow cytometry conducts quick, accurate retic counts and dyes to bind reticulocyte RNA, and automated analyzers are a revolution for this type of cell [24].

The variation of reference is solely dependent on techniques used for the counts. The availability of a new measure based on reticulocyte RNA content called immature reticulocyte fraction (IRF) has increased interest in automated reticulocyte analysis [25]. After the nucleus is ejected from orthochromatic erythroblasts, reticulocytes develop progressively over the course of 3 days in the bone marrow and 1 day in the peripheral circulation. Reticulocytes eventually become RNA-free red blood cells as they gradually lose their RNA, however a relatively small percentage

of RNA-rich, immature reticulocytes can be observed in the peripheral blood of healthy persons. However, depending on the analyzer being used, there are alternative expressions, which means that the reference intervals vary from lower to higher [25, 26]. The IRF is an early and sensitive indicator of erythropoiesis, regardless of how it is made, in fact, when red cell production rises, a greater proportion of immature reticulocytes becomes visible. Its weak but statistically significant positive correlation with the absolute reticulocyte count suggests that the IRF is an additional useful tool to evaluate erythropoietic activity. The two-dimensional matrices of IRF vs. the absolute reticulocyte count have the most clinical utility, particularly in the classification of anemia based on marrow response [25, 27]. In cases of normal or mild reticulocytosis, there are two subsets that exhibit positive covariance, which correspond to accelerated erythropoiesis and healthy subjects. In cases of marked reticulocytosis, on the other hand, the covariance is negative, indicating a gradual deceleration of erythropoiesis. It is therefore possible to hypothesize that the absolute reticulocyte count serves as a quantitative indicator of the efficacy of erythropoiesis, while the IRF serves as an index of acceleration, depending on the erythropoietic conditions [28, 29]. Therefore, this parameter is helpful in differentiating between (i) anemias caused by reduced marrow production (i.e., chronic renal disease), in which both values are found to be decreased (ii) anemias associated with an increase in erythropoiesis, such as acquired hemolytic anemias or blood loss, which results in an increase in both total reticulocytes and IRF, and (iii) anemias associated with acute infections or myelodysplastic syndromes, in which there is a dissociation between total reticulocyte count (normal or reduced) and the IRF, which can be increased [27, 30–33]. Because the increase in IRF occurs several days before the increase in total reticulocyte count, further applications include tracking the effectiveness of therapy in nutritional anemia (e.g., cobalamin, folates, and iron). When IV iron was administered to individuals with iron-deficient anemia, the amount increased on day 1 and kept rising until day 5, when it reached its maximum value. Reticulocyte counts >80 × $10^{10}$/L and reticulocyte counts/IRF ratios >7.7 are thought to be helpful in the screening process for moderate hereditary spherocytosis and traits [34].

### 2.3 Immature reticulocytes are sensitive and specific to low-dose erythropoietin treatment

After accelerated erythropoiesis, immature reticulocytes (IRs) rise in 36 hours. IRs are commonly expressed as the immature reticulocyte fraction (IRF), which is IR relative to the total number of reticulocytes. Erythropoietic stress is expected to raise the IRF because it causes the bone marrow to release IR early and to mature more slowly. In fact, stress erythropoiesis raises IRF in certain situations, such as anemia or Recombinant human erythropoietin (rhEpo) therapy, whereas it falls in situations when erythropoietic activity is reduced after autologous blood transfusion. This implies that IRF is a sensitive biomarker for rhEpo misuse. In humans, a single rhEpo injection of 150–300 IU/kg body weight (bw) dramatically increases IRF; however, individuals who abuse rhEpo are probably going to use lower doses. The IR and RBC count (IR/RBC) ratio is another possible biomarker. It was suggested as a biomarker for autologous blood transfusion in dried blood spots that were assessed by the CD71/Band-3 ratio. It suggests that the IR/RBC fraction is a sensitive biomarker to changes in erythropoiesis since, in contrast, the RBC count changes with an anticipated variation of less than 10% [35–42]. It is significant since many athletes spend more

than 3 weeks at altitudes of more than 2000 m above sea level. Since hypoxic exposure is known to impact erythropoietic activity, it is necessary to determine whether it confounds the variables that have been suggested [43].

World Anti-Doping Agency-accredited laboratories measure IRF using the Sysmex XN-series, which has a precision app. 8–10%. The applied methodology is single-fluorescence flow cytometry; that is, the cells are stained for RNA by a fluorescence marker, and the cell fraction with high and medium fluorescent intensity in relation to all stained cells represents IRF. However, the IR also expresses the transferrin receptor (CD71). CD71 expression progressively decreases during maturation in the blood and is undetectable in mature reticulocytes. We examined whether the identification of RNA and CD71 positive cells (i.e., IRs) by multi-fluorescence flow cytometry on the FACS Fortessa 3 laser platform could improve the precision (i.e., lower coefficient of variation) since measurement precision is crucial in anti-doping to prevent false-positive results. It has been shown in the past that identifying IRs using RNA and CD71 staining is a useful method for distinguishing between accelerated and repressed erythropoiesis [44–47].

### 2.4 Indicator of abnormality of erythropoiesis

It has been suggested that immature reticulocytes are the shift reticulocytes that correlate to Heilmeyer stages I and II. Recent work using flow cytometric analysis has demonstrated the utility of measuring reticulocyte maturity [48]. Research indicates that patients with specific forms of anemia, such as acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), aplastic anemia (AA), and megaloblastic anemia (MA), had a considerably higher proportion of immature reticulocytes even when the overall number of circulating reticulocytes was either normal or decreased. The clinical significance of alterations in reticulocyte maturity in patients with these illnesses is strongly supported by the research. Clonal hemopoiesis is seen in AML and MDS, and the anemia linked to these conditions results from dyserythropoiesis brought on by stem cell dysfunction. Despite being a self-replicating illness marked by a decrease in or malfunctioning of pluripotent stem cells, AA has been closely linked to dyserythropoiesis and clonally disordered hematopoiesis in recent studies. It is well-recognized that MA is caused by a defect in DNA synthesis, which leads to inefficient erythropoiesis and a certain level of extramedullary hemolysis. It is plausible to hypothesize that in individuals with specific hematologic illnesses, a rise in reticulocyte immaturity in conjunction with a reduced or normal reticulocyte count may indicate either dyserythropoiesis or inefficient erythropoiesis. Our understanding is that this is the first report to link reticulocyte immaturity to an aberrant erythropoiesis quality. Dyserythropoiesis is another clonal disease that manifests in PNH. Although we anticipated that patients with PNH would have reticulocyte immaturity similar to that of patients with AML, MDS, or AA, we only discovered a significant level of immaturity in correlation with an elevated reticulocyte count [49].

Our observations, which are similar to other publications, demonstrate that reticulocyte immaturity is elevated in hemolytic anemia and acute blood loss when erythropoiesis is encouraged, as seen by an increased absolute reticulocyte count. This rise might result from erythropoietin's improved stimulation of the bone marrow [49].

Very recently, Wells et al. demonstrated a correlation between blood ferritin concentrations and the mean fluorescence intensity of reticulocytes. This suggests that a patient's iron status affects reticulocyte immaturity. It may be necessary to provide clarification as the reticulocyte immaturity in iron deficiency anemia (IDA) patients

did not significantly increase, with ferritin concentrations of less than 11 pmol/l and a total iron binding capacity greater than 72 umol/l [49, 50]. Finally, the measurement of immature reticulocytes can be a diagnostic tool in the differential diagnosis of patients with hematological diseases and can be clinically beneficial for assessing qualitative alterations in erythropoiesis [49].

### 2.5 Association with hypertension and atherosclerosis

Since hematopoietic stem cells produced from bone marrow are thought to play a fundamental role in vascular homeostasis, bone marrow activity has been demonstrated to be intimately related to vascular maintenance. Age-related decreases in hematopoietic bone marrow function cause anemia in the elderly. As a result, erythropoietic activity based on reticulocyte counts may represent an elderly person's ability to maintain endothelium. The blood's antioxidant capability is mostly attributed to erythrocytes, and oxidative stress can be decreased by stimulating erythropoiesis. Since reticulocytes are immature erythrocytes, an increase in reticulocyte levels could be a sign of increased antioxidant activity that halts the progression of atherosclerosis, however, an increase in antioxidant activity could also be brought on by oxidative stress and inflammation, both of which are linked to hypertension. This mechanism may account for the notably positive correlation with hypertension and the inverse correlation with atherosclerosis. Moreover, it has been established that endothelial dysfunction is one of the primary pathways that result in atherosclerosis and glomerular damage (lower GFR). Renal anemia is the term used to describe reduced renal function; a risk factor linked to anemia. Renal function (GFR) was regarded as a confounding factor in our investigation. Nonetheless, there was a positive correlation between GFR and reticulocyte levels, and individuals with hypertension had significantly lower GFR values than those without the condition. These correlations corroborate the findings of comparable correlations between reticulocyte levels and atherosclerosis and hypertension [51].

### 2.6 A prognostic and diagnostic predictor for small cell lung cancer

Peripheral blood reticulocytes, which show reticulum network or granules as precipitated rough endoplasmic reticulum with accompanying polyribosomes, are a valuable clinical sign. Reticulocytes are discharged into the circulation during erythropoiesis when they progressively shed their RNA and develop into mature red blood cells [52]. The intensity of either fluorescence or light scattering/absorbance, which is dependent on RNA content, is used to assess reticulocyte maturity. According to Grazziutti et al. [53], reticulocytes are now classified as belonging to the low fluorescent region (LFR), middle fluorescent region (MFR), or high fluorescent region (HFR), which correlate to the lower, middle, or greater RNA concentration, respectively. More reproducible than HFR, the immature reticulocyte fraction (IRF) is a relatively novel reticulocyte statistic that encompasses MFR and HFR [54]. Research has demonstrated that as an early marker of bone marrow recovery or hematopoietic stem cell transplantation, an increase in IRF is preferable to other hematological parameters like absolute neutrophil count (ANC), immature platelet fraction (IPF), and reticulocyte counts [26, 55, 56]. IRF has been shown to be clinically useful in a number of situations, including evaluating bone marrow recuperation following chemotherapy [57]. One important method for evaluating the bone marrow's capacity to boost erythrocyte production in response to different kinds of anemias

is the reticulocyte count [58]. Therefore, in order to solve the problem of prognostic stratification, it is proposed that Reticulocyte Fraction Ratio (IMR) can be used as a quick and effective signal for stratifying the prognosis of small cell lung cancer.

### 2.7 Role of immature reticulocytes in acute lymphoblastic and acute myeloid leukemia

Many of the alterations that take place in the tumor microenvironment during acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) remain unknown despite tremendous advancements. Atomic force microscopy (AFM) was used to analyze the surface of juvenile reticulocytes in order to identify distinct alterations that occurred during the development of ALL and AML. The relevance of various atomic force microscopy methods for identifying subtle morphofunctional alterations in immature reticulocytes impacted by bone marrow tumor blasts. The linear dimensions of cells and the characteristics of the plasma membrane surface microrelief can be quantitatively assessed thanks to AFM imaging. These technologies hold great potential in enhancing our comprehension of the beginning and progression of malignant processes within the blood system, as well as evaluating the efficacy of current treatments. The reticulocytes' surface area, volume, width and height play important role in diagnosis and prognosis ALL patients. When examining the bone marrow activity during acute leukemia, the acquired data has significant predictive significance [59].

## 3. Conclusion

Reticulocytes, the immature red blood cells (RBCs) released from the bone marrow into the bloodstream, hold significant clinical value in diagnosing and managing various hematologic conditions. Their count and morphology provide critical insights into the state of erythropoiesis and overall bone marrow function, guiding clinicians in diagnosing, monitoring, and treating patients with a range of disorders.

### 3.1 Indicator of bone marrow activity

Reticulocytes are a direct reflection of bone marrow activity. A reticulocyte count is a valuable tool in assessing the bone marrow's response to anemia. In conditions of increased erythropoietic activity, such as hemolytic anemia or blood loss, the bone marrow compensates by releasing a higher number of reticulocytes into circulation. Conversely, in cases of bone marrow suppression or aplastic anemia, the reticulocyte count is typically low, indicating inadequate marrow response.

### 3.2 Diagnostic tool for anemia

The reticulocyte count aids in distinguishing between different types of anemia. An elevated reticulocyte count in an anemic patient suggests a responsive marrow, commonly seen in hemolytic anemia or acute blood loss. On the other hand, a low reticulocyte count in the context of anemia can point toward bone marrow failure, iron deficiency anemia, or chronic disease anemia, where there is insufficient production of new RBCs. This differentiation is crucial for determining the underlying cause of anemia and tailoring appropriate therapeutic strategies.

### 3.3 Monitoring response to treatment

In clinical practice, reticulocyte counts are routinely used to monitor the effectiveness of treatment for anemia. For instance, in patients receiving iron supplements for iron deficiency anemia, a rising reticulocyte count typically precedes an increase in overall hemoglobin levels, serving as an early indicator of treatment response. Similarly, in patients with vitamin B12 or folate deficiency, reticulocyte counts rise following appropriate supplementation, reflecting improved erythropoiesis.

### 3.4 Prognostic value in hematologic disorders

Reticulocyte counts also possess prognostic significance in various hematologic disorders. In sickle cell disease, for example, a sharp increase in reticulocyte count may signal an impending vaso-occlusive crisis, necessitating preemptive medical intervention. In leukemia and myelodysplastic syndromes, reticulocyte counts can provide information about bone marrow recovery post-chemotherapy or bone marrow transplant, influencing patient management and prognosis.

### 3.5 Indicator of treatment-induced bone marrow suppression

In oncology, reticulocyte counts serve as a marker for treatment-induced bone marrow suppression. Patients undergoing chemotherapy or radiation therapy often experience a decline in reticulocyte production due to the cytotoxic effects on bone marrow cells. Monitoring reticulocyte levels helps in assessing the degree of myelosuppression and planning for supportive measures such as transfusions or growth factor administration to mitigate the side effects.

### 3.6 Insights into hemolytic conditions

In hemolytic conditions, the reticulocyte count is a key parameter. Elevated reticulocytes indicate increased RBC turnover, prompting further investigations to identify the cause of hemolysis, such as autoimmune hemolytic anemia, hereditary spherocytosis, or glucose-6-phosphate dehydrogenase (G6PD) deficiency. The degree of reticulocytosis can also help gauge the severity of hemolysis and the adequacy of the bone marrow's compensatory response.

In summary, reticulocytes provide essential information about bone marrow activity, erythropoiesis, and the body's response to various hematologic challenges. Their clinical significance extends beyond simple enumeration; the context of reticulocyte counts, integrated with other laboratory findings and clinical data, enhances diagnostic accuracy, guides treatment decisions, and improves patient management. Thus, the reticulocyte count is an invaluable component in the clinical toolkit for managing hematologic conditions and monitoring therapeutic efficacy.

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## Conflict of interest

The authors declare no conflict of interest.

## Notes/thanks/other declarations

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Chapter 6

# Red Blood Cell Alloimmunization: Life-Threatening Response

*Mohammad Ali Jalali Far and Zeinab Eftekhar*

## Abstract

Alloimmunization is the formation of antibodies against non-self-antigens from a different member of the same species due to exposure to them via transfusion, pregnancy, or transplantation. Further to ABO(H) alloantigens, more alloantibody reactivity toward RBCs appeared as a result of transfusion evolution. Considering that nowadays RBC polymorphisms include more than 300 distinct alloantigens, alloantibodies produced against these antigens can cause various complications such as hemolytic disease of the fetus and newborn (HDFN) or hemolytic transfusion reactions (HTRs) which are related to significant morbidity and mortality. It seems that different factors can influence alloimmunization such as genetic factors, underlying diseases, infection, and inflammation. It is said that expanded antigen matching of RBCs is the only way to reduce transfusion-associated alloimmunization in the future but there is no way to fully eliminate the development and consequences of alloimmunization. So, it seems additional investigations are needed in this field.

**Keywords:** alloimmunization, transfusion, red blood cells, pregnancy, HDFN

## 1. Introduction

Alloimmunization is the formation of antibodies against non-self-antigens from a different member of the same species due to exposure to them via transfusion, pregnancy, or transplantation [1, 2]. The likelihood of alloimmunization can differ from population to population according to blood group antigens expression frequencies [3]. Despite many researches in this field, alloimmunization still remains a common and serious issue in blood transfusion and medical sciences [4]. Various complications such as hemolytic disease of the fetus and newborn (HDFN), erythroblastosis fetalis, or hemolytic transfusion reaction (HTR)s can happen through alloimmunization which can lead to significant morbidity and mortality [1, 4]. Because the increasing complexity of alloimmunization and the importance of improving blood transfusion safety, pregnancy-related care, and fetal/neonatal outcomes in patients, it seems additional investigations are needed to improve knowledge of the development and consequences of red blood cell (RBC) alloimmunization for earlier prevention, diagnosis, and effective treatment of it [5].

## 2. RBC alloantibody formation, detection, and evanescence

Alloimmunization can trigger an immune response, leading to the production of antibodies against the foreign RBC antigens [6]. A small number of blood recipients produce detectable alloantibodies because of having three conditions: (1) exposure to non-self RBC antigens, (2) sufficient dosage of antigen to provoke the immune system, and (3) having the human leukocyte antigen (HLA) to present those antigens [7, 8]. But unfortunately it has to be said that only 30% of induced RBC alloantibodies are detected which can be due to alloantibody evanescence (reduced over time) prior to the next alloantibody screen and/or insufficient sensitivity of commonly employed assays [5, 7]. Studies have indicated that around 70% of alloantibodies become undetectable just a few years after their initial formation [9]. To identify important alloantibodies, "screen" test is used, which is actually the indirect antiglobulin test (IAT) [7]. Traditional simple tube testing or newer methods such as solid-phase, gel technology, flow cytometry, or the enzyme-linked immunosorbent assay may help in completing antibody screening tests [1, 5].

## 3. RBC alloimmunization in the pregnant patient

Pregnancy is another common cause of alloimmunization, as maternal antibodies (IgG) which may be as a result of fetal/maternal hemorrhage during pregnancy/delivery, or via intrauterine transfusion (IUT), can cross the placenta and target fetal RBC surface antigens [1, 10]. Some factors such as prior major surgery, RBC or platelet transfusion, multiparity, prior male child, or operative removal of a prior placenta may be responsible for increased risk of alloimmunization [10]. It is said that HLA-DRB1*15-positive women are also more susceptible to antibody production [11]. In addition, a pregnant woman who has a history of drug use is at a higher risk of alloimmunization and it is probably because of needle-sharing [12]. Without intervention, maternal alloantibodies can cause hemolysis and suppress erythropoiesis, resulting in marked anemia and possibly immunosuppression in the fetus [1]. According to research, it is said that the major cause of fetal anemia is maternal RBC alloimmunization [13]. Up to 1 in 600 pregnancies are affected by maternal RBC alloimmunization and despite primary prevention strategies against RhD antigen, HDFN is mostly caused by anti-D alloantibodies [7]. Interestingly, some antibodies may not be clinically important because they are against antigens with low expression on RBC (anti-Lewis) or they are IgM antibodies that are incapable of crossing the placental barriers(anti-N) [10]. To check the presence or absence of maternal antibodies, IAT is recommended during pregnancy [13].

### 3.1 Hemolytic disease of the fetus and newborn

HDFN is a life-threatening disease that occurs due to the destruction of fetal erythrocytes by maternal IgG alloantibodies that persist for up to 6 months after birth and cause HDFN consequences till neonatal time [2, 14]. The risk of this disease increases in the second and third trimesters of pregnancy because of the increase in transplacental transfer [15]. It can be caused by more than 50 RBC alloantibodies [16]. It seems antigens that antibodies against them cause HDFN in order of importance are: D, c, K, E, Fya/Fyb, Jka/Jkb, and MNS [14]. Approximately 1.25% of pregnant women have clinically important RBC alloantibodies which

affect approximately 1/300 to 1/600 of live births by causing HDFN [2]. It is said about 83% of HDFNs that occur are due to previous pregnancies, 3% are due to previous transfusions, and 14% are undetermined [17]. The history of HDFN in the mother's previous pregnancy is very important because if there is HDFN in the previous pregnancy, the condition will be worse in the following one so previous obstetric history should be checked [2]. If the mother has clinically significant antibodies, the fetus should be examined for the expression of the relevant antigens [18]. Today, non-invasive techniques such as testing cell-free DNA from maternal plasma are used for this purpose [18]. Ultrasound-based techniques are also used for high-risk pregnancies to determine if the fetus is at risk of HDFN if required [19]. If after these investigations, the mother's antibodies are considered dangerous for the fetus, then intermittent monitoring like examining the severity of the disease using the antibody titer (which is considered 1:8 for anti-kell and 1:16 or 1:32 for others as a critical titer) is needed [1]. If the critical titer is reached because one of the most clinically important manifestations of HDFN is fetal and neonatal anemia, Noninvasive detection of moderate and severe anemia can be achieved through the use of Doppler ultrasonography, which relies on the observation of an elevation in the peak velocity of systolic blood flow in the middle cerebral artery [15, 20]. Finally, if needed, IUT, intravenous immune globulin (IVIg), or plasma exchange can be used as therapeutic measures [1].

## 4. Factors suggested to modify alloimmunization risk

The process of alloimmunization is influenced by various factors [21]. According to research, some factors such as the female gender due to increased vulnerability during pregnancy, miscarriages, abortions, and childbirth or maybe it is based on the hypothesis that women have a stronger immune system than men, pro-inflammatory cytokines (due to promote antigen presentation), longstanding infection, diabetes, allogeneic hematopoietic stem cell transplantation, acute chest syndrome, Vaso-occlusive crisis, and solid tumors increase the risk of antibody production and alloimmunization while some others like T regulatory cells (through suppressing immune responses), lymphoproliferative disease, leukemia (because of lymphocyte dysfunction and immunosuppression), and symptomatic atherosclerosis have the opposite effect on it [22–28]. In addition, it is said that an elevated count of reticulocytes in RBC units increases the possibility of alloimmunization in patients receiving them [29]. The genetics of the patient also play a role in the alloimmunization process, particularly with HLA because of its role in the regulation of the immune system [3, 22]. Certain HLA alleles are associated with an increased risk of alloimmunization [3]. It is also interesting that some HLA-IIs are associated with some specific antibody formation, for example, HLA-DRB1*04 with Anti-Fya, HLA-DRB1*11 & -DRB1*13 with Anti-K, and HLA*DRB1*15 with Rh system [3]. In addition to genetic factors, other environmental factors such as antigen disparity between patients and donors, age at first transfusion, and severity of underlying diseases may also contribute to the development of red blood cell alloimmunization [9, 22]. It is believed that factors such as the age and number of red blood cell units transfused and the type and amount of immunogenic antigens encountered during transfusion also may be responsible for modifying alloimmunization [8, 22]. Generally, the risk of alloimmunization depends on both the donor and recipient, which will be discussed below separately.

### 4.1 Donor factors

Genetic factors, length of RBC storage, contamination, and damage to RBCs are responsible for the increased risk of RBC alloimmunization [5, 9]. According to research, it seems that the age of RBC unit has a significant relationship with alloantibody formation, so older RBC units, due to having a higher amount of intracellular heme with a negative effect on the heme oxygenase system, lead to an increase in the level of oxidative stress, inflammation and as a result, alloimmunization [30]. Also, it is said, RBC units that obtained from male donors exhibit a greater susceptibility to storage-related degeneration and hemolysis probably because of testosterone [31].

### 4.2 Recipient factors

Recipients are divided into three groups based on the production of antibodies: (1) the individual who does not produce alloantibodies despite repeated exposure to foreign blood group antigens called "non-responder," (2) the one who produces just one antibody regardless of the number of exposures called "responder," and (3) an individual who produces more than one antibody independent of the number of exposures called "hyper responder" [8]. Various factors cause these differences, for example, some factors such as having certain genetic factors like TNF, MALT1, TLR1, STAT1, TANK, IKK1, IL-2, ADRA1b, IL-6, IL-1B, CTLA4, and some HLA variants including HLA-DRB1*04, -DRB1*15, and -DQB1*03, female sex, prior exposure, method of exposure, antigen dose, viral infection, autoimmunity, myelodysplastic syndrome (MDS), sickle cell disease (SCD), thalassemia, experiencing febrile

| Disease | Alloimmunization rate (%) | Effect on alloimmunization | Reason of effect | References |
|---|---|---|---|---|
| Sickle cell disease | 19–43 | Increase | Frequent blood transfusion reduces Treg suppressive function | [3, 6, 35] |
| Thalassemia major | 5–45 | Increase | Repeated RBC transfusions | [6, 36] |
| Myelodysplastic syndrome | 15–59 | Increase | Higher utilization of blood transfusions, changes in the immune system | [6, 8] |
| Chronic liver or renal failure | 1.3 | Decrease | Hampered (humoral) immune response, renal replacement therapy (RRT) mechanistically modulates RBC alloimmunization | [37, 38] |
| Inflammatory bowel disease | 8–9 | Increase | Inflammation | [6, 33] |
| Aplastic anemia | 11 | Increase | Repeated RBC transfusions | [39, 40] |

**Table 1.**
*A review of some diseases affecting the rate of alloimmunization.*

transfusion reactions, and inflammatory bowel disease (IBD) are responsible for increased risk of RBC alloimmunization [3, 5, 8, 9, 32–34]. In opposite, Some others like older age (it has been reported that individuals aged over 77 years are at a lower risk of blood group antigen alloimmunization probably because of immunosuppression), gram-negative infection, bone marrow failure, acute myeloid or lymphoid leukemia, immunosuppressive drugs, chronic liver or renal failure (CRF), and various genetic factors (like IL-10, TLR7, STAM, OX40L, IFNAR1, STAT4, IRF7, and FCGR2) can reduce the risk of alloimmunization [5, 9]. Some diseases that affect the rate of alloimmunization are summarized in **Table 1**.

## 5. Clinical significance of RBC alloimmunization

Various complications happen through alloimmunization which can lead to significant morbidity and mortality [4, 5]. Among these complications, alloimmunization has the greatest contributions to HDFN and delayed hemolytic transfusion reactions (DHTRs) [9, 41]. In addition, there are some diseases in which the high level of alloimmunization has brought consequences [42]. For example, in acute myeloid leukemia (AML), aplastic anemia (AA), hematopoietic progenitor cell transplant, non-Hodgkin lymphoma (NHL), solid tumors, and especially MDS and SCD patients due to the high-risk of alloimmunization, it is very difficult to find compatible blood and blood transfusions may be delayed in them, which can be dangerous [42].

Among other important changes that occur during alloimmunization, we can mention decreased CD4/CD8 ratio, increased B lymphocytes as well as CD8+ lymphocytes, and Treg lymphocyte deficiency which upset the balance of the immune system and bring consequences [43].

## 6. Clinical management

The easiest and most effective strategy to prevent alloimmunization is to limit blood transfusion and use it only in necessary cases [7]. Using AABB clinical guidelines can help us to make a more accurate diagnosis [44]. The next step to reduce the possibility of alloimmunization is to find the most antigenically similar RBC unit to the recipient by genotyping the recipient for various minor RBC antigens to pick up the most antigen-matched RBC units [8, 45]. Because this practice may not be economical in many cases, it is suggested that the prophylactic matching for antigens other than ABO and Rh be performed for people at risk such as thalassemia major, SCDs, AA, MDS, chronic myeloproliferative disease and other malignancy, CRF who require repeated RBC transfusions, and also pregnant women [8, 40, 46]. It should be noted that it is better to record and store the information obtained in the first hospital or center that the patient visited and make it available to other medical centers using electronic databases [7, 47]. This strategy in addition to limiting the transfusion record fragmentation and duplicated tests and procedures, helps to save time and money and increases the safety of blood transfusion; because if the antibodies are not detectable to a hospital for any reason, then the information collected by previous hospitals can be helpful [7, 48]. However, despite the advantages of this method, its use is still controversial and needs improvement due to the errors that have sometimes occurred [1, 48]. According to recent researches, leukoreduced RBC units may decrease the incidence of RBC alloimmunization [49]. Using immunosuppressants like

corticosteroids also has been shown a significant protective effect against alloimmunization [50]. The effect of splenectomy on alloimmunization is controversial, however, in a study by Evers et al., splenectomy was found to be significantly associated with protection against primary alloimmunization [51]. These strategies are used to prevent the formation of alloantibodies but if alloantibodies are formed, an action should be taken to limit their further development and destructive effects [8]. One approach to prevent the further spread of alloantibodies and reduce the rate of RBCs destruction is immunosuppression using IVIg and corticosteroids [9]. Also, it is said that the use of anti-CD20 antibodies, B cell depletion, or plasma cell targeting in humans has been associated with preventing the formation of new antibodies; however, their definitive effect on alloimmunization needs further investigation [5, 52]. In addition, C5 inhibitor and eculizumab may be advantageous in restricting alloantibody-mediated hemolysis [9].

## 7. Conclusions

Numerous influential factors play a role in the decrease or increase of alloimmunization, making it a multifactorial phenomenon [24]. Despite many researches that have been done in this field, the effect of some factors on alloimmunization is controversial and the researchers have not yet reached an agreement regarding the definitive effect of these factors on alloimmunization. For example, the effect of splenectomy on alloimmunization has been different in several studies, so that in some studies it has been introduced as a risk factor for alloimmunization and in others as a factor to reduce it [51, 53, 54]. The complexity of alloimmunization, along with the variable titer of antibodies during different times and the difficulty of identifying alloantibodies, has made it still have many hidden aspects [5]. Additionally, inadequate recognition of pregnancy alloimmunization causes HDFN to still remain as a serious complication [2, 55]. So, new mitigation and detection strategies and novel therapies of RBC alloimmunization are needed to improve transfusion and pregnancy safety and limit its associated morbidity and mortality, and also it is critical to conduct more investigations regarding better understanding of risk factors for alloantibodies development.

## Conflict of interest

The authors declare no conflict of interest.

## Abbreviations

| | |
|---|---|
| HLA | human leukocyte antigen |
| IAT | indirect antiglobulin test |
| HDFN | as hemolytic disease of the fetus and newborn |
| HTR | hemolytic transfusion reaction |
| RBC | red blood cell |
| IUT | intrauterine transfusion |
| MDS | myelodysplastic syndrome |
| AA | aplastic anemia |

| | |
|---|---|
| SCD | sickle cell disease |
| IVIg | intravenous immune globulin |
| DHTR | delayed hemolytic transfusion reactions |
| AML | acute myeloid leukemia |
| NHL | Non-Hodgkin lymphoma |
| IBD | inflammatory bowel disease |
| CRF | chronic renal failure |

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*Red Blood Cells - Functions and Significance* delves into two interesting aspects. Firstly, the unique structural components, their evolution through the ladders of different phyla, and their membrane with specific functions. The first section also emphasises the synergistic mechanisms of the membrane proteins towards the efficient functioning of these cells. Secondly, their pivotal role as biomarkers in different clinical conditions. The following section gives a comprehensive view of the clinical significance of red blood cells at various stages of their development and differentiation. The book highlights the efficient coordination between the cell's structural organisation and functions. The readers will also be intrigued by the red blood cells' unique structure and critical functions and their role in the early diagnosis of different clinical situations.

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