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Verlauf der Isoagglutinintiter bei Blutspendern:
Eine longitudinale, retrospektive Untersuchung über einen Zeitraum von vier Jahren

Isoagglutinin Titer Changes in Blood Donors:
A Longitudinal Retrospective Analysis Over Four Years

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Zusammenfassung

Die Isoagglutinine wurden vor über hundert Jahren (1) als integraler Bestandteil des AB0-Blutgruppensystems entdeckt und stellen bis heute eine wichtige Säule in der Transfusionsmedizin dar. Ihre Bedeutung hat sich im Laufe der Zeit auf weitere medizinische Disziplinen ausgeweitet, wie zum Beispiel auf AB0-Inkompatibilitäten in der Geburtsmedizin oder bei der Transplantation von Knochenmark und hämatopoetischen Stammzellen in der Hämatologie oder soliden Organen in der Transplantationsmedizin. (2, 3) Obwohl die Isoagglutinine bereits intensiv erforscht wurden, erfolgten bisherige Untersuchungen fast ausschließlich in Querschnittsstudien, ohne dass bisher vollständig geklärt werden konnte, ob und wie sich die Titer der Isoagglutinine im Laufe der Zeit verändern. (4)

Das Hauptziel dieser Dissertation bestand darin, die Veränderungen der Isoagglutinin-Titer bei Blutspendern über einen Zeitraum von vier Jahren zu analysieren. Dabei sollten die Titer nach Blutgruppe, Isoagglutinin-Spezifität, Isotyp und Art der Blutspende unterschieden und es sollte deren Korrelation mit Alter und Geschlecht analysiert werden. Weiterhin sollten sowohl Spender mit starken Titerschwankungen als auch die Prävalenz von Spendern mit hohem Titer, bezogen auf verschiedene kritische Titer Grenzwerte, identifiziert und bewertet werden. Letztlich sollte noch die Prävalenz von Spendern mit fehlenden IgG-Isoagglutininen analysiert werden. Zu diesem Zweck wurden in einer retrospektiven Längsschnittstudie Probenpaare von 1028 Vollblut- und Apherese-Thrombozyten-Spendern mit einer automatisierten Titrationsmethode analysiert, die sich durch minimale Ergebnisschwankungen auszeichnet und die in der Lage ist, zwischen den beiden klinisch relevanten Immunglobulin Isotypen IgG und IgM zu unterscheiden. Die vorliegende Untersuchung zeigte, dass die Veränderungen der Isoagglutinintiter, mit Ausnahme von Anti-A_1 IgM, über alle Blutgruppen und Studiengruppen statistisch signifikant waren. Die Titerveränderungen korrelierten weder mit dem Alter noch mit dem Geschlecht. Bei 4,6% der Blutspender wurden zudem starke Titerveränderungen von drei oder mehr Titerstufen festgestellt, wobei es sich bei der Mehrzahl (3,6%) um IgG Isoagglutinine handelte. Die Prävalenz hoher Titerstufen lag mit einem Titergrenzwert von 128 bei knapp über 20% und mit einem Titergrenzwert von 256 bei 10%. Die Prävalenz von Spendern ohne IgG Isoagglutinine betrug 20% der Studienpopulation.

Die Veränderungen der Isoagglutinintiter sind für die Definition eines Titergrenzwerts relevant, ab dem AB0-minorinkompatible Vollblutkonserven oder Thrombozytenkonzentrate ohne das Risiko einer hämolytischen Transfusionsreaktion transfundiert werden können. Die Häufigkeit, mit der Isoagglutinintiter bei Spendern mit niedrigem Titer überprüft werden sollten, hängt von der Wahrscheinlichkeit einer Titerveränderung ab. (4, 5) Die Festlegung eines kritischen Titer-Grenzwerts ist aufgrund der bekannten Titerschwankungen bereits schwierig und wird durch die meistens verwendetet manuelle Titrationsmethode weiter erschwert. Daher ist es schwierig, die Titrationsergebnisse verschiedener Studien zu vergleichen und sie mit

klinischen Daten zu korrelieren. (6) Transfusionsmedizinische Zentren verfügen normalerweise über eigene Protokolle für AB0-minorinkomaptible Transfusionen (7), allerdings sind nur in wenigen Ländern national anerkannte Titergrenzwerte definiert. Außer in der Transfusionsmedizin sind verlässliche Informationen über Veränderungen der Isoagglutinintiter auch bei AB0-inkompatiblen Nierentransplantationen erforderlich. (8) Auch hier liegen weder zu den kritischen Titern noch zur Vorhersage welcher Isoagglutinin-Isotyp auf das Ergebnis einer Organtransplantation mehr Einfluss hat, international anerkannten Protokolle vor. Dies könnte zumindest teilweise auf die methodenbedingten Schwankungen zurückzuführen sein. (9-12)

Das Design dieser Arbeit wurde so gewählt, dass methoden- und saisonabhängige Schwankungen minimiert werden. Um den Einfluss zufälliger Schwankungen auf die vorliegende Analyse auszuschließen, wurden starke Titerveränderungen als Unterschiede von mindestens drei Titerstufen definiert. Informationen über intrapersonale Veränderungen der Isoagglutinintiter über die Zeit, insbesondere wenn diese mit automatisierten Methoden gemessen wurden, liegen bisher kaum vor. Die vorliegende Arbeit untersucht die bislang meisten Probanden über den längsten bisher publizierten Zeitraum. Durch die Vielzahl der ausgewerteten Proben war es auch möglich, Probanden mit starken Titerveränderungen über die Zeit zu identifizieren und die Prävalenz von Spendern mit hohem Titer in Relation zu verschiedenen Titergrenzwerten zu untersuchen. Zum Vergleich mit der vorliegenden Untersuchung konnte nur einer kleinen dänischen Längsschnittstudie, die dieselbe Testmethode verwendet hat, herangezogen werden. In dieser dänischen Studie wurden, bis auf einen Probanden mit großer Titerschwankung (drei Titerstufen), nur minimale Titerschwankungen unter den 56 Probanden gefunden. (5) Übereinstimmend mit der dänischen Studie wurde kein Zusammenhang der Titerveränderungen mit dem Alter oder dem Geschlecht gefunden. Darüber hinaus ergab die vorliegende Untersuchung, dass nur 20% der Blutspender gar keine IgG-Isoagglutinine aufwiesen, was unabhängig vom Geschlecht oder der Spendenart war. Dieses Ergebnis lässt sich nicht ausschließlich durch Schwangerschaften oder Transfusionen als Antikörper stimulierende Faktoren erklären, sondern legt die Beteiligung weiterer stimulierender Faktoren für die Produktion von IgG Isoagglutininen nahe. Möglicherweise sind andere Faktoren, wie Ernährung, Probiotika und Umweltfaktoren, für Veränderungen der Isoagglutinin-Titer verantwortlich. Die vorliegenden Ergebnisse stellen eine verlässliche Datenquelle zu Isoagglutinintitern und zu Titerveränderungen dar, die bei der Erstellung von Vorgaben zur AB0-minorinkompatiblen Transfusion oder Organtransplantation herangezogen werden können. Darüber hinaus bieten die Ergebnisse eine zuverlässige Grundlage für Isoagglutinintiter-Vergleiche in zukünftigen Studien, insbesondere wenn die Dynamik von Titerschwankungen oder die Ursachen-Wirkungs-Beziehungen verschiedener Stimuli untersucht werden sollen.

List of Abbreviations

AHG	Anti-Human Globulin
CI	Confidence Interval
CMOS	Complementary Metal-Oxide Semiconductor
COVID-19	Coronavirus Disease 2019
DDT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
HDFN	Hemolytic Disease of the Fetus and Newborn
HLA	Human Leucocyte Antigen
HSCT	Human Stem Cell Transplantation
Ig	Immunoglobulin
ISBT	International Society of Blood Transfusion
K_m	Michaelis-Menten Constant
kDa	Kilodalton (Molecular Weight)
LISS	Low Ionic Strength Solution
LTOWB	Low Titer Group 0 Whole Blood
M	Molarity
PBS	Isotonic Phosphate Buffered Saline
PEI	Paul Ehrlich Institute
RBC	Red Blood Cell
RPM	Revolutions per Minute
SARS-CoV-1	Severe Acute Respiratory Syndrome Coronavirus Type 1
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus Type 2
TS	Titer Step
TSD	Titer Step Difference

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1 Introduction and Aim of the Dissertation

The isoagglutinins were discovered by Landsteiner during his experiments on the agglutinating effects of serum in 1900, just one year before his monumental discovery of the AB0 blood groups. (1) Their importance was immediately recognized in transfusion medicine, as they are naturally occurring antibodies and responsible for the fatal outcomes of early whole blood transfusions. (13) Today, blood transfusions are tailored to the specific needs of the patient, with AB0-compatible red blood cell or plasma components transfused separately. Nevertheless, the isoagglutinins and their titer must be considered when minor incompatible blood components such as platelet units or low titer blood group 0 whole blood must be transfused. Because of the short shelf life of platelet units, units of certain blood groups are in short supply and sometimes must to be transfused blood group AB0 non-identical. (14) Low-titer group 0 whole blood units from known low titer donors are used in various countries in military or civilian traumatology. (4) The importance of isoagglutinins extends beyond transfusion medicine to stem cell transplantation and kidney transplantation. In addition, isoagglutinin titer, especially of placenta crossing IgG isotype, can be of clinical importance in AB0 minor incompatible pregnancies. (13)

The recent interest in isoagglutinins is related to their titer and the probability of titer change. Although isoagglutinins have been studied for over a hundred years, they have been analyzed mostly in cross-sectional studies, and there is scarce information available on intrapersonal changes in titer over time. (5) Blood donors known as low titer must be titrated regularly based on the likelihood of titer change, to maintain their low titer status and be able to donate low titer blood components. (4) A few recent longitudinal studies examined changes over short periods of time in populations that were either small or composed mainly of blood group 0 individuals, using mostly manual titration methods. In these studies, only minimal isoagglutinin titer changes were observed. (4, 5, 8, 15, 16) Nevertheless, acute increases in titer have been reported over the years, particularly in the context of transfusion reactions. (6, 17, 18) The factors causing titer changes and the extent of their influence are not completely elucidated. (4) It has been considered that the main factors influencing the isoagglutinin titers are AB0 incompatible pregnancies and blood transfusions, and that the isoagglutinin titers decrease with increasing age. (19) Nevertheless, titer changes have been reported in association with dietary supplements such as probiotics (18), with diet, possibly through the composition of gut microbiota (20, 21), and with several early vaccines (15).

In addition to isoagglutinin titer changes, a safe critical titer cutoff must be defined in relation to AB0 minor incompatible transfusion and organ transplantations to minimize hemolytic transfusion reactions and graft rejections. (5, 8) Transfusion and transplantation institutions

usually have internal protocols in place to regulate AB0 minor incompatibility. (7) However, to date, there is no internationally accepted critical titer. (15)

Establishing a critical titer cutoff for AB0 incompatibility is challenging because isoagglutinin titration results, commonly obtained by manual methods, are known to vary due to inter- and intra-personal biases. (22, 23). Automated titration methods are used in fewer laboratories, probably because of the higher costs. (24, 25) The titration results obtained by different methods are difficult to compare and subsequently correlate with clinical data. In addition, most methods cannot accurately distinguish between subclasses of isoagglutinins without laborious sample preparation. Nevertheless, the determination of isoagglutinin isotype is clinically relevant in obstetrics, transfusion and transplantation medicine. (13)

The main objective of this dissertation was to analyze longitudinal changes of isoagglutinin titers in blood donors over a four-year period by blood group, isoagglutinin specificity, isotype and donation type, and their correlation with age and gender. In addition, the aim of the study was to identify and evaluate blood donors with large titer changes and the prevalence of donors with high titer isoagglutinins in relation to various critical titer cutoffs. Furthermore, the prevalence of blood donors lacking IgG isoagglutinins was to be analyzed. For this purpose, sample pairs from 1028 whole blood and apheresis platelet donors were to be analyzed in a retrospective longitudinal study. The study design accounted for possible seasonal titer fluctuations, as blood samples were collected during the same period of the year. An important feature of the study was the use of an automated titration method known for minimal result variation. (26-28) Moreover, this automatic method enabled the differentiation between the two clinically important immunoglobulin isotypes, IgG and IgM, and provided accurate and consistent results. (29, 30)

2 Literature Discussion

2.1 Discovery of Isoagglutinins

The isoagglutinins are regular alloantibodies produced by individuals lacking the corresponding antigen on the surface of their erythrocytes (31). The isoagglutinins were discovered in 1900 by Landsteiner, an Austrian pathologist, at the University of Vienna. He observed that human serum was capable to agglutinate erythrocytes from both humans and animals. This discovery was published as a footnote to his paper in German *"Zur Kenntnis der Antifermentativen, Lytischen und Agglutinierenden Wirkungen des Blutserums und der Lymphe"*, meaning *"To the Knowledge of the Anti-Fermentative, Lytic and Agglutinating Effects of Blood Serum and Lymph"*. (1) A year later he published the discovery of three blood groups, which he first named A, B and C (32). A forth blood group, called blood group AB, was discovered by von Decastello and Sturli in 1902. (33) Later, blood group C was renamed O from the German "ohne" meaning without (34) to indicate the absence of antigens A and B on the surface of erythrocytes. However, the phenotype O is referred to as 0 (number zero) in Germany and therefore in the present dissertation.

2.2 AB0 Blood Group System

The AB0 group system includes two common antigens: A and B, which define the four major blood groups phenotypes A, B, AB and 0 (35). In addition to antigens, the AB0 blood group system includes two regular antibodies called isoagglutinins, anti-A and anti-B. Isoagglutinins anti-A are normally present in sera from blood group 0 and B individuals, whereas isoagglutinins anti-B are present in sera from blood group 0 and A individuals. Sera from blood group AB individuals are usually free of isoagglutinins. (19) This simplified understanding of the AB0 blood group system is presented in **Table 1**.

Table 1 *AB0 Blood Group System*

AB0 Phenotype	Antigens	Isoagglutinins
0	none	anti-A and anti-B
A	A	anti-B
B	B	anti-A
AB	A and B	none

Note. Reference (19)

Based on antigen expression on the erythrocyte surface, the blood groups of AB0 system can be further divided into subgroups. The most common are A_1, A_2, B, A_1B, A_2B and 0. In order to distinguish between antigen, phenotype and gene, a conventional terminology was adopted using subscripts, superscripts and italicized letters, where for example A1 indicates the antigen, A_1 represents the phenotype and A^1 denotes the allele. (36, 37)

2.2.1 Molecular Genetics

The production of antigens of the AB0 blood group system is influenced by three genes: the *ABO* gene, located on chromosome 9, and the *FUT1* and *FUT2* genes, located on chromosome 19 (19). These genes encode enzymes that further catalyze the biosynthesis of the antigens as shown in **Table 2** (38).

Table 2 *Some Glycosyltransferase Genes and the Enzymes They Produce*

Locus	Allele	Transferase
ABO	*A*	α1,3-*N*-acetyl-D-galactosaminyltransferase
	B	α1,3-D-galactosyltransferase
	O	none
FUT1 (H)	*H*	α1,2-L-fucosyltransferase
	h	none
FUT2 (SE)	*Se*	α1,2-L-fucosyltransferase
	se	none

Note. Reference (19)

The genomic organization of the *ABO* gene, determined in 1995 by Yamamoto and Bennett (39), consists of seven exons, with the last two, exons 6 and 7 containing the majority of the coding sequence (19). There are five common alleles described for the *ABO* gene: A^1, A^2, *B*, O^1 and O^2 (19). The alleles *A* and *B* are autosomal codominant (40), whereas the *O* alleles are amorph and autosomal recessive (41). The allele A^1 is dominant over A^2 allele (19).

The nucleotide sequence of allele A^1 (common *A*, ISBT *ABO*A1.01*) is used as reference when describing the other alleles. The allele A^1 encodes the enzyme A_1-transferase (19). Compared to allele A^1, allele A^2 (ISBT *ABO*A2.01*) has one nucleotide substitution (c.467C>T, resulting in amino acid substitution p.Pro156Leu) and a nucleotide deletion (c.1061delC) that disrupts the subsequent stop codon of an A^1 allele, resulting in a product with an 21 amino acids addition to the carboxyl-terminus (19, 38, 42). The allele A^2 encodes the enzyme A_2-transferase. (19). Compared to allele A^1, allele *B* (ISBT *ABO*B.01*) has seven nucleotide substitutions (c.297A>G, c.526C>G, c.657C>T, c.703G>A, c.796C>A, c.803G>C and c.930G>A) which result in only four amino acid replacements (p.Arg176Gly, p.Gly235Ser, p.Leu266Met, p.Gly268Ala) (19, 38, 43). These four amino acid substitutions change the specificity of the produced enzyme from A- to B-glycosyltransferase (44). Allele O^1 (O01 or ISBT ABO*O.01.01) is identical to allele A^1, except for one nucleotide deletion at position 261 (c.261delG) (38), which results in a translation stop signal at the codon for amino acid 116 and lack of production of an active enzyme (19). Another common allele, O^1-variant or O^{1v} (O02 or ISBT ABO*O.01.02), has additionally to the nucleotide 261 deletion another nine nucleotide differences (c.106G>T, c.188G>A, c.189C>T, c.220C>T, c.297A>G, c.646T>A, c.681G>A, c.771C>T; c.829G>A) compared to allele A^1 (19, 38, 45). Compared to allele A^1,

allele O^2 (O03 or ISBT ABO*O.02.01) does not possess the nucleotide deletion at position 261, but rather five nucleotide substitutions (c.53G>T, c.220C>T, c.297A>G, c.526C>G, c.802G>A) which result in four amino acid replacements (p.Arg18Leu, p.Pro74Ser, p.Arg176Gly and p.Gly268Arg) (38). The last amino acid substitution is probably responsible for the production of the inactive enzyme (19, 46, 47).

The *ABO* allele frequencies determined in the German population were 0.214 for allele A^1, 0.068 for allele A^2, 0.051 for allele *B*, 0.650 for allele O^1 and 0.017 for allele O^2. (19, 48) The most common *ABO* genotypes, predicted phenotypes, as well as phenotype frequency in Germany are presented in **Table 3**.

Table 3 *Common ABO Genotypes and Phenotypes, and Phenotype Frequency*

Genotype	Phenotype	Phenotype Frequency in Germany	
A^1/A^1	A_1	A	43.26%
A^1/A^2			
A^1/O			
A^2/A^2	A_2		
A^2/O			
B/B	B	B	10.71%
B/O			
A^1/B	A_1B	AB	4.86%
A^2/B	A_2B		
O/O	0	0	41.21%
Note. Reference (19)		(49)	

A relatively rare allele group of *ABO* gene is represented by *cisAB*, a chimera of alleles *A* and *B* caused either by a mutation of an *A* or *B* gene or by an unequal crossing-over, resulting in adjacent *A* and *B* genes (19). These alleles encode either a glycosyltransferase capable of both A and B activities (50, 51) or two separate enzymes (52). The cisAB phenotype was described in 1964 when an unusual inheritance pattern was observed in two generations with 0 phenotype parents of A_2B children (19, 53). The name cisAB proposed in 1973 helps differentiate the phenotype from the common transAB (54). The most common allele *cisAB01* (50) (ISBT *ABO*cisAB.01*) has two nucleotide substitutions (c.467C>T, c.803G>C) which cause two amino acid substitutions (p.Pro156Leu, p.Gly268Ala) (38).

Another rare allele group is represented by the *BA* alleles which are responsible for the B(A) phenotype. The first *BA* allele described in 1993 (ISBT *ABO*BA.01*) had only five of the seven nucleotide substitutions of a *B* allele, with nucleotides 657 and 703 remaining unsubstituted (55). Consequently, there are only three amino acid substitutions present in the produced B-transferase, position 235 being identical to an A-transferase (38). The B(A) erythrocytes possess antigen B and only fine traces of antigen A (35).

The A- and B-transferases encoded by the *ABO* alleles need the H antigen as acceptor substrate for the synthesis of A and B antigens (19). The H antigen is the indirect product of the genes *FUT1* and *FUT2.* Each of these genes encodes an enzyme, a fucosyltransferase which catalyzes the biosynthesis of antigen H in different tissues and secretions. (19).
The *FUT1* gene (reference allele *FUT1*01* or *H*) has four exons and the *FUT2* gene (reference allele *FUT2*01* or *Se*) has two exons (38), but only one exon encodes the enzyme (exon 4 of *FUT1* and exon 2 of *FUT2*) (19). The *FUT1* gene regulates the biosynthesis of H antigen expressed on glycolipids and glycoproteins of tissues of endodermal and mesodermal origin (including erythrocytes) meanwhile the *FUT2* gene regulates the presence of H antigen on glycoproteins of ectodermal origin and in secretions (19, 56). 80% of Europeans are antigen secretors, possessing the *Se* gene (19). Both genes encodes a similar α1,2-L-fucosyltransferase (H- and Se-transferase) which catalyze the addition of L-fucose to the terminal galactose of precursor disaccharides, called peripheral core structures (19, 35).

There were five types of peripheral core structures identified: Type 1 (Galβ1→3GlcNAcβ1→R), Type 2 (Galβ1→4GlcNAcβ1→R), Type 3 (Galβ1→3GalNAcα1→R), Type 4 (Galβ1→3GalNAcβ1→R) and Type 6 (Galβ1→4Glcβ1→R) (19, 39). Type 1 antigens are found on tissues derived from endoderm and in secretions. Type 2 antigens are present on red cells, platelets, on tissues derived from ectoderm and mesoderm, and in secretions. Type 3 chain antigens, such as repetitive Type 3 chains are found on glycolipids of blood group A erythrocytes. Type 4 chain antigens are present on glycolipids in abundant quantities in kidneys and in small quantities on erythrocytes. Type 6 chain antigens are found in milk and urine. (19) Due to the wide distribution of the H antigen on different tissues and cell types in the human body, and consequently of A and B antigens, the ABO and H systems are also named histo-blood groups (37).

2.2.2 Antigens and Phenotypes

The antigens in AB0 blood group system are carbohydrates and, as presented above, they are indirect products of the responsible genes (19). On normal erythrocytes there are up to one million antigens belonging to AB0 blood group system per cell (41). The International Society of Blood Transfusion has listed four antigens as belonging to the AB0 blood group. They are A, B, A,B and A1 and are presented in **Table 4** (57).

Table 4 *ISBT Numerical Notation of AB0 Blood Group System and Antigens*

Blood Group System		Antigen Number			
001	ABO	001	002	003	004
		A	B	A,B	A1

Note. Reference (57)

The biosynthesis of A antigen is catalyzed in the final step by A-transferase, α1,3-*N*-acetyl-D-galactosaminyltransferase, which adds *N*-acetyl-D-galactosamine to the fucosylated galactose of H antigen (19). The A_1- and A_2-transferases, produced by the A^1 and A^2 alleles respectively, are qualitatively different, as they have different pH optima, cation requirement and K_m value, with A_2-transferase having a lower activity (58). Therefore, there are quantitative differences, like the number of antigen sites per cell, as well as qualitative differences between A_1 and A_2 erythrocytes (19). Per cell, the number of A antigen on A_1 and A_1B erythrocytes is 8 to $12*10^5$ and 5 to $9*10^5$ respectively, meanwhile on A_2 or A_2B cells there are 1 to $4*10^5$ and $1*10^5$ antigen sites, respectively (19). One of the qualitative differences between A_1 and A_2 erythrocytes is represented by the presence of repetitive Type 3 A structures on A_1, but not on A_2 cells (19, 59, 60). The A_2 phenotype is the most frequent A weak phenotype, having higher H antigen sites that were not transformed in antigen A (41). The phenotyping of A subgroups, however, is based only on the quantitative difference and is tested with the help of *Dolichos biflorus* lectin (19). This lectin, a plant extract capable to agglutinate erythrocytes, recognizes α-linked *N*-acetylgalactosamine and, when appropriately diluted, agglutinates A_1 cells but not weak A phenotypes (41).

Antigen A1 is a distinct antigen from antigen A and represents a repetitive Type 3 A structure present on erythrocytes of A_1 phenotype (35). The addition of a galactose to the terminal *N*-acetyl-D-galactosamine of an Type 2 A antigen transforms it into a peripheral core structure of Type 3 and after further fucosylation into Type 3 H antigen (19). A_1-transferase can synthetize the antigen A1 by adding an *N*-acetyl-D-galactosamine to the Type 3 H antigen. Weaker enzymes like A_2-transferase cannot catalyze the biosynthesis of antigen A1 (35). Subgroup A_1 cells possess antigens A and A1, whereas A_2 cells have only the A antigen (19).

The biosynthesis of B antigen is catalyzed by B-transferase, α1,3-D-galactosyltransferase, which adds D-galactose to the fucosylated galactose of H antigen (19).

In case of homozygosity for *O* allele, a nonfunctional protein is produced and the H antigen remains unchanged, resulting the 0 phenotype (37). Fucose, N-acetyl-D-galactosamine and galactose are the immunodominat sugars of antigens H, A and B, respectively, and their loss represents the loss of antigen activity (19).

In the absence of H- or Se-transferase (homozygosity for both *FUT1*- and *FUT2*-null alleles), the H antigen cannot be synthetized and consequently neither A or B antigens, even if in the presence of A- or B-transferases. This rare autosomal recessive phenotype is known as the Bombay phenotype. (19) The para-Bombay phenotype however has an active *FUT2* gene and expresses H antigen in secretions and some tissues (41).

The A,B antigen is a common epitope which is present on A, as well as on B antigens and is distinct from the common structural motif representing antigen H (Fucα1-2Gal) (61). The antigen A,B is present in A, B and AB phenotypes and is recognized by a single immunoglobulin anti-A,B, but not by the mixture of separate anti-A and anti-B. (19, 61) The antigen was experimentally demonstrated in 2005 (61).

The antigens of the AB0 blood group system are expressed, additionally to erythrocytes, on lymphocytes and on platelets. The expression on lymphocytes is not intrinsic but rather acquired from plasma in secretors and under the control of the *FUT2* gene (19). The expression of the antigens on platelets and megakaryocytes is primarily regulated by the *FUT1* gene (62), but they can also be adsorbed from plasma in secretors under the control of the *FUT2* gene (19). Granulocytes and monocytes do not express antigenic structures of the AB0 blood group system regardless of the secretor status (19). In addition to the hematopoietic tissue, the histo-blood group antigens are also expressed on various tissues (63). As Type 2 structures and under the control of *FUT1* gene, they are expressed on epidermis, primary sensory neurons, vascular endothelium, renal glomeruli and convoluted tubules. In digestive and respiratory mucosae and renal urinary epithelium, the antigens can be of Type 1 or 2, primarily under the control of the *FUT2* gene (19).

2.2.3 Isoagglutinins

In addition to antigens, the AB0 blood group system includes alloantibodies that are produced prior to any stimulation by human antigens. The antibodies are called isoagglutinins and they are produced by humans lacking the corresponding antigen. Hence, the serological AB0 blood group typing entails the determination of antigens, forward typing, as well as the determination of antibodies in the reverse typing. There are two common antibodies in the AB0 blood group system, anti-A and anti-B. (41)

The isoagglutinins are secreted by B1 B lymphocytes that are one of the three main classes of B lymphocytes. B1 B lymphocytes originate from B1 progenitors found in the fetal liver and they continue to self-renew after the perinatal period. The antibodies are directed against carbohydrate antigens of commensal microflora, which are similar to the antigens in the AB0 blood group system. Carbohydrate and phospholipid antigens can stimulate antibody production without the involvement of T Lymphocytes and are called T-independent antigens. It was demonstrated in mice that B1 B cells produce IgM antibodies against T-independent antigens. (64) A positive correlation between B1 B lymphocytes and IgM and IgG isoagglutinins was also reported in humans. (65)

The development and production of isoagglutinins was linked to intestinal flora, especially to Gram-negative microorganisms. Early studies demonstrated that many Gram-negative bacteria express substances with A- and B-like antigen activity (66). The production of isoagglutinins, either de novo or titer increase, was demonstrated after stimulation with inactive bacteria, such as *Escherichia coli* O86 (a Gram-negative high B- and faint A-active bacterial strain) in chickens and also in humans. (67, 68)

The isoagglutinins are soluble immunoglobulins belonging to the isotypes IgM, IgG and IgA (19). Immunoglobulin G is a monomer antibody with the basic structure of four-chain immunoglobulin with two identical heavy chains and two identical light chains connected by disulfide bonds. IgG antibodies have two identical antigen-binding sites and are of four subclasses with molecular masses of 150kDa (IgG_1, IgG_2, IgG_4) and 170 kDa (IgG_3). Depending on the subclass, IgG antibodies are capable to cross the placenta and activate complement. Immunoglobulin M is a pentameric molecule composed of five basic Y-shaped structures joined together by a joining chain, with a total of ten antigen-binding sites. IgM antibodies are large molecules with a molecular masse of 970 kDa and can therefore cause direct agglutination but cannot cross the placenta. Immunoglobulin A can exist as a monomer, dimer, trimer or multimer. IgA antibodies are present in serum as a monomer, while in secretions they exist in a dimeric form with molecular masse of 390 kDa. IgA antibodies do not cross the placenta. Two molecules of IgA are joined together by a joining chain, similar to IgM. (69)

The antibody response to non-protein antigens such as the antigens of AB0 blood group system is primarily based on IgM production. (70) Early studies on isoagglutinins reported that in individuals without further immunization events, such as pregnancy or blood transfusion, the isoagglutinins belong almost exclusively to the IgM class. (71) In other reports, IgG isoagglutinins were found in all non-immunized subjects examined. These individuals, who participated in an immunization program that used human A and B antigens for the manufacture of blood grouping reagents, had IgG but no IgA isoagglutinins present before the immunization event. After immunization, IgG titer increased and all donors also produced IgA isoagglutinins. (19, 72) Some of the characteristics of different classes and subclasses of isoagglutinins are listed in **Table 5** and **Table 6**.

Table 5 *Characteristics of the Different Isoagglutinin Isotypes*

Characteristic	**IgM**	**IgG**	**IgA**
Sera of non-immunized donors	yes	sometimes	rarely
Sera of immunized donors	yes	usually	usually
Erythrocyte agglutination	yes	yes	yes
Hemolytic	yes	yes	no
Complement binding	yes	yes	no
Titer increased in antiglobulin test	no	yes	yes
Thermal optimum	4˚C	4-37°C	
Activity destroyed by dithiothreitol	yes	no	partially
Activity destroyed by heating	yes	no	no

Note. Reference (19)

The IgG isoagglutinins are predominantly belonging to IgG_1 and IgG_2 subclasses and rarely to subclasses IgG_3 and IgG_4 (19, 73). The same was observed in sera of group 0 mothers who gave birth to blood group A or B children; IgG_2 isoagglutinin titer was higher compared to the other subclasses and IgG_3 and IgG_4 were present in only 40% of the analyzed cases. (19, 74) Isoagglutinins present in different secretions such as saliva, milk, tears, cervical secretions, or cysts are IgA. (19)

Isoagglutinins are almost never found in neonatal sera (19) and when present, they are mostly IgG of maternal origin (75). Isoagglutinins are produced in the first six months of life after immunization with A and B antigens existing in nature and reach adult concentrations by 5 to 10 years of age. (41, 76) Early studies reported a decrease in isoagglutinin titer with age, but later studies have not confirmed this observation (76, 77).

In addition to the separate isoagglutinins anti-A and anti-B, sera from many individuals of blood group 0 contain a cross-reacting antibody named anti-A,B. This antibody reacts with the A,B epitope, a structure common to both A and B antigens, and is predominantly of the IgG isotype, rarely IgM and IgA. (19) Anti-A,B does not interact with the H antigen. (61) It can

be demonstrated by incubating A erythrocytes with group 0 sera, followed by the elution of the adsorbed antibodies. The eluate obtained will agglutinate both A and B erythrocytes. The same will happen if B erythrocytes are incubated with 0 sera; the eluted antibodies will agglutinate both B and A erythrocytes. (19)

Table 6 *Characteristics of IgG Subclasses*

Characteristic		**IgG_1**	**IgG_2**	**IgG_3**	**IgG_4**
Placenta crossing		++	+	++	-
Complement activation	Classic pathway	++	+	++	-
	Alternative pathway	+	+	+	+
Note. Reference (69)					

In addition to anti-A isoagglutinins, sera from blood group B individuals contain anti-A1 antibodies. Erythrocytes A_1 agglutinate with both antibodies, anti-A and anti-A1, whereas A_2 erythrocytes agglutinate only with anti-A. Adsorption of group B sera with A_2 erythrocytes initially removes only anti-A and eventually also anti-A_1 after continued adsorption. (19) Anti-A1 antibodies can also be present in sera of A_2 (1%-2%) and A_2B (22%-26%) individuals. (19, 37) However, anti-A1 antibodies are not clinically relevant. (35)

Alongside AB0 alloantibodies, AB0 autoantibodies could also be found. Auto-anti-A and auto-anti-B are rare and can occasionally cause autoimmune hemolytic anemia. (19) A fatal fulminant intravascular hemolysis and kidney failure has been reported in association with auto-anti-A. (78) The AB0 autoantibodies can be of the IgM and IgG isotype. (19)

Furthermore, anti-A or anti-B antibodies can be produced by passenger lymphocytes of graft origin and cause severe and even fatal hemolysis. (19) Such antibodies, typically IgG, have been reported after solid organ transplantation (kidney, liver, hear, lung, pancreas or spleen) (79) or after minor incompatible bone marrow and peripheral blood stem cell transplantations (80-82). They appear about seven to 10 days after transplantation and persist for about a month. (19)

2.2.4 Isoagglutinin Titer Influencing Factors

The baseline isoagglutinin titer of an individual depends on a variety of parameters. Some of these are genetic, such as AB0 blood group and sex. Others are either environmental factors or influenced by them, such as age, gender, nutrition, infectious diseases, vaccines, pregnancies, or blood transfusions. In a study examining the association between genetics and isoagglutinin levels in relatives from 74 families, it was found that only 20%-30% of the variation could be explained and linked to genetic makeup. (83)

Age. Aging has been associated with a decline in immune responses. (84, 85) Currently it is recognized that not all immune responses decline with age and that some can even increase. Therefore, the changes are now described as a progressive occurrence of dysregulation, characterized by decreased cell-mediated and persevered humoral immune responses. Although B lymphocyte functions are preserved with a relative increase in IgA and IgG production, the affinity of the antibodies produced decreases with age. (86) Various factors have been associated with decreased immune response in elderly individuals, such as nutritional status, macro- and micronutrient deficits, and the presence of subclinical diseases. (84, 86) Early studies of age-related isoagglutinin titer changes reported a gradual decline in titer. However, these findings could not be supported by later studies. (76, 77) Lower isoagglutinin titers have been reported in very elderly, healthy individuals over 90 years of age, but, some of them also reached high titers. (76) A lower isoagglutinin affinity with a relatively unchanged titer has been demonstrated in healthy elderly individuals. (85)

Sex and Gender. Not only sex specific biological factors, but also gender related social factors can influence immune responses. Biological differences between women and men, such as the hormonal differences are also reflected on the function of B lymphocytes. Estrogen increases while testosterone inhibits the production of IgM and IgG immunoglobulins. (87) In pregnancy, the elevated levels of progesterone appear to suppress the cell-mediated immune response and enhance the humoral immune response. (86) Furthermore, gender represents an important influencer of lifestyle. Exposure to different antigens, prioritization of health needs, domestic or physical activities can elicit different immune response in females and males. (87) It is known that isoagglutinin titers are higher in females compared to males. (88)

Nutrition and Environment Factors. Nutritional status is closely related to changes in immune responses. It has been shown that both quantitative and qualitative changes in various macronutrients can affect the immune response even in healthy individuals. (86) Furthermore, in addition to diet, environmental factors can also influence the composition of intestinal flora (21) and thus affect isoagglutinin production. (20) The consumption of processed foods in developed countries is also reflected in gut flora composition (21) and in the isoagglutinin titer

(20). It was found that isoagglutinin levels in the Japanese population decreased over a 15-year period due to a more westernized lifestyle compared to the titers in the Thai and Laotian populations, who consume more natural foods. (20) In one study, the microbial composition of intestinal flora was investigated by analyzing stool samples of healthy individuals living in three different environments: Amerindians from the Amazonas of Venezuela, rural Malawian population, and metropolitan population of United States of America. One of the differences between the three population groups was that the two rural populations had a natural diet compared to the metropolitan population. The study reported significant differences in the microbial composition of gut flora in both children and adults between the urban and the rural populations. It was also found that genetically unrelated but cohabiting family members showed similarities in microbial composition across cultures. Moreover, differences related to age and geographical location have also been reported. (21)

Probiotics. Probiotics are dietary supplements association with isoagglutinin stimulation. An increase in anti-B isoagglutinin titer was demonstrated in a case report examining hemolytic transfusion reactions to platelet units. (18) Probiotics are “live microorganisms which when administered in adequate amounts confer a health benefit on the host” as defined by the joint expert consultation of the Food and Agriculture Organization of the United Nations and the World Health Organization (89). Microorganisms used as probiotic may belong to genera such as *Lactobacillus, Bifidobacterium, Saccharomyces, Enterococcus, Streptococcus, Pediococcus, Leuconostoc, Bacillus, or Escherichia* (90, 91). The idea that regular consumption of beneficial microbes along with the food intake might be related to good health was introduced by Metchnikoff in 1907 after observing the longevity of Bulgarian peasants which consumed regularly fermented dairy products. He related this observation with the “Bulgarian bacillus”. (92) The use of probiotics has shown some health benefits in the prevention and treatment of some gastrointestinal conditions, dental disorders or conditions related to allergy. (93, 94) Probiotic consumption was associated with immune system stimulation and enhanced antibody response in children and adults, immunoglobulin enhancement. (95-98) A double-blind, placebo-controlled study conducted in children of low socioeconomic status showed that probiotic supplementation had no immune-stimulating benefit. These results have been attributed to increased early microbial exposure in those children. Furthermore, there was no significant difference in the isoagglutinin levels between the control and experimental groups. (99)

Vaccines. Many of the earlier versions of biologically derived vaccines were reported to have AB0 blood group antigen-like activity. The substances responsible were either biological extracts of bacteria or viruses, or substances used in the manufacturing process. Vaccines such as influenza, pneumococcal or tetanus vaccines could stimulate IgG isoagglutinin production and cause hemolytic transfusion reactions when administered to blood donors or

even hemolytic disease of the newborn when administered blood group 0 to pregnant women. However, the new vaccines, which are either biosynthetic or highly purified, show no effect on the isoagglutinin titer. (15) A study published in 2016 reported a subtle increase in IgG isoagglutinin titer after vaccination with *Streptococcus pneumoniae* type 23 polysaccharide. However, the increase was only detectable with surface plasmon resonance, a technology of increased sensitivity. This titer change was so subtle that it could not be visualized using conventional agglutination methods. (100)

Infectious Diseases. AB0 blood group antigen-like activity has been reported over the years for various bacteria, particularly gram-negative bacteria such as *Escherichia coli* (66), viruses such as *Streptococcus pneumoniae* (100-102), and parasites such as *Ascaris suum, Ascaris lumbricoides*, *Necator americanus*, *Trichinella spiralis*, *Schistosoma mansoni, Taenia solium* (103, 104). Several infections with these microorganisms have been reported to be accompanied by increases in isoagglutinin titers. (41) Higher isoagglutinin titers have also been reported in patients with chronic malaria and visceral larva migrans (103, 104).

Seasonal Changes. Seasonal and circadian fluctuations are known for several immune parameters. In a study that analyzing various immune parameters related to seasonality it was found that the levels of circulating lymphocytes, neutrophils and C-reactive protein, with the exception of monocytes, showed variation. Moreover, no association was found between these changes and population traits, lifestyle, or environment in that study. (105) Furthermore, seasonal variation in isoagglutinin titer was observed in a retrospective longitudinal study that analyzed changes in IgM isoagglutinin titer in an active military population of blood donors over an 18-month period. It was observed that anti-A IgM titers were higher in spring and lower in autumn than in the winter months, and that anti-B IgM titers were lower in spring and autumn and higher in summer than in the winter months. (4)

2.3 Clinical Importance of Isoagglutinins

The importance of isoagglutinins is reflected by their mandatory determination as part of the AB0 blood group determination, which is regulated in Germany by the national Scientific Guideline for Hemotherapy (106). The isoagglutinins are of clinical importance in transfusion medicine, obstetrics, stem cell and organ transplantations. (13) In case of AB0 incompatibility, isoagglutinins meet the corresponding antigens, form antigen-antibody complexes and can cause hemolysis. The severity of hemolysis is influenced, among other factors, by the quantity and quality of isoagglutinins. (107)

Blood Transfusion. Before the discovery of isoagglutinins and AB0 blood groups, blood transfusions posed a clinical hazard. Due to the existence of preformed antibodies against AB0 antigens, hemolytic reactions may occur after the first contact with the corresponding antigen. The consequences of incompatible AB0 blood transfusions can be fatal, which is why blood group compatibility became the basis of blood transfusion. (107) The compatibility in AB0 blood group system is shown in **Table 7.**

Table 7 *AB0 Blood Group Transfusion Compatibility*

	AB0 Blood Group Compatibility	
Patient	Red Cells / Major Compatibility	Plasma / Minor Compatibility
0	0	0, A, B and AB
A	A and 0	A and AB
B	B and 0	B and AB
AB	AB, A, B and 0	AB

Note. Reference (108)

In emergency situations, when the patient’s blood group is unknown, universal group 0 red blood cell and group AB plasma units can be transfused. However, in non-critical situations, blood transfusions can be planned and the patient should be transfused with blood components of the same blood group. Incompatibility problems can arise when mistakes are made during the transfusion-related process. (107) These errors include mislabeled patient or donor blood samples, errors in blood group typing or cross-matching, errors in labeling blood components, errors in patient identification prior to transfusion, including misinterpretation of bedside compatibility tests. (109, 110) Haemovigilance systems are in place in many countries to monitor and analyze adverse events related to the entire blood transfusion process (111). In Germany, haemovigilance reporting is regulated by the German Medicines Act and the German Transfusion Act. Serious adverse events and serious adverse reactions related to transfusion are reported to Paul-Ehrlich-Institute (PEI), which is the competent authority in Germany. (112) In its haemovigilance report, the PEI reported that $3.3*10^{6}$ red cell units, $6.3*10^{5}$ plasma units and $4.7*10^{5}$ platelet units were transfused in 2020. The incidence of hemolytic reactions per 10^{6} transfused units was estimated to be 5.8 for red blood cell units,

4.2 for platelet units and zero for plasma. (113) In the United Kingdom, the Serious Hazards of Transfusion (SHOT) haemovigilance system analyzes transfusion-related serious adverse reactions and events. SHOT data show that errors accounted for the majority, with 81.3% of reports in 2021. Between 2016 and 2021, 1778 near-miss events were reported in addition to 19 AB0 incompatible red blood cell transfusions, indicating a large potential for incorrect transfusions. A near-miss event is a deviation from standard guidelines that could have resulted in an incorrect transfusion but was detected before the transfusion was performed (114). Most near-miss events were due to wrong blood in tube, where the blood sample was drown from the wrong patient. These mistakes can only be detected if the laboratory has a historical record of the patient's blood group. (109)

Platelet Transfusion. The only blood components that can be transfused out-of-group in non-critical situations when AB0 identical products are not available are platelet units. Platelet units can be manufactured either by apheresis from a single donor or by pooling buffy coats from four to six whole blood donations. Platelets can be stored either in plasma or in platelet additive solution (PAS), which reduces the plasma content by about 70% (115). Moreover, platelet units can be pathogen reduced, a process that has no effect on plasma content. Depending on the manufacturing process, a platelet concentrate can contain up to 350 ml plasma. An important aspect that makes transfusion of platelet concentrates even more complex is their short shelf life of about 5 days due to storage at room temperature (20-24°C). As a result, platelet units are produced sparingly and are not always available in all blood groups. (14)Therefore, blood group identical transfusion of platelet units is not always possible. AB0 minor incompatible platelet transfusions, due to passive infusion of isoagglutinins can induce hemolytic transfusion reactions. It has been estimated that between 10% and 40% of platelet transfusions are minor incompatible, depending on the institution (116) with a 1 to 9000 risk of hemolytic reactions (117). However, the true incidence of passively mediated hemolytic transfusion reactions is unknown because they are not easily recognized, especially in outpatients. (18) AB0 minor incompatible platelet transfusions are usually well tolerated because the infused isoagglutinins are diluted throughout the patient's total blood volume. Nevertheless, severe hemolytic reactions can occur when platelet units containing high titer isoagglutinins are transfused, when large amounts of incompatible plasma are transfused, or when the patient's total blood volume is small, such as in neonates. (118) The incidence of these transfusion reactions also depends on the type of platelet unit transfused, apheresis or pooled, stored in plasma or PAS, and on the number of units transfused, which are usually regulated by the hospital's internal transfusion protocol, if one exists. Two other important considerations in minor incompatible transfusions are the use of low-titer units donated by known low-titer donors and the critical isoagglutinin titer cutoff used. In addition, the severity

of hemolytic transfusion reactions also depends on the clinical condition of the patient, with delayed transfusion reactions often going undetected due to clinical bias. (116, 119, 120)
In Germany, the national Scientific Guideline for Hemotherapy recommends transfusion of AB0-identical platelet units and, if not possible, AB0 compatible (minor incompatible) transfusion, taking into account either the volume of transfused plasma or the quantity of isoagglutinins. (106)

Whole Blood Transfusion. The transfusion of whole blood has been practiced in several countries around the world since the First World War, especially in the military sector. It is advocated that whole blood provides a balanced resuscitation with simplified logistics and reduced donor exposure by using only one bag instead of three separate blood components (red blood cells, plasma, and platelet units). Furthermore, only a minimal amount of inert fluid is infused into the patient compared to the larger volume of additive solution contained in each blood component. In addition to AB0-identical whole blood transfusion, the use of low-titer group 0 whole blood (LTOWB) has been introduced and is dependent on the availability of low titer donors. In recent years, the transfusion of LTOWB used in military resuscitation of trauma patients, has also been adopted in civilian trauma. (121-123) Several international civilian trauma centers incorporated LTOWB into their transfusion procedures in 2018 after the American Association of Blood Banks (AABB) standards approved this type of transfusion. (124) In Germany, whole blood is generally used only for autologous transfusions.

Acute Hemolytic Transfusion Reactions are a rare complication of transfusion today, but they can be fatal. The blood group most commonly associated with acute hemolytic transfusion reactions is blood group AB0. The acute hemolytic reaction is usually caused by IgM isoagglutinins, but IgG antibodies ca also be involved, although usually in delayed reactions. The IgM isoagglutinins, which are large pentameric molecules, can activate the classical complement pathway directly in the intravascular space after binding to the corresponding antigen alone. During the activation cascade, C5b-9 membrane attack complexes destroy the erythrocyte membrane and induce intravascular hemolysis. Intravascular hemolysis can also be caused by IgG_1 and IgG_3 isoagglutinins. However, IgG isoagglutinins are less effective in binding and activating complement. When the complement cascade is activated, it is usually incomplete up to the formation of C3b molecules. The erythrocytes coated with either IgG isoagglutinins or C3b molecules are extravascular hemolyzed by the reticuloendothelial system in the spleen and liver. (107, 125) Acute hemolytic transfusion reactions occur usually within 24 hours of transfusion and are characterized by fever, abdominal, back or flank pain, hemoglobinuria. Several other signs and symptoms such as chills, nausea, diarrhea, and increase in bilirubin, hypotension, or tachycardia may accompany it. (126) Severe hemolytic reactions can result in disseminated intravascular coagulation, renal failure and shock. The severity of the reaction depends of the quantity of

blood transfused, antigen density, complement activation, reticuloendothelial system, and antibody characteristics, such as isotype, subclass and titer. (125) In AB0 minor incompatible transfusions, the isoagglutinin titer is finite.

Hemolytic Disease of the Fetus and Newborn (HDFN) is caused by maternal IgG antibodies that can cross the placenta and are directed against fetal blood group antigens. Destruction of IgG-sensitized fetal erythrocytes occurs in the fetal spleen and can result in hyperbilirubinemia, anemia, and in severe forms, hydrops fetalis and death. (2) HDFN caused by AB0 incompatibility involves maternal isoagglutinins of IgG class. Although 15% to 25% of pregnancies are AB0-incompatible, the prevalence of clinically significant HDFN is low, less than one percent. (127) HDFN caused by isoagglutinins usually affects the neonate and rarely the fetus and is characterized by hyperbilirubinemia without significant anemia. (128) This is mainly because the expression of A and B antigens on neonatal erythrocytes is not yet fully developed. (127) During pregnancy, the excess bilirubin is cleared by the maternal circulation. However, because of the liver of the newborn is immature, it cannot conjugate the excess bilirubin after birth, resulting in kernicterus. (2) Most cases can be treated with phototherapy, and exchange transfusion is rarely needed. (127) HDFN due to AB0 incompatibility is mostly observed in non-group 0 newborns of group 0 mothers with high antenatal IgG isoagglutinins. HDFN caused by anti-A isoagglutinins is more frequent than that caused by anti-B. Nevertheless, rare cases of A mothers with high anti-B titer have been reported. (127, 128) The isoagglutinin titers may increase during AB0-incompatible pregnancies. (129)

Allogenic Hematopoietic Stem Cell Transplantation (HSCT) represents the transplantation of multipotent stem cells from a healthy donor into a patient with a high-risk hematological malignancy. Donor selection is based on the human leukocyte antigen (HLA) compatibility. AB0 compatibility is not mandatory, and up to 50% of HSCTs are AB0 major-, minor- or bidirectional incompatible. (130) Although extremely rare, transplant-related severe hemolysis and death have been reported after AB0 minor incompatible HSCT. Hemolysis may result from passive transfer of isoagglutinins or from de novo production of isoagglutinins by the transferred B-lymphocytes. (131) The latter phenomenon is known as passenger lymphocyte syndrome, in which B lymphocytes transferred with the graft begin to produce isoagglutinins against patient’s A or B antigens. Passenger lymphocyte syndrome can also occur after solid organ transplantation. (132) AB0 major incompatible HSCT may be complicated by delayed regeneration of donor erythropoiesis, known as pure red cell aplasia, due to host isoagglutinins directed against the transplanted cells. (131) Usually, the isoagglutinins disappear after the conditioning therapy received by the patient prior to transplantation and are replaced by antibodies produced by engrafted B cells. Hemolysis may occur if patient’s isoagglutinins persist after transplantation. (130) Nevertheless, the isoagglutinins can be removed

successfully by plasmapheresis or immunoadsorption. (131, 133) Isoagglutinin titers between 1 and 8 are usually associated with successful engraftment. (134)

Solid Organ Transplantation is the treatment of choice for patients with terminal organ failure. Patient and graft survival has improved significantly in the last years due to advances in immunosuppressive therapy and surgical procedures. (135) However, there is a discrepancy between the increasing number of patients requiring transplantation and the organs available. Therefore, various desensitization protocols have been implemented in the effort to overcome the immunological barrier of AB0 incompatibility and enable AB0 incompatible transplantation from living donor. (136-138) The complications of AB0-incompatible solid organ transplantation involve hyperacute and acute antibody mediated rejection. These complications occur due to patient isoagglutinins directed against donor blood group antigens present on the graft endothelial cells, followed by complement activation, intravascular thrombosis and tissue infarction. (8, 139) Research on AB0-incompatible solid organ transplantations show that the majority of successful graft transplantations were kidneys. Other solid organ transplantation, such as lung and heart transplantations, have had less favorable outcomes with a high rate of hyperacute rejection or graft failure. However, AB0-incompatible hepatic transplantations have been relatively successful in pediatric patients due to the immature immune system of young children. (135, 140) The management of isoagglutinin titers pre- and post-transplant is imperative in AB0-incompatible transplantations for the prevention of early rejection and maintaining graft function. (140) Plasma exchange and AB0 antigen-specific immunoadsorption are important tools in the desensitization and isoagglutinin depletion regimens. The aim of apheresis is to reduce the isoagglutinin level to a titer between 8 and 32. However, the critical isoagglutinin titer and test methods vary by institution. (140, 141) Several reports have emphasized the importance of reliable titer results, due to the known wide inter-laboratory variability in titration. (142, 143)

Association With Infectious Diseases. The association between intestinal flora and isoagglutinin production is well established. It has been hypothesized that isoagglutinins may play a modest protective role against enteric bacteria which express A- or B-like antigens. The assumption that IgM isoagglutinins might promote phagocytosis of bacteria was demonstrated in vitro with human anti-B isoagglutinins, which facilitated the phagocytosis of *Escherichia coli* O86 (144). In a prospective study, the association between bacterial culture results of pediatric patients and AB0 blood group was analyzed and a higher risk of infection with enteric microorganisms was reported for patients of blood group B and AB (145). Another study reported a higher incidence of *Escherichia coli* sepsis in hospitalized patients of group B and AB. The presence of anti-B isoagglutinins in blood group 0 and A individuals appeared to provide protection against microorganisms presenting B-like antigens, such as *Escherichia*

coli. (146) Moreover, the composition of gut microbiota could be influenced by the AB0 blood group of the individual. In a Finnish study, a difference in composition was found in individuals of group B and AB compared to individuals of group 0 and A (147). (41)

Human Immunodeficiency Virus (HIV) infection is caused by an enveloped retrovirus. The association with blood group AB0 is due to the ability of the virus to accept host antigens belonging to the AB0 blood group system to be added to its envelope. In vitro, isoagglutinins have neutralized HIV presenting the corresponding blood group antigens. This indicates that AB0 major incompatibility between a new potential host and the infected patient may provide some protection. A retrospective Brazilian study examining the incidence of HIV infection in blood donors over a 16-year period found a slightly higher rate of infection in blood group B donors. (41, 148)

The most recent association was between AB0 blood group and infectious diseases was coronavirus disease 2019 (COVID-19). Here it was also hypothesized that the isoagglutinins might have a protective role. (149) The pathogen responsible for the disease is SARS-CoV-2, an enveloped coronavirus. The analysis of risk factors for COVID-19 disease indicated that there might be an association between blood group AB0 and susceptibility and severity of infection. A literature review published in 2021 by the ISBT group of experts in transfusion medicine and hematology suggests that the risk of severe disease might be lower in individuals of blood group 0. The same observation was made during the SARS-CoV-1 epidemic. One of the proposed mechanisms of association involves isoagglutinins as neutralizing antibodies. Isoagglutinins could bind the corresponding antigen expressed on the viral envelope and prevent infection of lung epithelial cells. (41, 150) It was found that isoagglutinin levels were significantly lower in patients infected with COVID-19 compared to healthy individuals. This may suggest that low isoagglutinin levels could be a risk factor for infection. (149, 151)

The protective role of isoagglutinins was also hypothesized in influenza infection in several studies in the 1960s and 1970s. Influenza virus is an enveloped, single-stranded RNA orthomyxovirus that is highly pathogenic. The virus has been involved in several pandemic outbreaks, such as the Spanish flu in 1918. After the Influenza pandemic in 2009, the World Health Organization initiated a literature review that revealed conflicting data: high risk of infection was reported for all three blood groups having isoagglutinins - 0, A and B. Therefore, the association hypothesis could not be supported by the available data. (41, 152)

Another infection in which isoagglutinins may play a protective role is schistosomiasis. This is a parasitic disease caused by trematodes of the genus *Schistosoma*. The infection occurs after contact with infected water, with freshwater snails acting as intermediate hosts. It has been reported that blood group A poses a higher risk for infection. The hypothesis for the protective mechanism involves anti-A isoagglutinins, which would block specific epitopes of female trematodes necessary for mating. (41)

2.4 Isoagglutinin Determination

In immunohematology, the isoagglutinins are determined by two common methods: haemagglutination and the indirect Coombs test. Usually, the isoagglutinins tested are anti-A and anti-B of the IgM and IgG isotypes.

2.4.1 Haemagglutination

Haemagglutination represents the clumping of erythrocytes that possess the antigen corresponding to the present antibody, resulting in the formation of a visible aggregation of erythrocytes. Macroscopically visible haemagglutination is mediated by IgM antibodies, which are large molecules capable of agglutinating erythrocytes at room temperature without additional support. The reaction can be performed in physiological saline without additives such as bovine serum albumin, enzymes such as papain or secondary antibodies such as anti-human globulin. (35, 69)

Haemagglutination is the standard method used in AB0 blood group typing, which includes forward and reverse blood group determination. In forward typing, antigens are determined by incubating the erythrocytes to be tested with reagents containing anti-A, Anti-B or anti-AB antibodies. In reverse typing, isoagglutinins are determined by incubating test erythrocytes of groups A_1, A_2 (optional), B, and 0 with the serum to be tested. Reverse blood typing detects isoagglutinins of the IgM isotype. The typical results for the four common AB0 blood groups are presented in **Table 8**. (35)

Table 8 *Forward and Reverse AB0 Blood Grouping Reactions of AB0 Phenotypes*

Antigens on Erythrocytes + Reagents			Isoagglutinins in Serum + Test Erythrocytes				AB0 Phenotype
Anti-A	Anti-B	Anti-AB	A_1	A_2	B	0	
-	-	-	+	+	+	-	0
+	-	+	-	-	+	-	A
-	+	+	+	+	-	-	B
+	+	+	-	-	-	-	AB

Note. Reference: (35)

Reverse blood grouping can be performed in tubes, on column agglutination cards or on plates, as seen in **Figure 1.** In the tube method, plasma is mixed with a suspension of test erythrocytes in a test tube. The tubes are then centrifuged to bring the isoagglutinins closer to the antigens present on the surface of test erythrocytes to facilitate agglutination, and then gently shaken to resuspend the erythrocytes. If a solid clump has formed in the tube, this indicates the presence of the corresponding isoagglutinin. Haemagglutination on plates or microplates is similar to the tube method. Instead of individual tubes, plates with multiple wells are used so that multiple tests can be performed simultaneously. (35, 69)

Figure 1 *Reverse Blood Grouping Reactions of Blood Group 0*

Note. The isoagglutinins were tested manually in tubes, on plate and by column agglutination. The serum to be tested was incubated at room temperature with test erythrocytes of group A_1, A_2, B and 0, respectively.

The column agglutination method is performed on cards with usually 6 columns containing usually a neutral gel suspension, to which plasma and a suspension of test erythrocytes are added. After an incubation period, the cards are centrifuged and individual erythrocytes migrate to the bottom of the column, while agglutinated erythrocytes cannot transit through the gel and remain at the top. (153) The test reactions are graded according to the strength of agglutination, with 0 representing a negative reaction and from 1+ to 4+ representing a positive reaction. (110)

2.4.2 Coombs Test

The Coombs test, also known as the antiglobulin test, is a haemagglutination assay that detects IgG antibodies that are either bound to surface antigens of erythrocytes (the direct Coombs test) or free in plasma (the indirect Coombs test). Because IgG antibodies are unable to agglutinate erythrocytes themselves, a secondary antibody, anti-human globulin (AHG), is used to visualize the antigen-antibody reaction. The IgG isotype, which are incomplete antibodies can be determined with the tube method, by column agglutination or solid phase assays. (35)

The indirect Coombs tube assay begins with sensitization of the test erythrocytes with the antibody-containing plasma, which are placed in a medium that facilitates agglutination, such as low ionic strength saline (LISS), and incubated at 37°C. The test erythrocytes are then washed to remove excess antibody, followed by incubation with AHG to facilitate agglutination, and finally by centrifugation. This manual method is very laborious and is rarely used today. (108)

Usually, IgG isoagglutinins are determined by column agglutination or with solid phase red cell adherence assays. The determination of IgG isoagglutinins is not part of the routine AB0 blood grouping. They are determined in the diagnosis of hemolytic disease of the newborn, in stem cell or organ transplantation, after AB0 incompatible transfusion reactions, or in crossmatching. The column agglutination method is performed on cards with columns already containing LISS and AHG. Serum and test erythrocytes are added to the columns, incubated at 37°C,

and subsequently centrifuged. In the presence of IgG isoagglutinins, the agglutinated erythrocytes remain at the top of the column. (154, 155) This method is usually performed manually, but can also be used on automated platforms. The solid phase red cell adherence method is usually performed on an automated platform on microplates coated with anti-erythrocyte antibodies. Initially, test erythrocytes are bound to the wells of the microplate, then the serum or plasma sample is added. The antigen-antibody reaction is then visualized by adding indicator red cells coated with anti-human IgG. (156)

2.4.3 Titration

In addition to qualitative analysis, the isoagglutinins can also be determined semi-quantitatively by titration. Titration of isoagglutinins plays an important role in immunohematology and transfusion medicine, as it makes the transfusion of minor incompatible platelet units and low-titer group 0 whole blood a safer procedure. (5, 120) Furthermore, titration of isoagglutinins is part of prenatal care, diagnosis of hemolytic disease of the newborn, as well as AB0 incompatible hematopoietic stem cell and solid organ transplantation. (13, 110)

In antibody titration, a series of tubes, usually ten, is prepared, with one volume of saline solution dispensed in all except the first tube. An equal volume of sample (serum or plasma) is then added in the first two tubes. Using a clean pipette, the content of the second tube must be mixed several times and then transferred into the next tube, and so on. A clean pipette must be used for every dilution step. One volume of the diluted sample should be taken the last tube and kept for possible further dilutions. The first tube contains one volume of the undiluted sample, the second tube containing a dilution of 1:2 meaning one volume of plasma in a final volume of two, and so on. Thereafter, the agglutination methods described above are used to identify the IgM and IgG isoagglutinins in the dilution series prepared. The titer is then expressed as the reciprocal of the highest dilution showing a macroscopic agglutination of 1+. (110, 157)

When comparing the two manual titration methods, gel column agglutination is less time-consuming than the tube method and more sensitive, usually resulting in one dilution higher. (9) Furthermore, a good correlation was found between the automated IgM titration on microplates and manual column agglutination when performed accurately. (30) The titration of IgG isoagglutinins is problematic because it may be affected by IgM antibodies. A high IgM titer can falsify IgG titration by overestimating the result. This is due to a strong agglutination reaction of IgM isoagglutinins that remains stable at 37°C. (158) Various methods can be used to inactivate the IgM isoagglutinins and facilitate the titration of IgG antibodies, such as sample treatment with Dithiothreitol (DDT), heat, or the use of NeutrAB® solution. When titrating IgG agglutinins by the tube method, the use of DTT-treated plasma is recommended to reduce the impact of IgM isoagglutinins. The sample (serum or plasma) must be incubated with

0.01 M DDT at 37°C for about 30 to 60 min, mixing regularly. DDT inactivates IgM antibodies by cleaving the disulphide bonds that hold the IgM molecule in pentameric form, whereas having a minimal effect on IgG antibodies. It is unclear whether this reagent can be used in column agglutination, and it is possible that plasma treated with DDT may give false positive reactions in column agglutination. (158, 159) Heat inactivation is a method that allows the determination of IgG antibodies by inactivating IgM antibodies. The method is usually performed at a temperatures of 63°C for approximately 10 minutes. Heat inactivation is less time consuming and does not requires additional reagents. (160) Both heat inactivation and DTT are effective methods for reducing IgM antibodies but they may interfere with IgG binding. (161) Another method for IgM inactivation is the NeutrAB® assay. The NeutrAB® reagent contains water soluble antigens A and B. During incubation of the sample with the reagent, the soluble antigens neutralize IgM isoagglutinins. (162)

An important method for the quantification of IgG antibodies is the solid phase red cell adherence method. This method allows separate quantification of IgG isoagglutinins without the interference of IgM antibodies and without the use of a chemical treatment. (24) IgG titration results obtained by the solid phase method correlate with column agglutination results obtained with samples treated with DDT. It was also found that the IgG titer difference between column agglutination results and the solid phase results correlated with the increase of IgM titer in these samples. (29, 30)

2.4.4 Reproducibility of Titration Methods

Titrations is the common method used in immunohematology to investigate isoagglutinin concentration. However, it is well known that antibody titration, more specifically manual titration in tubes or by column agglutination, is a technique with low reproducibility, both interlaboratory and inter-operator. Differences in dilution techniques and agglutination methods between laboratories contribute discrepancies in results. Furthermore, the most significant cause of variation is known to be differences in assessment of agglutination strength by personnel from the same laboratory (operator bias). (23) Moreover, several variables in the technique used, such as phenotype and concentration of test erythrocytes, diluent used, reuse of pipette tips for more than one dilution, incubation time and temperature, centrifugation force and time, may affect the results. (157)

Standardization of antibody titration is a challenge. The use of a uniform and detailed procedure might reduce the result variation not only between laboratories, but also inter-operator by limiting the chances for technique variation. Such a procedure was introduced in the United States of America in 2008 by the College of American Pathologists and Biomedical Excellence for Safer Transfusion Committee. The study on which the uniform procedure was based demonstrated the importance of clear definitions for endpoint dilution and reaction grading. In this study, 35 laboratories (11 European, 22 North American, one South American and one Australian) were provided with an identical testing kit containing serum samples and test erythrocytes. A statistically significant reduction in variation was observed when an additional grade, weak plus (barely visible agglutinates) instead of 1+ (small agglutinates) was used as the titration endpoint. This showed once again that the main cause of discrepancy is operator subjectivity. (23) Another study, conducted a few years after the implementation of the uniform method, analyzed data from proficiency testing reported to the College of American Pathologists between 2009 and 2013. This study had a significantly higher number of participants compared than the 2008 study (a median of 574 and 1100 responses for anti-A isoagglutinin and anti-D antibody titers, respectively, over a five-year period). However, the outcome was different, and despite the use of uniform methods, the analysis did not reveal a statistically reduction in the variance of titers between laboratories. (22) Nevertheless, the standardization of antibody titration could be achieved by using automated methods. Currently, different automated platforms are used for isoagglutinin titration. The most commonly used are automated column agglutination (IgM and IgG), solid phase red cell adherence (IgG) and direct agglutination on microplates (IgM). Comparison to manual methods, automated assays have high interlaboratory and inter-instrument reproducibility, in addition to known reduced turnaround time. However, the use of automated methods may not be feasible for small laboratories due to financial limitations, which may explain the widespread use of labor intensive manual titration. (24, 25)

3 Materials and Methods

3.1 Materials

3.1.1 Blood Samples

The Transfusion Center in Mainz has a large number of active blood donors, whole blood and platelet donors who donate regularly over many years. Donor screening and deferral are performed using a donor questionnaire approved by Paul-Ehrlich-Institute (PEI) and in accordance with the current requirements of the German Hemotherapy Guidelines (163).
In this analysis were included blood donations collected in 2017 from donors of the Transfusion Center who returned within ± 14 days during the same period in 2021. Both, whole blood and apheresis platelet donors were included. All four common AB0 blood groups were included. Testing was performed on residual plasma after completion of the mandatory donor testing.
The 2017 blood donations took place between April 24 and July 04. All samples were collected in tubes containing EDTA gel (S-Monovette® K2 EDTA Gel 7.5 ml) and immediately centrifuged (10 min at 4000 RPM, Rotina 380 centrifuge). The plasma was then aliquoted within 12 hours of collection. Two 900 µl aliquots were subsequently frozen and stored at -24 ± 1°C as counter samples for mandatory transfusion-transmitted infection tracing. After the statutory retention period had expired and before disposal, the plasma samples required for this investigation were set aside until the titration assays were performed. The storage continued at -24 ± 1°C. Before testing, the samples were thawed at room temperature.
The 2021 blood donations took place between April 29 and June 21. The samples were collected in tubes containing EDTA gel (S-Monovette® K2 EDTA Gel 7.5 ml) and immediately centrifuged (10 min at 4000 RPM, Rotina 380 centrifuge). After completion of mandatory donor testing, the samples were refrigerated until the next day and up to 72 hours on weekends, and then frozen in 5 ml tubes (5 ml SARSTEDT tubes) at -24 ± 1°C until titration testing was performed.

3.1.2 Laboratory Equipment

All laboratory equipment belonged to the Transfusion Center. This research received no external funding. Following laboratory equipment was used:

- Blood Group Analyzer NEO Iris® (Immucor Medizinische Diagnostik GmbH, Dreieich, Germany)
- Capture-R® SELECT Solid Phase System for the Immobilization of Human Erythrocytes (Immucor Medizinische Diagnostik GmbH, Dreieich, Germany)
- Capture-R® Ready Indicator Red Cells (Immucor Medizinische Diagnostik GmbH, Dreieich, Germany)
- Capture® LISS (Immucor Medizinische Diagnostik GmbH, Dreieich, Germany)
- Referencells® Group A1 and B (Immucor Medizinische Diagnostik GmbH, Dreieich, Germany)
- Galileo System Liquid Concentrate (Immucor Medizinische Diagnostik GmbH, Dreieich, Germany)
- Galileo Microplates (Barcoded) (Immucor Medizinische Diagnostik GmbH, Dreieich, Germany)
- S-Monovette® K2 EDTA Gel 7.5 ml (Sarstedt AG & Co. KG, Nümbrecht, Germany)
- Tubes 5 ml ø 13 mm (Sarstedt AG & Co. KG, Nümbrecht, Germany)
- Push-In Plugs for tubes ø 13 mm (SARSTEDT AG & Co. KG, Nümbrecht, Germany)
- Centrifuge Rotina 380 (Hettich GmbH & Co. KG, Tuttlingen, Germany)
- Centrifuge Universal 30 RF (Hettich GmbH & Co. KG, Tuttlingen, Germany)
- Eppendorf Reference Pipette 10 - 100 µl (Eppendorf Vertrieb Deutschland GmbH, Wesseling, Germany)

3.2 Methods

For all donations, blood group AB0 was routinely confirmed at the time of donation as part of the release of blood component. All samples were titrated for IgM isoagglutinins against A_1 and B erythrocytes, including the group AB samples, to confirm reverse typing of the AB0 group. The reverse typing allowed verification of the accuracy of sample identification.

Table 9 *Titration Assays by AB0 Blood Group*

Method	Assay Abbreviation	Blood Group 0	Blood Group A	Blood Group B	Blood Group AB
NEO Iris®	LT_IgM_A_1	x	x	x	x
NEO Iris®	LT_IgM_B	x	x	x	x
NEO Iris®	LT_IgG_A_1	x		x	
NEO Iris®	LT_IgG_B	x	x		
NEO Iris®	HT_IgG_A_1 (if needed)	x		x	
NEO Iris®	HT_IgG_B (if needed)	x	x		
manual	High-IgM-A_1 (if needed)	x		x	
manual	High-IgM-B (if needed)	x	x		

IgG isoagglutinins were tested according to the AB0 blood group of the sample as shown in **Table 9**: blood group 0 samples was titrated for IgG anti-A_1 and anti-B, blood group A samples for IgG anti-B and blood group B samples for IgG anti-A_1. The isoagglutinin titration was carried out on three NEO Iris® blood group automates from Immucor.
IgM titration was performed with NEO Iris® using low titer range assays that could determine a titer of up to 128 as presented in **Table 10**. Samples with a positive reaction at the 1:128 dilution were then tested manually following the NEO Iris® IgM assay protocol and an extended range of doubling dilutions, up to 1:2048. The manual assay plates were loaded on NEO Iris® and its software graded the agglutination reaction, according to its protocol. The last positive reaction was assigned as assay result.

Table 10 *AB0 Titration Assays on NEO Iris® for IgM Determination*

Antibody	Assay description	Abbreviation	Titer range	Microplates
IgM	low titer range assays 8-well titration	LT_IgM_A_1 LT_IgM_B	1–128 (doubling dilutions)	Galileo Microplate on NEO Iris®
	high titer range assays 12-well titration	High-IgM-A_1 High-IgM-B	1–2048 (doubling dilutions)	Galileo Microplate manual
Note. Reference (164)				

The automated system determined an IgG titer of up to 4096 using two assays: a low titer test that could determine a titer of up to 128 and a high titer test that could determine a titer of up to 4096. The samples were initially tested using the low range assays. When the 1:128 dilution showed a positive reaction, the samples were additionally tested with the high range assays as presented in **Table 11**. Depending on AB0 blood group and sample titer, one sample could

be tested with up to eight assays as shown in **Table 9**. Not all assays of a sample were run on the same automate. Some samples showed a titer of 64 in the high titer assay following a positive reaction at the 1:128 dilution with a low range assay. A titer of 128 was assigned to these samples. Invalid samples were retested. If the result remained invalid, the samples were excluded from the study. Daily quality controls were performed on each NEO Iris® prior to samples testing. The low titer assay required 100 µl of plasma. The high titer assays required 15 µl plasma. The manual high titer assay required 100 µl sample.

Table 11 *AB0 Titration Assays on NEO Iris® for IgG Determination*

Antibody	Assay description	Abbreviation	Titer range	Microplates
IgG	low titer range assays 8-well titration	LT_IgG_A_1 LT_IgG_B	1–128 (doubling dilutions)	Capture-R® Select on NEO Iris®
	high titer range assays 8-well titration	HT_IgG_A_1 HT_IgG_B	16–4096 (doubling dilutions)	

Note. Reference (164)

3.2.1 Blood Grouping System

NEO Iris® is a robotic instrument designed to automate standard immunohematology assays and operate independently for periods of time. NEO Iris® is programmed to move microplates and fluids (reagents and blood samples) to different areas (incubator bays, microplate washing station, centrifuge and plate reader) for a given assay in the correct sequence. The plate reader captures images of the microplates with the help of CMOS (complementary metal-oxide semiconductor) cameras. Based on a multi feature image analysis and predefined criteria, the NEO Iris® software calculates a reaction value for each well and then assigns a result to the assays. (164, 165) The software version used was 1.8.2.0-2.11.

Reagent Red Blood Cells for AB0 Serum Grouping: Referencells® - 2 is a set of two vials of A_1 and B test erythrocytes. The vials contain a 2%-4% suspension of pooled cells that are negative for C, D and E antigens. The cells are suspended in a buffered solution to slow down hemolysis and loss of antigenicity. Additionally to adenosine and adenine, the solution also contains EDTA and three antibiotics. A_1 and B test erythrocytes are used in AB0 reverse grouping as part of AB0 typing. A_1 or B erythrocytes can detect the presence of anti-A or anti-B isoagglutinins. (166) Depending of test environment, IgM or IgG antibodies can be detected.

Capture-R® Select Solid Phase System consists of polystyrene microwell strips. A Capture-R® plate contains 12 strips à 8 microwells. The microwells are coated with two antibodies, a goat anti-murine antibody and an anti-RBC antibody, capable to immobilize erythrocytes to the microwell surface. The solid phase system is used to detect IgG antibodies against erythrocyte antigens. (156)

Capture® LISS is a low ionic strength solution, which, if added to an antibody detection test, reduces the ionic strength and thus, enhances antigen-antibody interactions and reduces incubation time. Furthermore, Capture® LISS contains a dye, bromocresol purple, which has a purple blue color that changes in turquoise in the presence of plasma. In this manner, Capture® LISS potentiates the detection of IgG antibodies and indicates the plasma sample addition. (167)

Capture-R® Ready Indicator Red Cells are single donor or pooled erythrocytes which are coated with murine monoclonal anti-human IgG. In this manner, the indicator cells bind to IgG antibodies that were previously bound to immobilized test erythrocytes. The indicator cells are suspended in a solution containing adenine, adenosine and antibiotics for preservation and reagent stability. (168)

Galileo System Liquid Concentrate is a phosphate buffered saline, tenfold concentrate. Before use, the concentrate is diluted with deionized water. It is used in the pipetting system for washing the probes and sample dilution in some assays. Furthermore, it is used as wash buffer in the washing steps of Capture solid phase adherence assays. (169)

Galileo Microplates (Barcoded) are untreated polystyrene microplates with 96 U-shaped wells. (170)

The reagents necessary for each assay are presented in **Table 12**.

Table 12 *Assay Reagent Component Grid*

	Assay							
Reagents and microplates	LT_IgG_A_1	LT_IgG_B	HT_IgG_A_1	HT_IgG_B	LT_IgM_A_1	LT_IgM_B	High-IgM-A_1	High-IgM-B
Capture-R® Select	x	x	x	x				
Untreated Microplates (Barcoded)					x	x	x	x
Reagent Red Blood Cells (Referencells – Group A_1)	x		x		x		x	
Reagent Red Blood cells (Referencells – Group B)		x		x		x		x
Capture-R® Ready Indicator Red Cells	x	x	x	x				
Capture® LISS	x	x	x	x				
PBS	x	x	x	x	x	x	x	x

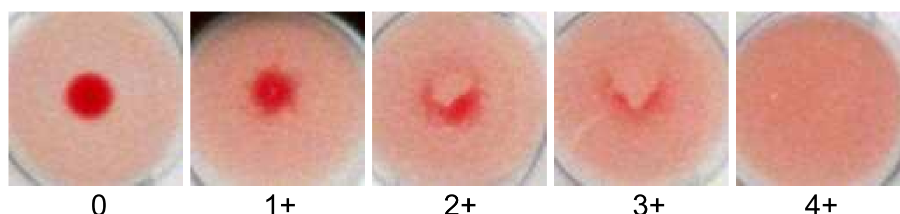
Note. Reference (164)

3.2.2 Titration Assays

Automated AB0 Titrations - Solid Phase Red Cell Adherence Assays

The assay performed were LT_IgG_A_1, LT_IgG_B, HT_IgG_A_1 and HT_IgG_B, with the version 1.02 25Mar19. The assays were performed on NEO Iris®, fully automated. The necessary reagents are described in **Table 12** and were loaded according to specifications onto the instrument before testing. The assays were assigned manually to each sample. Initially, PBS and test erythrocytes A_1 or B (Referencells®) were pipetted into coated microwells strips (Capture-R® Select Solid Phase System) followed by centrifugation. In this way, the cells adhered as a monolayer to the microwell. The excess was subsequently removed in a washing step. Following, the sample was added and the doubling dilutions were made. LISS was pipetted into the microwell, to highlight the addition of plasma. If plasma was present, LISS changed color from purple blue in turquoise. Subsequently, the strips were incubated at 39°C. In this manner, if present, anti-A or anti-B antibodies (IgG) interacted with A or B antigens of the test cells, forming an antigen-antibody complex. The plasma-LISS mixture was afterwards washed from the strip. Lastly, indicator cells (Capture-R® Ready Indicator Red Cells) were added to the microwells followed by centrifugation. The centrifugation step brought the indicator cells (anti-IgG) in close contact to the anti-A or anti-B antibodies (IgG), which were bound to the adhered A_1 or B test erythrocytes on the surface of the microwells. Due to the anti-IgG-IgG complexes that were formed, the indicator cells remained immobilized to the microwell walls as a second erythrocyte layer (positive reaction) as shown in **Figure 2**. In the absence of isoagglutinins, no anti-IgG-IgG complexes were formed and the indicator cells pelleted to the bottom of the microwells (negative reaction). (166, 168) An image of the microplate was captured with NEO Iris's camera and the software calculated a reaction value for each well based on a multi feature image analysis. A result and an interpretation were then assigned to the wells based on predefined criteria associated with the calculated reaction value. (164)

Figure 2 *Capture® Grading Chart*



Note. Grades from 1+ up to 4+ represent positive results. Grade 0 represents a negative result.

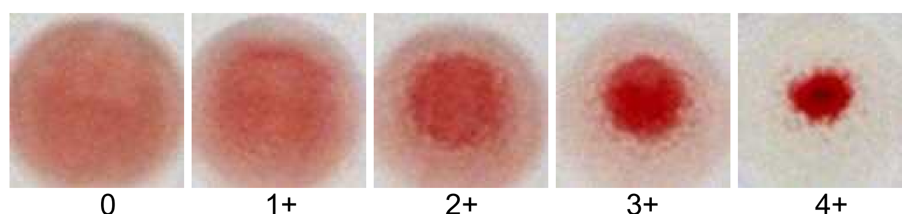
Automated AB0 Titrations - Haemagglutination Assays

The assay performed were LT_IgM_A_1 and LT_IgM_B, with the version 2.02 25Mar19.

The assays were performed on NEO Iris®, fully automated. The necessary reagents are described in **Table 12** and were loaded according to specifications onto the instrument before

testing. The assays were assigned manually to each sample. Initially, PBS was pipetted into uncoated microwells (Galileo Microplates). The sample was added and the doubling dilutions were made. After adding the test erythrocytes A_1 or B (Referencells®) the microplate was incubated at 20°C and then centrifuged. The test erythrocytes agglutinated in the presence of IgM antibodies due to antigen-antibody complexes and formed a cell button on the bottom of the microwell (positive reaction). The haemagglutination grading chart is shown in **Figure 3**. In the absence of IgM antibodies, the test erythrocytes did not agglutinate and remained dispersed in the well (negative reaction). (166) The software of NEO Iris® calculated the reaction value for each well and assigned a result based on predefined criteria associated with the calculated reaction value. (164)

Figure 3 *Haemagglutination in Microwells Grading Chart*



0 1+ 2+ 3+ 4+

Note. Grades from 1+ up to 4+ represent positive results. Grade 0 represents a negative result.

Manual AB0 Titrations - Haemagglutination Assays

The assay performed were High-IgM-A_1 and High-IgM-B. The necessary reagents are described in **Table 12**. The assays were performed manually, following the automated assay protocol of the low haemagglutination assays and extending the range of doubling dilutions up to 1:4096. The reaction values were calculated with NEO Iris® software and the titration result was assigned manually. The assay was performed on Galileo Microplates. A volume of 50 µl PBS was dispensed in each of the wells 2 to 12. In the first well, 100 µl sample plasma was pipetted. With a new pipette tip, 50 µl sample plasma was transferred from well 1 into well 2 and mixed with the existing 50 µl PBS. Subsequently, the remaining doubling dilutions were made. After the last dilution was made, 50 µl diluted plasma was discarded out of microwell 12. The plate was then rested for 30 seconds. Afterwards, 15 µl test erythrocytes A_1 or B (Referencells®) were added into each microwell. The plate was subsequently incubated for 10 minutes at 20°C, then centrifuged for 60 seconds at 250 g revolutions per minute (Centrifuge Universal 30 RF). Afterwards, the plate was tapped manually to suspend the cell buttons and immediately loaded on NEO Iris®. The camera captured a picture of the microplate and the NEO Iris® software assigned a reaction values to each well. Subsequently, grades were assigned for every well and the assay result was set at the last positive reaction, as showed in **Figure 3**. In this manner, the same interpretation method was used for the IgM assays so that the automated results would correlate to the manual results.

3.2.3 Statistical Analysis

The recording of data, assay results and donor information, was performed in a custom-made Excel 2016 spreadsheet (Microsoft Office Professional Plus 2016, Microsoft Corporation, Redmond, Washington, United States of America). The following donor data were collected in pseudonymised form: blood group, isoagglutinin titer, donation type, age and gender.

Isoagglutinin analysis was performed by blood group. In addition, each blood group was divided into study groups: male and female, and whole blood and apheresis platelet donors. The platelet group included all donors who donated platelets at least once.

Furthermore, the isoagglutinin titer was expressed as a titer step (TS) using $\log_2$ as suggested by Vennes in 1954 (171). For example, titer 2 (2^1) was coded as 1, titer 4 (2^2) was coded as 2, and so on. A negative agglutination reaction was coded as -1 titer step. TS correlation to titer is presented in **Table 13**. Moreover, isoagglutinin titer changes were analyzed by titer step difference (TSD). TSD was calculated as follows: TS in 2017 was subtracted from TS in 2021. In addition, an individual standard deviation (*SD)* was calculated for the titer step results from 2017 and 2021 of each donor. The individual *SD*s were pooled together to calculate overall pooled *SD*s by blood group and overall by isoagglutinin. Analysis of titer values, calculation of TS, TSD, individual and pooled *SD* were performed in Excel.

In this analysis, the default age was considered the age in 2021.

Table 13 *Correlation Between Titer and Titer Step*

Titer Step	-1	0	1	2	3	4	5	6	7	8	9	10	11
Titer	negative	1	2	4	8	16	32	64	128	256	512	1024	2048

Statistical data analysis was performed with IBM® SPSS® Statistics Version 23 (IBM Corporation, Armonk, New York, United States of America).

Wilcoxon signed-rank test was carried out to investigate the statistical significance of the titer step changes between 2017 and 2021. The level of significance was set at α = 5%. The critical *Z*-value for a 5% level of significance is $|Z|$ => 1.96 for a two-tailed test. The effect size was calculated by dividing the absolute value of *Z* by the square root of the number of pairs and compared to Cohen’s classification (0.1 - small, 0.3 - medium, 0.5 - large effect) (172).

Multiple linear regression was performed to investigate whether age and gender were associated with the difference in titer step. Isoagglutinin TSD was used as the dependent variable. The variable age was centered by subtracting the mean age from each age value. The variable gender was coded -1 for male and 1 for female. In multiple linear regression, mean TSD was adjusted with age and gender.

4 Results

4.1 Donor Population

A total of 1073 blood donors who donated both in 2017 (April 24 to July 04) and the same period in 2021 (April 29 to June 21) were included in this study. Each donor donated during the same period of the year, with ±14 days between donation dates. Of the 1073 blood donors, 43.3% had blood group 0 (465 donors), 40.9% had blood group A (439 donors), 11.6% had blood group B (124 donors) and 4.2% had blood group AB (45 donors). As expected, blood group AB donors had negative titration results for IgM anti-A_1 and anti-B. Therefore, the 45 donors of blood group AB were excluded from the following analysis.

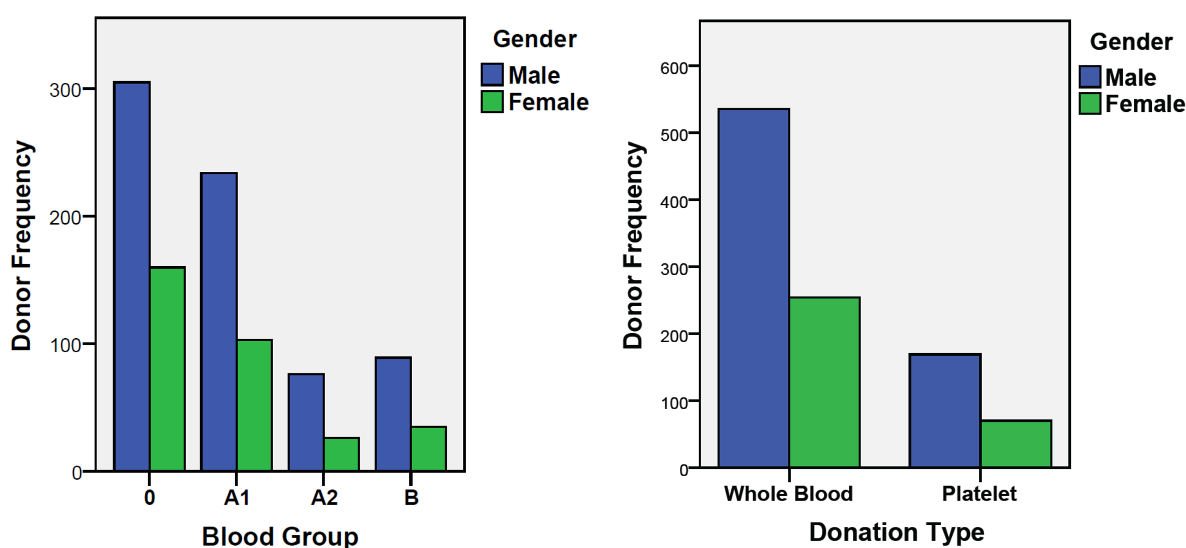
A total of 1028 donors of blood groups 0, A and B were analyzed. The distribution by blood group was 44.4% blood group 0, 42.7% blood group A and 12.1% blood group B donors. Divided by gender, 68.5% were male (704 donors) and 31.5% were female (324 donors). Regarding the type of donation, the population was divided into two groups, of which 76.7% were whole blood donors (789 donors) and 23.3% were platelet donors (239 donors). The platelet group included donors who had donated platelets in either 2017 or 2021. The distribution by blood group, donation type and gender is presented in **Table 14** and **Figure 4**.

Table 14 *Donor Distribution by Blood Group, Gender and Donation Type*

	Blood Group 0		**Blood Group A**		**Blood Group B**	
	Whole Blood	Platelet	Whole Blood	Platelet	Whole Blood	Platelet
Male	241	64	226	84	68	21
Female	125	35	97	32	32	3

Note. N = 1028

Figure 4 *Donor Distribution by Blood Group, Gender and Donation Type*



Note. Number of Donors by Blood Group, Donation Type and Gender

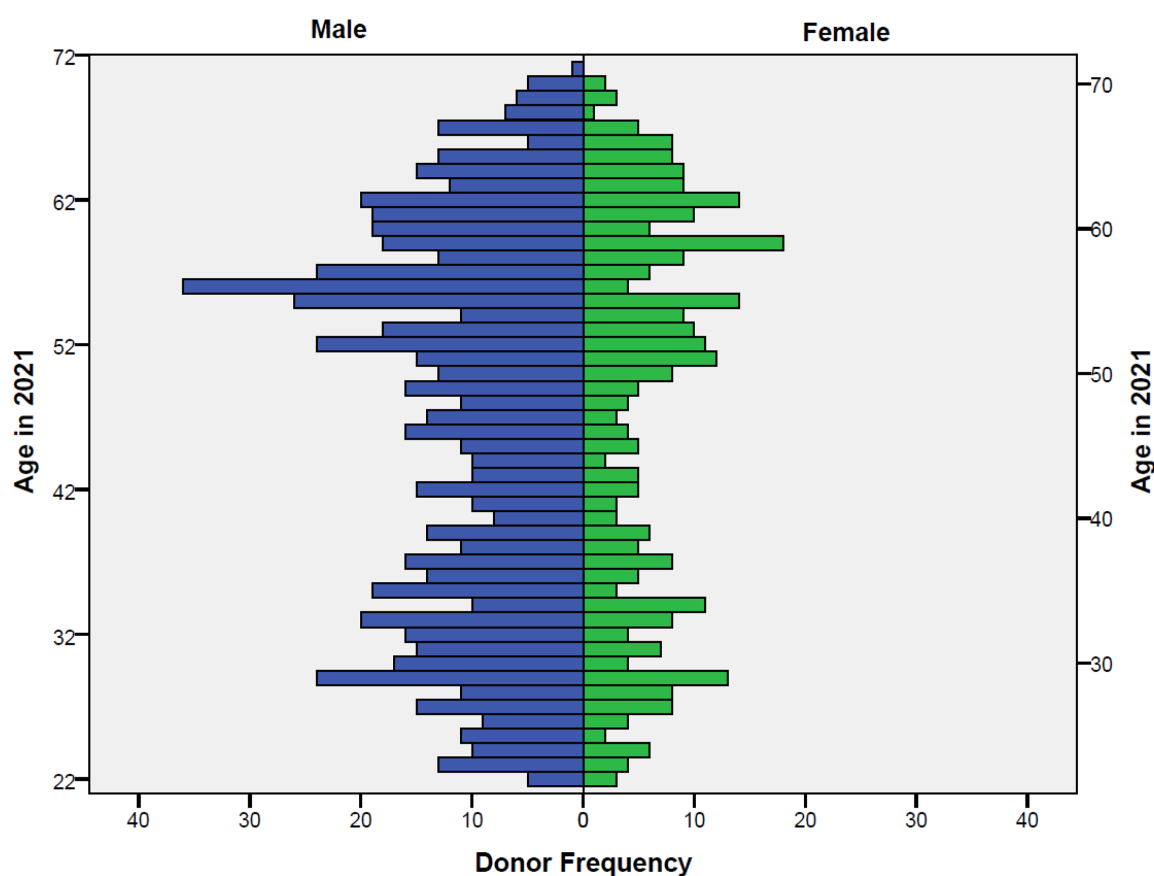
The ages of the donors in 2021 ranged from 22 to 71 years (median (*Md*) = 49, mean (*M*) = 46.78, standard deviation (*SD*) = 13.31). The age parameters stratified by gender, donation type and blood group are presented in **Table 15**. The population age distribution of the population stratified by gender is presented in **Figure 5**. The donor distribution and age parameters within the blood groups stratified by gender and type of donation, are shown in the Appendix section in **Table 28**.

Table 15 *Donor Age*

Study Groups	Age in 2021					
	n	*Min*	*Max*	*Md*	*M*	*SD*
Male	704	22	71	48	46.40	13.20
Female	324	22	70	51	47.60	13.51
Whole Blood	789	22	71	51	47.59	13.62
Platelet	239	22	66	44	44.08	11.85
Blood Group 0	465	22	71	49	46.71	13.43
Blood Group A	439	22	70	49	46.47	13.29
Blood Group B	124	23	70	51	48.10	12.95

Note. *N* = 1028. Age of the donors in 2021.

Figure 5 *Donor Distribution by Age and Gender*



Note. Pyramid histogram – number of donors by gender and age in 2021.

4.2 Isoagglutinin Titer

The isoagglutinin titers ranged over the years from 1 to 256 for IgM isoagglutinins and from negative to 2048 for IgG isoagglutinins. The highest isoagglutinin mean titer was observed in blood group 0 donors for anti-A_1 IgG, followed by anti-B IgG. The lowest mean isoagglutinin titer was found in blood group A donors for anti-B IgG, followed by anti-A_1 IgG in blood group B donors. Isoagglutinin titer results for 2017 and 2021 and overall titer values for the two years combined are presented in **Table 16**.

Table 16 *Isoagglutinin Titer*

Blood Group	Titer 2017				Titer 2021				Titer Overall	
	Min	*Max*	*Md*	*M*	*Min*	*Max*	*Md*	*M*	*Md*	*M*
Anti-A_1 IgM										
0	1	256	16	22.48	1	256	16	24.85	16	23.67
B	2	128	8	16.98	2	128	16	18.48	16	17.73
Anti-A_1 IgG										
0	0	2048	64	116.8	0	1024	64	102.3	64	109.54
B	0	64	2	5.18	0	32	4	6.26	2	5.72
Anti-B IgM										
0	1	128	8	14.17	1	256	8	15.47	8	14.82
A	1	128	8	12.4	1	256	8	13.39	8	12.90
Anti-B IgG										
0	0	2048	16	48.31	0	2048	32	63.39	16	55.85
A	0	512	0	3.3	0	512	1	3.96	1	3.63

Note. The overall titers represent the results of the two years combined.

All donors had quantifiable IgM isoagglutinins corresponding to their blood group. However, 28.6% (294 donors) of all 1028 donors had negative IgG results for at least one of the two 2017 or 2021 samples. Of them, the majority of 20.7% (213 donors) remained negative in both years, 5.6% (57 donors) changed to positive and 2.3% (24 donors) changed to negative. These donors are presented **Table 17** by blood group and isoagglutinin.

Table 17 *Donors Lacking IgG Isoagglutinins by Change Over the Years*

	Change to Negative	Remain Negative	Change to Positive
Blood Group 0			
Anti-A_1 IgG	1 (0.2)	7 (1.5)	4 (0.9)
Anti-B IgG	3 (0.7)	2 (0.4)	2 (0.4)
Blood Group A			
Anti-B IgG	16 (3.7)	188 (42.8)	42 (9.6)
Blood Group B			
Anti-A_1 IgG	4 (3.2)	16 (12.9)	9 (7.3)

Note. Data are reported as number (% of the respective blood group).

The stratification of donors who remained IgG negative over the years by blood group, gender and type of donation is presented in **Table 18**.

Table 18 *Donors Lacking IgG in Both Years by Blood Group, Gender and Donation Type*

	Blood Group 0		Blood Group A		Blood Group B	
	Whole Blood	Platelet	Whole Blood	Platelet	Whole Blood	Platelet
Male	2 / 1	1 / 1	94	38	12	2
Female	3 / 0	1 / 0	38	18	2	0

Note. Data are reported as number. For group 0, data are reported as anti-A_1 / anti-B. None of the group 0 donors had both IgG isoagglutinins negative.

Relative to each blood group, the distribution of the donors who remained IgG negative over the years was 42.8% of group A donors (188 of 439), 12.9% of group B donors (16 of 124) and 1.9% of group 0 donors (9 of 465).

Relative to each gender study group, the distribution of the donors who remained IgG negative over the years was 21.5% of male donors (115 of 704) and 19.1% of female donors (62 of 324). Of the 62 female donors, 29 (27 of group A and 2 of group B) were up to 49 years old, which is the upper limit of childbearing age in birth statistics (173).

Relative to each donation type, the distribution of the donors who remained IgG negative over the years was 19.3% of the whole blood donors (152 of 789) and 25.5% of the platelet donors (61 of 239).

The donors that remained IgG negative over the years had IgM titers below 128, except for three donors with anti-A_1 IgM titers of 128.

4.3 Isoagglutinin Titer Step

The isoagglutinin titers were converted to titer steps (TS) for statistical analysis, as shown in **Table 13**.

4.3.1 Distribution of Titer Steps

The isoagglutinin TS ranged over the years from 0 to 8 for IgM isoagglutinins and from -1 to 11 for IgG isoagglutinins. Here as well, the highest isoagglutinin mean TS was observed in blood group 0 donors for anti-A_1 IgG, followed by anti-B IgG. The lowest mean isoagglutinin TS was found in blood group A donors for anti-B IgG, followed by anti-A_1 IgG in blood group B donors. Isoagglutinin titer step results for 2017 and 2021 and overall titer steps for the two years combined are presented in **Table 19**.

Table 19 *Isoagglutinin Titer Step*

Blood Group	TS 2017					TS 2021					TS Overall	
	Min	*Max*	*Md*	*M*	*SD*	*Min*	*Max*	*Md*	*M*	*SD*	*M*	Pooled *SD*
						Anti-A_1 IgM						
0	0	8	4	3.99	1.19	0	8	4	4.05	1.28	4.02	0.37
B	1	7	3	3.56	1.16	1	7	4	3.65	1.24	3.60	0.33
Overall	0	8	4	3.90	1.20	0	8	4	3.96	1.28	3.81	0.36
						Anti-A_1 IgG						
0	-1	11	6	5.24	2.19	-1	10	6	5.64	1.98	5.53	0.53
B	-1	6	1	1.25	1.73	-1	5	2	1.64	1.80	1.44	0.52
Overall	-1	11	5	4.54	2.71	-1	10	5	4.80	2.54	3.49	0.53
						Anti-B IgM						
0	0	7	3	3.16	1.36	0	8	3	3.31	1.29	3.24	0.39
A	0	7	3	2.96	1.40	0	8	3	3.09	1.34	3.02	0.39
Overall	0	7	3	3.06	1.38	0	8	3	3.20	1.32	3.13	0.39
						Anti-B IgG						
0	-1	11	4	4.22	2.03	-1	11	5	4.48	2.03	4.35	0.61
A	-1	9	-1	0.15	1.55	-1	9	0	0.38	1.69	0.27	0.45
Overall	-1	11	2	2.24	2.72	-1	11	2	2.49	2.78	2.31	0.54

Note. The pooled *SD* for each blood group was calculated based on the individual *SD*s. The overall pooled *SD* was calculated based on the pooled *SDs* of the respective blood groups.

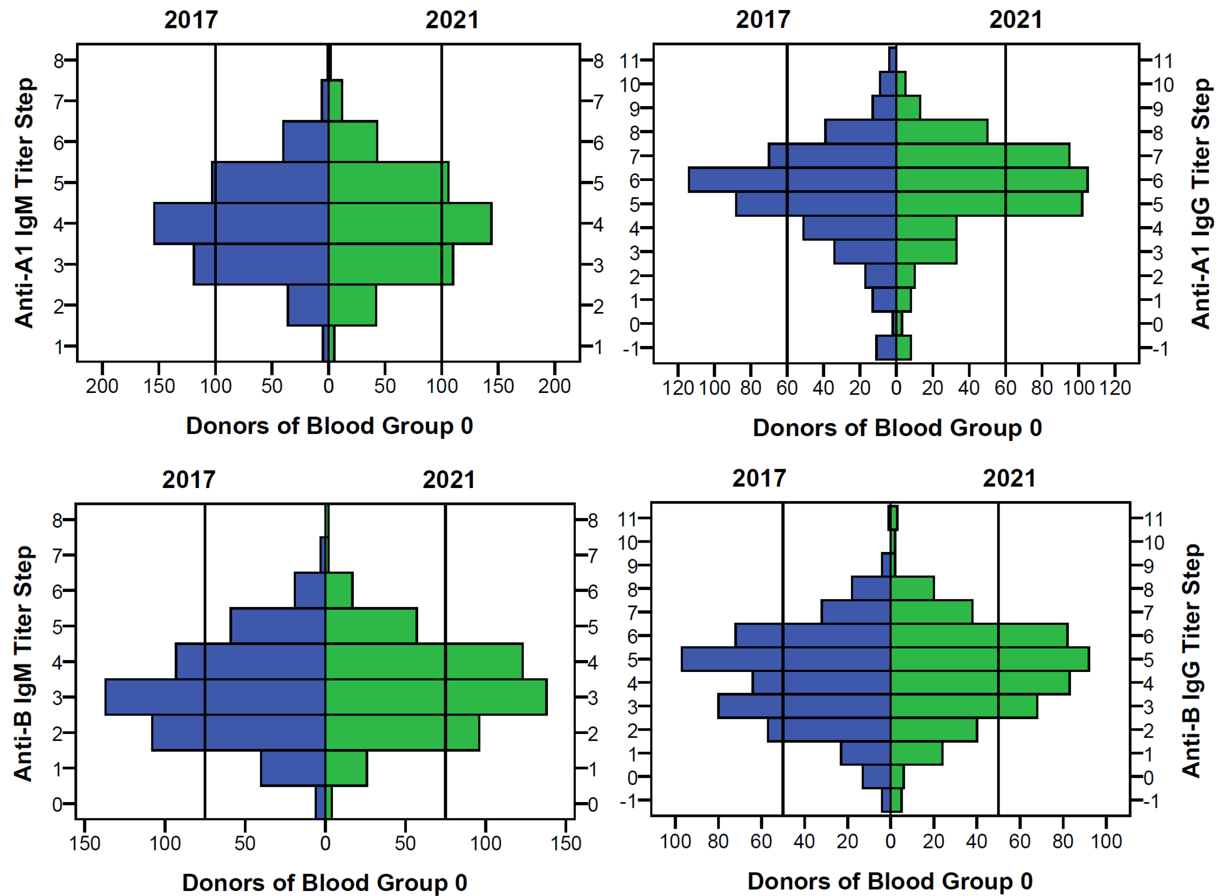
The mean TS of each isoagglutinin showed a slight increase in 2021 compared with 2017.

The descriptive TS parameters within blood groups, stratified by antibody specificity, isotype, gender and type of donation are presented in **Table 29** in the Appendix section.

Blood Group 0

The TS distribution of blood group 0 donors in 2017 compared with 2021 is shown as comparative histograms in **Figure 6**. These histograms also show the slight increase in titer in 2021 compared to 2017.

Figure 6 *Isoagglutinin Titer Step Distribution in Blood Group 0 Donors*

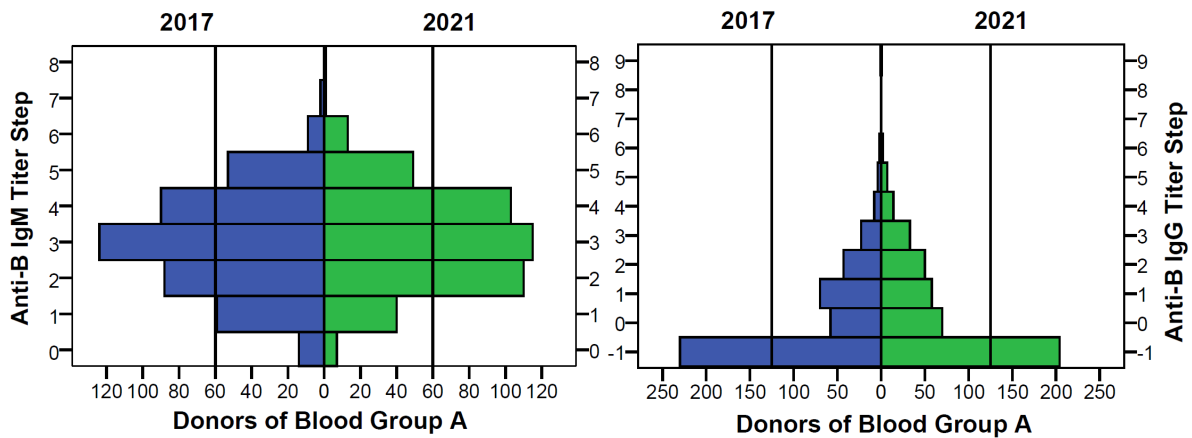


Note. Isoagglutinin TS comparison between 2017 and 2021. Data are presented as number.

Blood Group A

The TS distribution of blood group A donors in 2017 compared with 2021 is shown as comparative histograms **Figure 7**. These histograms also show the slight increase in titer in 2021 compared to 2017.

Figure 7 *Isoagglutinin Titer Step Distribution in Blood Group A Donors*

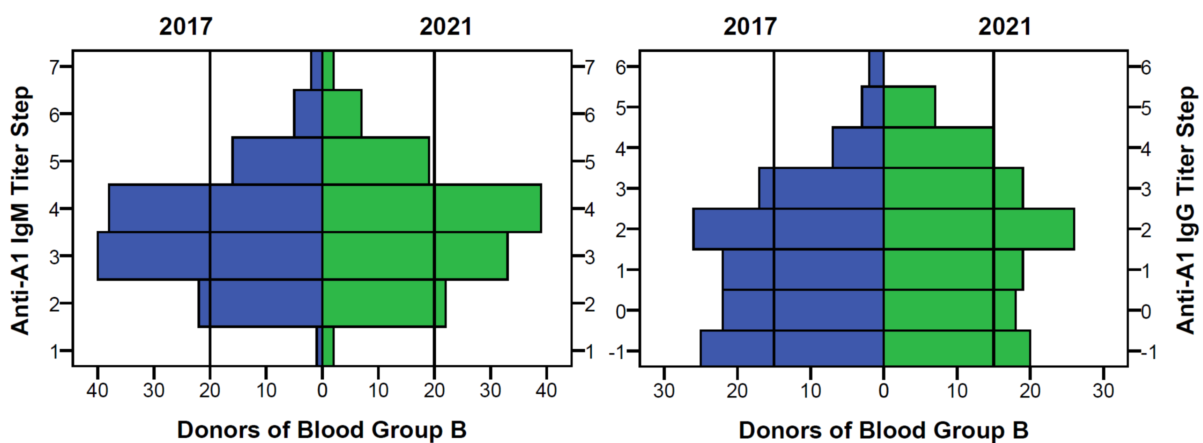


Note. Isoagglutinin TS comparison between 2017 and 2021. Data are presented as number.

Blood Group B

The TS distribution of blood group B donors in 2017 compared with 2021 is shown as comparative histograms **Figure 8**. These histograms also show the slight increase in titer in 2021 compared to 2017.

Figure 8 *Isoagglutinin Titer Step Distribution in Blood Group B Donors*



Note. Isoagglutinin TS comparison between 2017 and 2021. Data are presented as number.

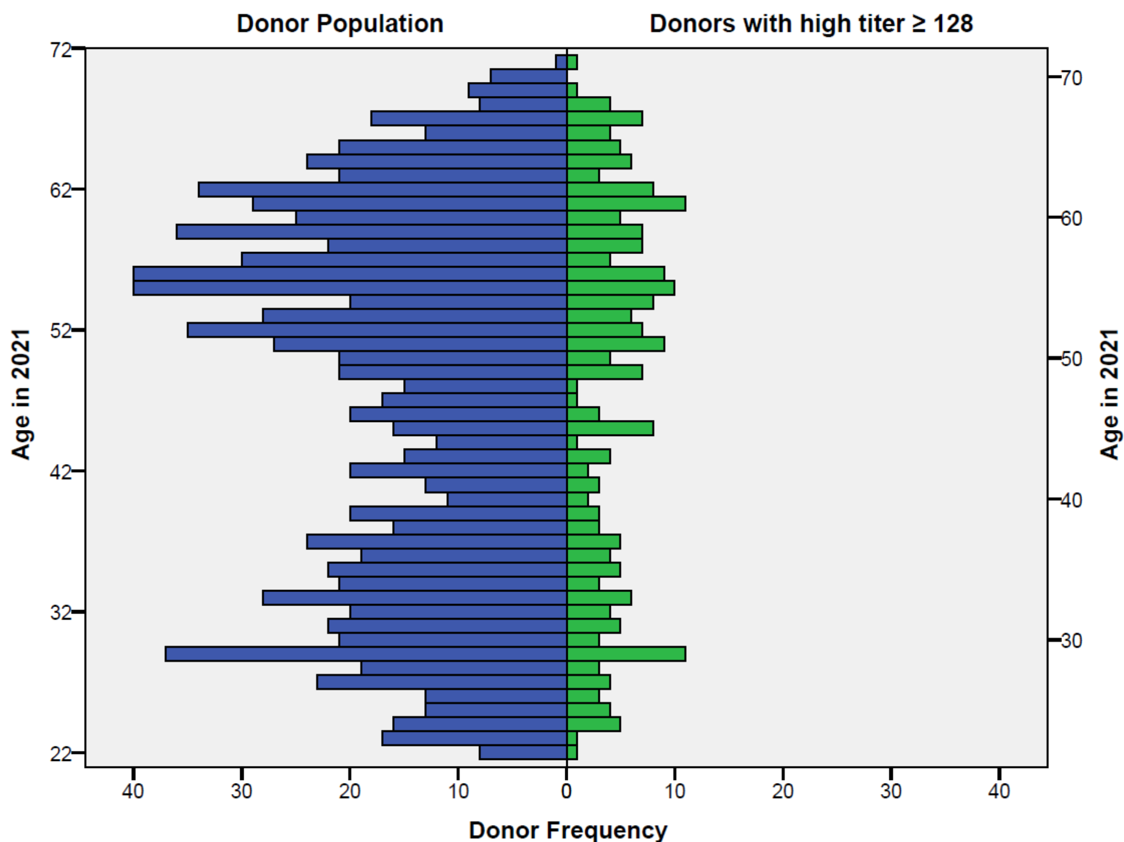
4.3.2 Isoagglutinin Titer Changes Around Titer Step 7

Considering TS 7 (titer 128) as cutoff for a high titer, 22.5% (231 donors) of all 1028 donors had had at least one a high titer isoagglutinin over the years.

Relative to each gender study group, the distribution of the donors with titers ≥ TS 7 was 19.0% of male donors (124 of 704) and 29.9% of female donors (97 of 324).
Relative to each donation type, the distribution was 22.7% of whole blood donors (179 of 789) and 40.6% of platelet donors (97 of 239).
Relative to each blood group, the distribution was 47.7% of group 0 (222 of 465), 1.1% of blood group A (5 of 439) and 3.2% of group B donors (4 of 124).
The age of these donors ranged from 22 to 71 years (*Md* = 51, *M* = 47.99, *SD* = 13.35).

The distribution by age of the donors with high titers in comparison to the study population is presented in **Figure 9**. The distribution of donors with titers ≥ TS 7 by blood group, gender and donation type is presented **Table 20**.

Figure 9 *High Titer Donor Distribution by Age and Titer Step 7*



Note. TS 7 represents titer value 128. Age distribution of high titer donors compared with all donors.

The 231 donors had in total 300 high titer isoagglutinins. Multiple high titer isoagglutinins were only observed in blood group 0 donors.

Relative to the overall population, 3.0% of all donors (31 of 1028, 32 antibodies) had high IgM titers and 20.8% (214 of 1028, 268 antibodies) had high IgG titers.

Regarding group 0 high titer donors with multiple high isoagglutinins, 59 had two high titer isoagglutinins and five donors had simultaneously three high titer isoagglutinins.
The combinations of two isoagglutinins were: anti-A_1 IgG and anti-B IgG for 49 donors, anti-A_1 IgM and anti-A_1 IgG for 6 donors, anti-B IgM and anti-A_1 IgG for two donors, anti-B IgM and anti-B IgG for one donor, and anti-A_1 IgM and anti-B IgM for another donor.
The combinations of three isoagglutinin were: anti-A_1 IgM, anti-A_1 IgG and anti-B IgG for three donors and anti-B IgM, anti-A_1 IgG and anti-B IgG for two donors.

Table 20 *High Titer Donors by Blood Group, Gender, Donation Type and Titer Step 7*

	Blood group 0		**Blood group A**		**Blood group B**	
	Whole Blood	Platelet	Whole Blood	Platelet	Whole Blood	Platelet
Male	103	26	1	0	3	1
Female	68	25	4	0	0	0

Note. Data are reported as number. TS 7 encodes titer 128.

Regarding titer changes around the TS 7 cutoff, and relative to all high titer isoagglutinins, 52.0% of them remained high over the years (156 of 300), 16.3% changed to a low titer (49 of 300) and 31.7% changed to a high titer (95 of 300), The distribution by blood group and isoagglutinin around the TS 7 is shown in **Table 21**.

Regarding titer changes around the TS 7 cutoff and high titer donors, and relative to the overall population, 8.4% of all donors (86 of 1028, 95 antibodies) converted to high titer donors, 3.0% (31 of 1028) converted to low titer donors and 11.1% (114 of 1028) remained high titer.

Table 21 *Changes of High Titer Isoagglutinins Around Titer Step 7*

	High Titer Isoagglutinins Considering Cutoff TS 7		
	Change to Low Titer	Remain High Titer	Change to High Titer
		Blood Group 0	
Anti-A_1 IgM	4 (0.9)	3 (0.7)	11 (2.4)
Anti-B IgM	2 (0.4)	1 (0.2)	3 (0.7)
Anti-A_1 IgG	21 (4.5)	114 (24.5)	49 (10.5)
Anti-B IgG	18 (3.9)	37 (8.0)	28 (6.0)
		Blood Group A	
Anti-B IgM	2 (0.5)		2 (0.5)
Anti-B IgG		1 (0.2)	
		Blood Group B	
Anti-A_1 IgM	2 (1.6)		2 (1.6)

Note. Data are reported as number (% of the respective blood group). 64 blood group 0 donors had multiple high titer antibodies. Anti-A_1 IgG of group B had no high titer. TS 7 encodes titer 128.

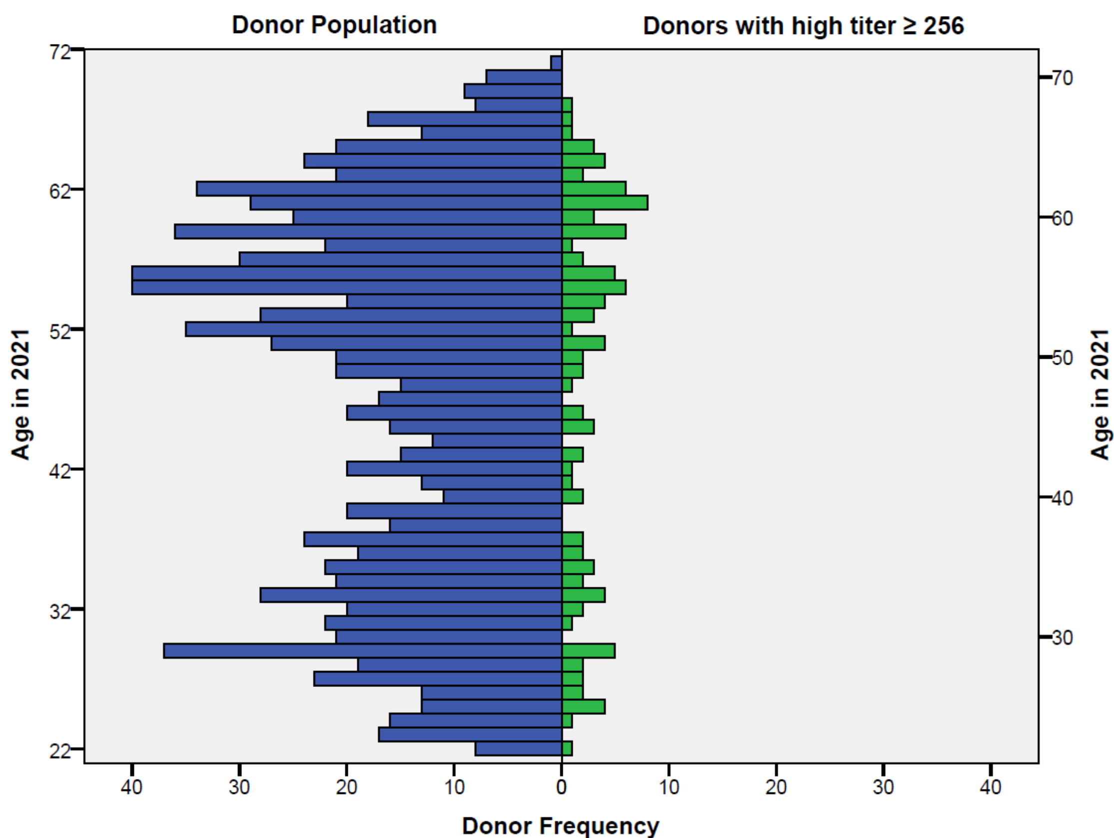
4.3.3 Isoagglutinin Titer Changes Around Titer Step 8

Considering the critical titer one titer step higher at TS 8 (titer 256), 110 donors (10.7% of all 1028 donors) had at least one high titer isoagglutinin.

Relative to each gender study group, the distribution of the donors with titers ≥ TS 8 was 8.9% of male donors (63 of 704) and 14.2% of female donors (46 of 324).
Relative to each donation type, the distribution was 10.9% of whole blood donors (86 of 789) and 10.0% of platelet donors (24 of 239).
Relative to each blood group, the distribution was 23.2% of group 0 (108 of 465), 0.5% of blood group A donors (2 of 439).
The age of these donors ranged from 22 to 68 years (*Md* = 53, *M* = 48.24, *SD* = 13.32).

The distribution of the donors with titers ≥ TS 8 by blood group, gender and donation type is presented **Table 22** and the distribution by age in comparison to the study population is presented in **Figure 10**.

Figure 10 *High Titer Donor Distribution by Age and Titer Step 8*



Note. TS 8 represents titer value 256. Age distribution of high titer donors compared with all donors.

The 110 donors had in total 128 high titer isoagglutinins. Multiple high titer isoagglutinins were only observed in blood group 0 donors.

Relative to the overall population, 0.2% of all donors (2 of 1028, 32 antibodies) had high IgM titers and 0.3% (3 of 1028, 268 antibodies) had high IgG titers.

Regarding group 0 high titer donors with multiple high isoagglutinins, 19 donors had two high titer isoagglutinins. The isoagglutinin combinations were anti-A_1 IgG and anti-B IgG for 15 donors, anti-A_1 IgM and anti-A_1 IgG for 2 donors and anti-B IgM and anti-B IgG for one donor.

Table 22 *High Titer Donors by Blood Group, Gender, Donation Type and Titer Step 8*

	Blood Group 0		**Blood Group A**		**Blood Group B**	
	Whole Blood	Platelet	Whole Blood	Platelet	Whole Blood	Platelet
Male	51	11	1	0	0	0
Female	33	13	1	0	0	0

Note. Data are reported as number. TS 8 encodes titer 256.

Regarding titer changes around the TS 8 cutoff, and relative to all high titer isoagglutinins, 48.4% of them remained high over the years (62 of 128), 21.1% changed a low titer (27 of 128) and 30.5% changed to a high titer (39 of 128), The distribution by blood group and isoagglutinin around the TS 8 is shown in **Table 23**.

Regarding titer changes around the TS 8 cutoff and high titer donors, and relative to the overall population, 3.4% of all donors (35 of 1028, 95 antibodies) converted to high titer donors, 2.1% (22 of 1028) converted to low titer donors and 5.2% (53 of 1028) remained high titer donors.

Table 23 *Changes of High Titer Isoagglutinins Around Titer Step 8*

	High Titer Isoagglutinins Considering the Cutoff TS 8		
	Changed to Low Titer	Remain High Titer	Change to High Titer
		Blood Group 0	
Anti-A_1 IgM		1 (0.2)	1 (0.2)
Anti-B IgM			2 (0.4)
Anti-A_1 IgG	22 (4.7)	43 (9.3)	25 (5.4)
Anti-B IgG	5 (1.1)	17 (3.7)	10 (2.2)
		Blood Group A	
Anti-B IgM			1 (0.2)
Anti-B IgG		1 (0.2)	

Note. Data are reported as number (% of the respective blood group). 19 blood group 0 donors had multiple high titer antibodies. There were no group B high titer donors. TS 8 encodes titer 256.

4.3.4 Wilcoxon Signed-Rank Test

Wilcoxon signed-rank test was carried out to compare TS 2017 to TS 2021. Detailed results of the Wilcoxon signed-rank test are presented in the Appendix section in **Table 30**.

Blood Group 0

Anti-A_1 IgM changes of blood group 0 were statistically non-significant.

Anti-A_1 IgG, anti-B IgM and IgG changes were statistically significant with a small effect size, $r = 0.149$, $r = 0.140$ and $r = 0.148$, respectively.

Blood Group 0 Anti-A_1 IgM: $p = .128$ IgG: $p = 5.26E\text{-}6$

Blood Group 0 Anti-B IgM: $p = 1.84E\text{-}5$ IgG: $p = 6.78E\text{-}6$

Changes by Gender

In the male study group, anti-A_1 IgM changes were statistically non-significant. Anti-A_1 IgG, anti-B IgM and IgG changed statistically significant with a small effect size, $r = 0.189$, $r = 0.128$ and $r = 0.146$, respectively.

In the female study group, anti-A_1 IgM and IgG changes were statistically non-significant. Anti-B IgM and IgG titer changes were statistically significant with a small effect size, $r = 0.164$ and $r = 0.151$, respectively.

Blood Group 0 Male Anti-A_1 IgM: $p = .387$ IgG: $p = 3.11E\text{-}6$

Blood Group 0 Male Anti-B IgM: $p = .002$ IgG: $p = 3.12E\text{-}4$

Blood Group 0 Female Anti-A_1 IgM: $p = .166$ IgG: $p = .226$

Blood Group 0 Female Anti-B IgM: $p = .003$ IgG: $p = .007$

Changes by Donation type

In the whole blood study group, anti-A_1 IgM changes were statistically non-significant. Anti-A_1 IgG, anti-B IgM and IgG changes were statistically significant with a small effect size, $r = 0.188$, $r = 0.132$ and $r = 0.142$, respectively.

In the platelet study group, anti-A_1 IgM and IgG changes were statistically non-significant. Anti-B IgM and IgG titer changes were statistically significant with a small effect size, $r = 0.169$ and $r = 0.165$, respectively.

Blood Group 0 Whole Blood Anti-A_1 IgM: $p = .267$ IgG: $p = 3.57E\text{-}7$

Blood Group 0 Whole Blood Anti-B IgM: $p = 3.57E\text{-}4$ IgG: $p = 1.18E\text{-}4$

Blood Group 0 Platelets Anti-A_1 IgM: $p = .266$ IgG: $p = .813$

Blood Group 0 Platelets Anti-B IgM: $p = .017$ IgG: $p = .020$

Blood Group A

Anti-B IgM and IgG changes were statistically significant with a small effect size, $r = 0.121$ and $r = 0.171$, respectively.

Blood Group A   Anti-B   IgM: $p = 3.52E{-4}$ IgG: $p = 4.22E{-7}$

Changes by Gender

In the male study group, anti-B IgM and IgG changes were statistically significant with a small effect size, $r = 0.148$ and $r = 0.146$, respectively.

In the female study group, anti-B IgM changes were statistically non-significant, whereas anti-B IgG titer changes were statistically significant with a small effect size, $r = 0.231$.

Blood Group A Male   Anti-B   IgM: $p = 2.19E{-4}$ IgG: $p = 2.88E{-4}$

Blood Group A Female   Anti-B   IgM: $p = .449$ IgG: $p = 2.06E{-4}$

Changes by Donation type

In the whole blood study group, anti-B IgM and IgG changes were statistically significant with a small effect size, $r = 0.103$ and $r = 0.162$, respectively.

In the platelet study group, anti-B IgM and IgG changes were statistically significant with a small effect size, $r = 0.165$ and $r = 0.203$, respectively.

Blood Group A Whole Blood   Anti-B   IgM: $p = .009$ IgG: $p = 3.86E{-5}$

Blood Group A Platelets   Anti-B   IgM: $p = .012$ IgG: $p = .002$

Blood Group B

Anti-A_1 IgM changes were statistically non-significant.

Anti-A_1 IgG changes were statistically significant with a small effect size, $r = 0.264$.

Blood Group B   Anti-A_1 IgM: $p = .130$ IgG: $p = 3.11E{-5}$

Changes by Gender

In the male study group, anti-A_1 IgM changes were statistically non-significant, whereas anti-A_1 IgG changes were statistically significant with a small effect size, $r = 0.299$.

In the female study group, anti-A_1 IgM and IgG changes were statistically non-significant.

Blood Group B Male   Anti-A_1 IgM: $p = .194$ IgG: $p = 6.47E{-5}$

Blood Group B Female   Anti-A_1 IgM: $p = .439$ IgG: $p = .147$

Changes by Donation type

In the whole blood study group, anti-A_1 IgM changes were statistically non-significant, whereas anti-A_1 IgG changes were statistically significant with a small effect size, $r = 0.286$.

In the platelet study group, anti-A_1 IgM changes were statistically significant with a moderate effect size, $r = 0.365$, whereas anti-A_1 IgG changes were statistically non-significant.

Blood Group B Whole Blood   Anti-A_1 IgM: $p = .647$ IgG: $p = 5.15E{-5}$

Blood Group B Platelets   Anti-A_1 IgM: $p = .016$*   IgG: $p = .268$

4.4 Titer Step Difference Between 2021 and 2017

TSD was calculated by subtracting TS in 2017 from the TS in 2021. A negative TSD represents a decrease in titer and a positive TSD represents a titer increase.

4.4.1 Distribution of Titer Step Difference

TSD was calculated by blood group and isoagglutinin, and by isoagglutinin for the overall population, as shown in **Table 24**. The overall TSD of each isoagglutinin was calculated of all donors that produced the respective isoagglutinin. There were 589 donors tested for anti-A_1 isoagglutinins and 904 donors tested for anti-B isoagglutinins.

The TSD ranged from -4 to +3 for IgM isoagglutinins and from -3 to +6 for IgG isoagglutinins. The majority of donors had no titer changes when stratified by blood group and isoagglutinin, with the exception of blood group 0 donors for anti-B IgG, where the majority had positive TSD indicating a titer increase. The percentage of positive TSD was larger than the percentage of negative TSD, indicating a titer increase, as well as the mean TSD which had positive values for all isoagglutinins.

Table 24 *Isoagglutinin Titer Step Difference*

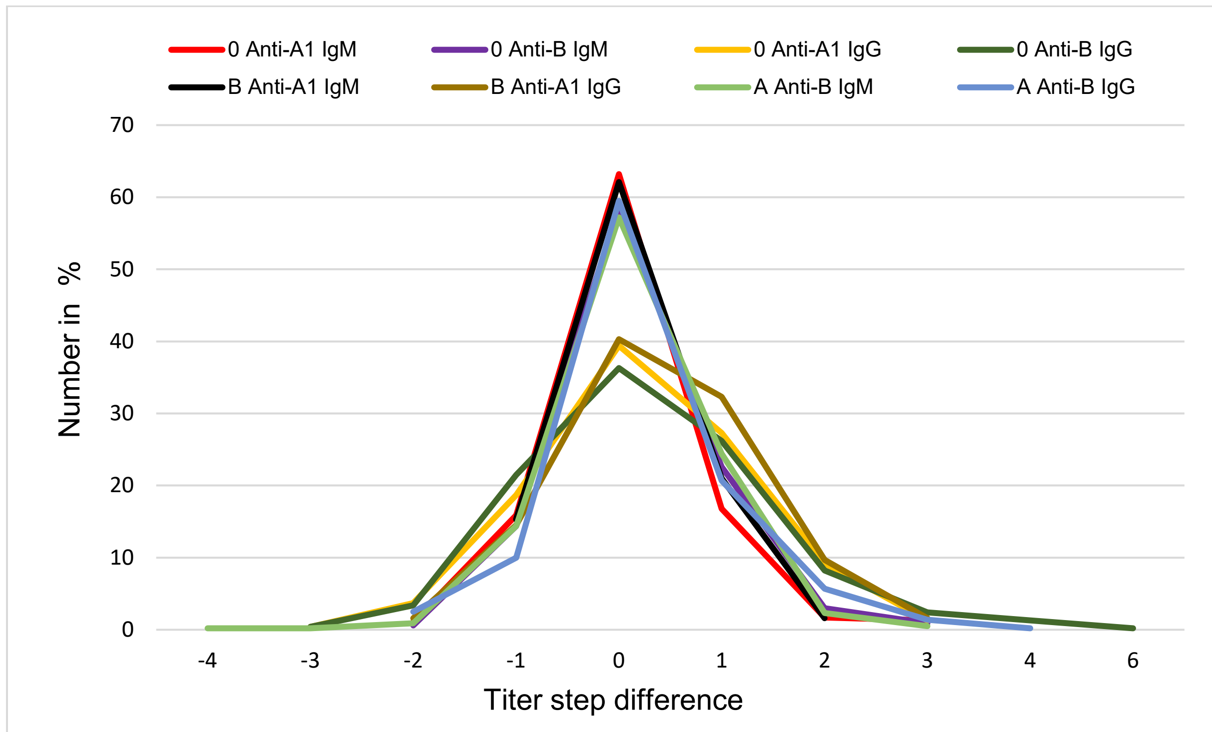
Blood Group	*n*	Titer Step Difference										
		Min	*Max*	*Md*	*M*	*SD*	0	±1	±2	≥±3	Negative	Positive
							Anti-A_1 IgM					
0	465	-2	+3	0	0.06	0.74	63.2	32.7	2.8	1.3	17.0	19.8
B	124	-1	+2	0	0.09	0.65	62.1	36.3	1.6	0.0	15.3	22.6
Overall	589	-2	+3	0	0.07	0.72	63.0	33.5	2.5	1.0	16.6	20.4
							Anti-A_1 IgG					
0	465	-3	+3	0	0.22	1.04	39.4	46.0	12.9	1.7	22.8	37.8
B	124	-2	+3	0	0.39	0.96	40.3	46.8	11.3	1.6	16.1	43.6
Overall	589	-3	+3	0	0.26	1.03	39.6	46.2	12.5	1.7	21.3	39.1
							Anti-B IgM					
0	465	-2	+3	0	0.15	0.76	58.5	37.0	3.6	0.9	15.0	26.5
A	439	-4	+3	0	0.13	0.77	57.1	38.8	3.2	0.9	15.7	27.2
Overall	904	-4	+3	0	0.14	0.76	57.9	37.8	3.4	0.9	15.4	26.7
							Anti-B IgG					
0	465	-3	+6	0	0.26	1.19	36.4	47.7	11.6	4.3	25.3	38.3
A	439	-2	+4	0	0.22	0.86	59.5	30.7	8.2	1.6	12.5	28.0
Overall	904	-3	+6	0	0.24	1.04	47.5	39.5	10.0	3.0	19.1	33.4

Note. *N* = 1028. TSD of -2 represents a titer decrease of 2 titer steps. TSD was calculated by blood group and overall by isoagglutinin.

The largest percentage of negative TSD was found for anti-B IgM of blood group A. The largest percentage of positive TSD was found for anti-B IgG of blood group 0.

TSD distribution by blood group and isoagglutinin is presented in **Figure 11**.

Figure 11 *Isoagglutinin Titer Step Difference by Blood Group*



Note. Data are presented in %

TSD distribution within blood groups stratified by gender and donation type is presented in **Table 32** in the Appendix section.

4.4.2 Donors With Large Titer Step Difference

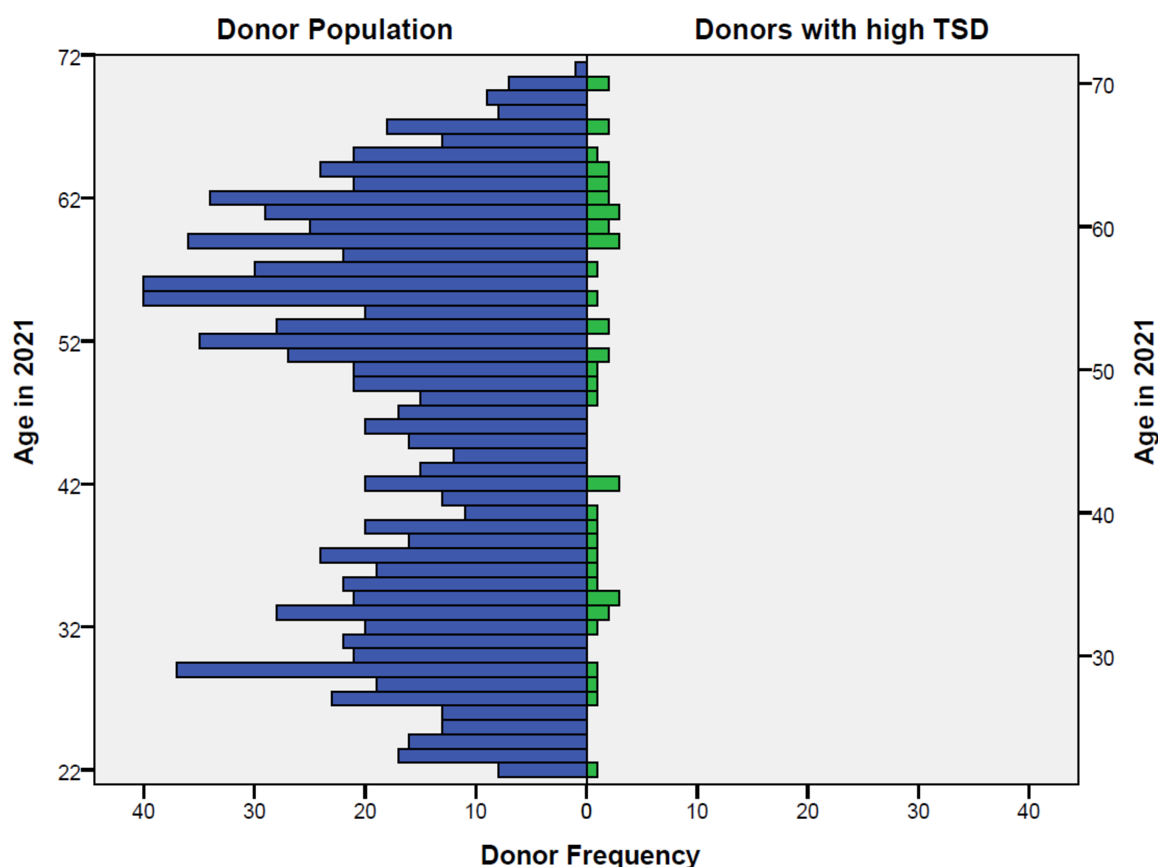
TSD greater than or equal to ±3 was considered large TSD.

The analysis showed that 4.6% of all donors (47 of 1028) had large TSD. Relative to each gender group, the distribution of donors with large TSD was 4.6% of male donors (32 of 704) and 4.6% of female donors (15 of 324). Relative to each donation type, the distribution was 4.4% of whole blood donors (35 of 789) and 5.0% of platelet donors (12 of 239).

The age of the donors with large TSD ranged from 22 to 70 years (*Md* = 51, *M* = 49.38, *SD* = 13.50). The distribution by age in comparison to the study population is presented in **Figure 12**.

Relative to the study population, 0.6% (5 donors) showed a negative TSD (titer decrease), whereas 4.0% (41 donors) had positive TSD (titer increase).

Figure 12 *Large Titer Step Difference Donor Distribution by Age*



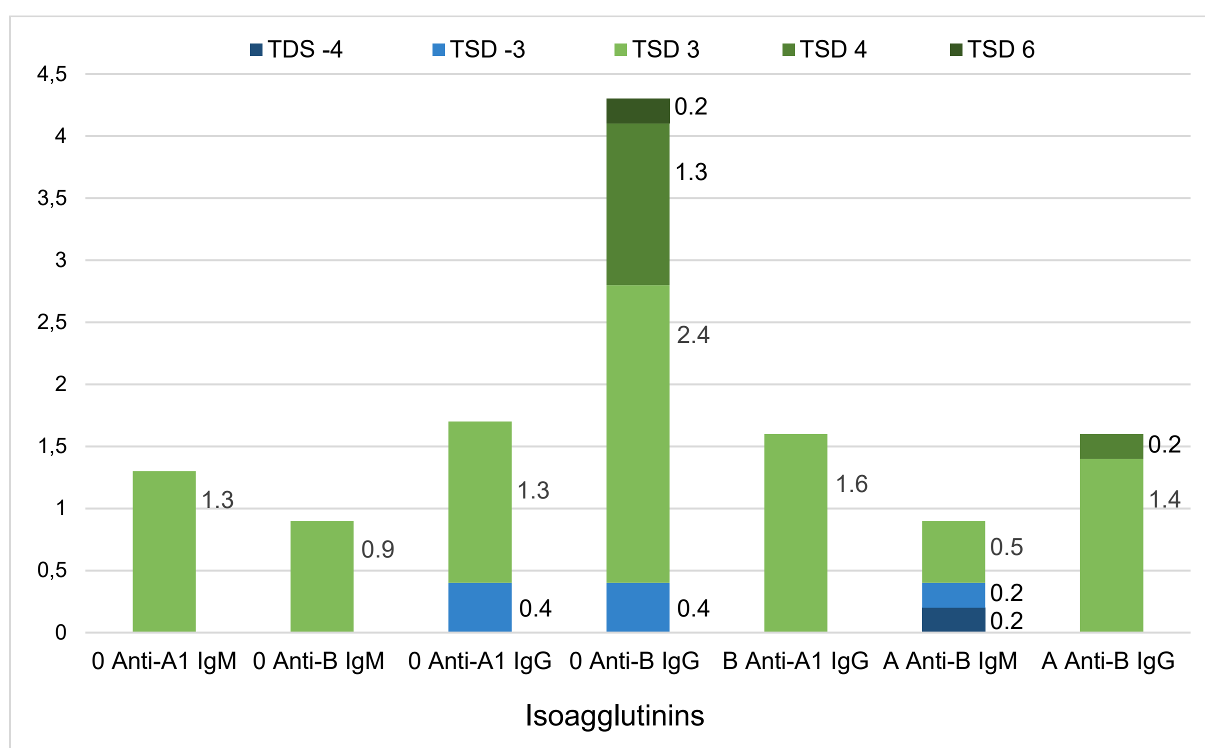
Note. Age distribution of donors with large titer step difference compared with all donors.

Regarding the Distribution by Blood Group of donors with large TSD, 7.3% belonged to blood group 0 (34 of 1028 donors), 2.5% to blood group A (11 of 1028) and 1.6% to blood group B (2 of 1028 donors). These donors had in total 51 isoagglutinins with large TSD. Of them, 38 isoagglutinins belonged to group 0 donors, 11 to group A and 2 to group B. The isoagglutinins with large TSD are presented by blood group in **Figure 13**.

Regarding the Isoagglutinin Isotype, of the 51 isoagglutinins with large TSD, the majority of 37 antibodies were IgG (37 donors, 3.6% of all donors), with 27 anti-B IgG and 10 anti-A_1 IgG. The rest of 14 antibodies were IgM (13 donors, 1.3%), with 6 anti-A1 IgM and 8 anti-B IgM.

Regarding the Number of Isoagglutinins with large TSD per donor, the majority of the donors (44, 93.6%) had only one isoagglutinin with large TSD. The donors with negative TSD (titer decrease) had exclusively one large TSD antibody. There were three donors with positive TSD (titer increase) with multiple large TSD isoagglutinins, all of blood group 0. Two of them had two large TSD antibodies and one donor had three large TSD antibodies. The two donors were female, of age 33 and 36, respectively, with large TSD for anti-B IgM (TSD +3, titer increase to TS 8 for both donors) and anti-B IgG (TSD +4, titer increase to TS 5 and TS 11, respectively). The third donor was male, age 64, with large TSD for anti-A_1 IgM (TSD 3, titer increase to TS 7), anti-B IgM (TSD +3, titer increase to TS 4) and anti-B IgG (TSD +4, titer increase to 6).

Figure 13 *Large Isoagglutinin Titer Step Difference by Blood Group*

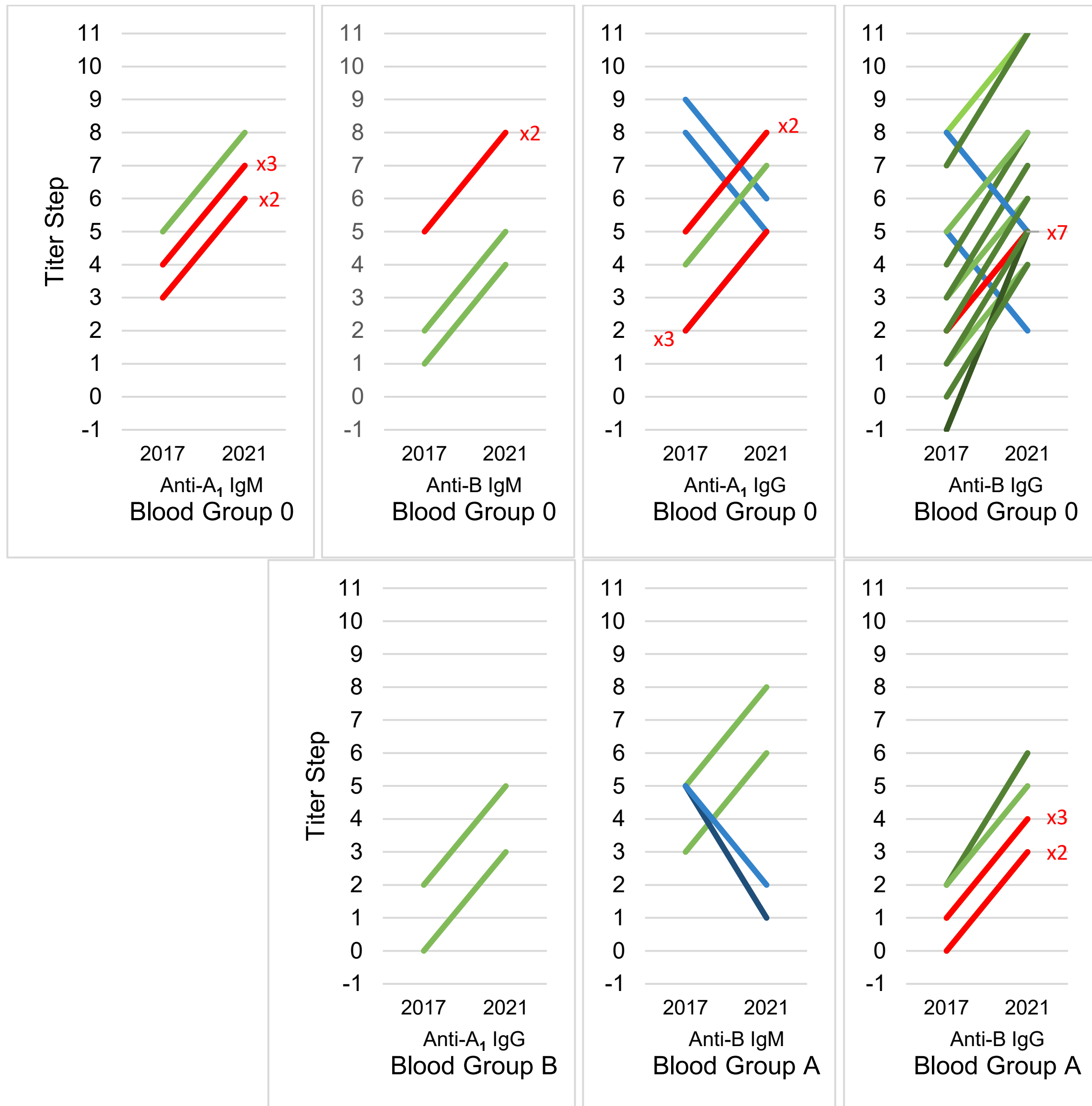


Note. Data are presented as % after stratification by blood group, antibody specificity and isotype. There were no ±5 or -6 TSD. Anti-A_1 IgM of group B had no large TSD. Three donors had more than one isoagglutinin with large TSD. The green columns represent increase in titer. The blue columns represent decrease in titer.

Regarding TSD Amplitude, -4 TSD was found for one isoagglutinin (one donor, 0.1% of all donors), -3 TSD for five isoagglutinins (5 donors, 0.5%), +3 TDS for 37 isoagglutinins (33 donors, 3.2%), +4 TSD for seven isoagglutinins (7 donors, 0.7%) and +6 TSD for one

isoagglutinin (one donor, 0.1%). The distribution of large TSD isoagglutinins by TS change is presented in **Figure 14**.

Figure 14 *Titer Changes of Isoagglutinins With Large Titer Step Difference*



Note. TS changes from 2017 to 2021 of the antibodies with large TSD stratified by blood group specificity and isotype. Anti-A_1 IgM of blood group B had no large TSD. The red lines indicate multiple donors with the same TS changes; the number of donors is indicated on the diagram. The green lines represent increase in titer. The blue lines represent decrease in titer.

The donors with very large TSD of +6 and ±4 are presented hereafter.

The donor with TSD of +6 was female, age 27, group 0, with high titer difference for anti-B IgG and titer increase to TS 5.

TSD of +4 was found exclusively for anti-B IgG, for six donors of group 0 and one of group A. Three of the group 0 donors were already presented in the previous section as donors with multiple large TSD. The other three donors were male, age 29, 53 and 61, respectively, with titer increase to TS 4, 7 and 8, respectively. The donor of blood group A was male, age 57, titer increase to TS 6.

TSD of -4 was found for one donor, male, age 38, blood group 0, anti-B IgM, titer decrease to TS 1.
The isoagglutinins with large TSD by blood group and isoagglutinin is presented in **Table 25**.

Table 25 *Isoagglutinins With Large Titer Step Difference by Blood Group*

	Titer Step Difference 2021-2017				
	-4	-3	3	4	6
			Blood Group 0		
Anti-A_1 IgM			6 (1.3)		
Anti-B IgM			4 (0.9)		
Anti-A_1 IgG		2 (0.4)	6 (1.3)		
Anti-B IgG		2 (0.4)	11 (2.4)	6 (1.3)	1 (0.2)
			Blood Group A		
Anti-B IgM	1 (0.2)	1 (0.2)	2 (0.5)		
Anti-B IgG			6 (1.4)	1 (0.2)	
			Blood Group B		
Anti-A_1 IgG			2 (1.6)		

Note. Data are reported as number (% in the respective blood group). There are no samples with 5 titer steps difference. Anti-A_1 IgM of blood group B had no extreme TSD.

Regarding Changes Around Cutoff TS 7 of Isoagglutinins With Large TSD, the majority of the antibodies remained below cutoff (33 antibodies, 32 donors, 3.1% of all donors), followed by antibodies with titer increase (13 antibodies, 12 donors, 1.2%), titer decrease (3 donors, 3 antibodies, 0.3%) and antibodies which remained at high titer (2 antibodies, 2 donors, 0.2%). The changes of isoagglutinins with large TSD around the TS 7 cutoff by blood group are shown in **Table 26**.

Table 26 *Changes of Isoagglutinins With Large Titer Step Difference Around Titer Step 7*

	Isoagglutinins With Large Titer Step Difference			
	Remain < TS 7	Decrease < TS 7	Increase ≥ TS 7	Remain ≥ TS 7
		Blood Group 0		
Anti-A_1 IgM	2 (0.4)		4 (0.9)	
Anti-B IgM	2 (0.4)		2 (0.4)	
Anti-A_1 IgG	3 (0.7)	2 (0.4)	3 (0.7)	
Anti-B IgG	14 (3.0)	1 (0.2)	3 (0.7)	2 (0.4)
		Blood Group A		
Anti-B IgM	3 (0.7)		1 (0.2)	
Anti-B IgG	7 (1.6)			
		Blood Group B		
Anti-A_1 IgG	2 (1.6)			

Note. Data are reported as number (% in the respective blood group). Anti-A_1 IgM of blood group B showed no large TSD. TS 7 represents titer 128.

Regarding Changes Around Cutoff TS 8 of Isoagglutinins With Large TSD, the majority of the antibodies remained under cutoff (38 antibodies, 36 donors, 3.5% of all donors), followed by antibodies with titer increase (9 antibodies, 8 donors, 0.8%), titer decrease (3 antibodies, 3 donors, 0.3%) and antibodies which remained at high titer (one antibody, one donors, 0.1%). The changes of isoagglutinins with large TSD around the TS 8 cutoff by blood group are presented in **Table 27**.

Table 27 *Changes of Isoagglutinins With Large Titer Step Difference Around Titer Step 8*

	Isoagglutinins With Large Titer Step Difference			
	Remain < TS 8	Decrease < TS 8	Increase ≥ TS 8	Remain ≥ TS 8
	Blood Group 0			
Anti-A_1 IgM	5 (1.1)		1 (0.2)	
Anti-B IgM	2 (0.4)		2 (0.4)	
Anti-A_1 IgG	4 (0.9)	2 (0.4)	2 (0.4)	
Anti-B IgG	15 (3.2)	1 (0.2)	3 (0.7)	1 (0.2)
	Blood Group A			
Anti-B IgM	3 (0.7)		1 (0.2)	
Anti-B IgG	7 (1.6)			
	Blood Group B			
Anti-A_1 IgG	2 (1.6)			

Note. Data are reported as number (% in the respective blood group). Anti-A_1 IgM of blood group B showed no large TSD. TS 8 represents titer 256.

4.4.3 Regression Analysis

A multiple linear regression was carried out to investigate whether age and gender are associated with the difference in titer step. The multiple linear regression performed also an adjustment with age and gender on the TSD.

Blood Group 0

Anti-A_1 IgM. The regression performed for anti-A_1 IgM TSD showed no significant association with age and gender, *p* = .266. The adjusted mean of anti-A_1 IgM TSD was statistically non-significant, *p* = .063.

Anti-A_1 IgG. The regression performed for anti-A_1 IgG TSD was statistically significant, R^2 = .018, *F*(2, 462) = 4.136; *p* = .017. Age (*B* = -.008, *p* = .036) was significantly associated with the difference in titer step, while gender was not (*B* = -.095, *p* = .061). Although statistically significant, this regression model could only explain the TSD variance for 1.8% (8 of 465 donors) of the samples. The adjusted mean of anti-A_1 IgG TSD was statistically significant (*B* = .194, *p* = 1.38E-4).

Anti-B IgM. The regression performed for anti-B IgM TSD showed no significant association with age and gender, *p* = .783. The adjusted mean of anti-B IgM TSD was statistically significant, *p* = 1.35E-5.

Anti-B IgG. The regression performed for anti-B IgG TSD showed no significant association with age and gender, *p* = .502. The adjusted mean of anti-B IgG TSD was statistically significant, *p* = 4.50E-6.

Blood Group A

Anti-B IgM. The regression performed for anti-B IgM TSD showed no significant association with age and gender, *p* = .402. The adjusted mean of anti-B IgM TSD was statistically significant (*p* = .010).

Anti-B IgG. The regression performed for Anti-B IgG TSD showed no significant association with age and gender, *p* = .469. The adjusted mean of anti-B IgG TSD of group A was statistically significant (*p* = 1.46E-7).

Blood Group B

Anti-A_1 IgM. The regression performed for anti-A_1 IgM TSD showed no significant association with age and gender, *p* = .631. The adjusted mean of anti-A_1 IgM TSD of group B was statistically non-significant (*p* = .226).

Anti-A_1 IgG. The regression performed for Anti-A_1 IgG TSD showed no significant association with age and gender, *p* = .709. The adjusted mean of anti-A_1 IgG TSD of group B was statistically significant (*p* = 4.27E-4).

The mean TSD, which was adjusted with age and gender, was statistically significant and corresponded to the results of the Wilcoxon test. Multiple linear regression results for blood groups 0, A and B donors are presented in **Table 31** in the Appendix section.

4.5 Result Summary

The results indicate that the isoagglutinin titers changed statistically significant over the course of four years, with the exception of anti-A_1 IgM. For all study groups the mean isoagglutinin titer increased slightly in value in 2021 compared to 2017. Furthermore, it was observed that the difference in titer between 2021 and 2017 ranged from - 4 to + 6 titer steps, with the majority of the donors remaining within a difference of two titer steps. Large titer changes (≥ ± 3 TSD) were observed in 4.6% (47 of 1028) of the donors. Of these, 1.2% (12 of 1028) and 0.8% (8 of 1028) had large titer changes that exceeded the high critical titer cutoffs of 128 and 256, respectively. The majority of donors with large titer changes belonged to blood group 0 and had an anti-B IgG titer that increased by 3 titer steps.

It was observed that 22.5% (231 of 1028) and 10.7% (110 of 1028) of donors had at least one high isoagglutinin titer, considering the critical titer cutoffs of 128 and 256, respectively. The majority of these donors belonged to blood group 0.

In the analysis of the IgG isotype, it was observed that 20.7% (213 of 1028) of the donors lacked IgG isoagglutinins. The distribution was maintained after stratification by gender and by donation type. However, the distribution by blood group differed, with 1.9% of blood group 0 donors (9 of 465), 42.8% of blood group A (188 of 439) and 12.9% of blood group B donors (16 of 124) having no IgG isoagglutinins.

The results showed that the isoagglutinin titer changes over the four-year period were statistically significant across all blood groups, isoagglutinin specificities and isotypes, with the exception of anti-A_1 IgM. The statistical significance was demonstrated with two statistical tools, the Wilcoxon signed-rank test and the multiple linear regression. Furthermore, the regression analysis showed that the difference in titer could not be associated with age or gender, with one exception. Anti-A_1 IgG titer changes showed an association with age, but not with gender, for only 1.8% (8 of 465) of the blood group 0 donors.

The significance of the changes analyzed by study group showed that the male and whole blood study groups carried the statistical significance of the overall population, as they represented the majority of donors. Furthermore, it was seen that the female donors had only anti-B statistical significance changes, IgM and IgG in blood group 0, and IgG in blood group A. Likewise, in the platelet study group only anti-B, IgM and IgG, had statistical significant changes. Furthermore, in contrast with the entire tested population, the few blood group B platelet donors (24 donors) showed significant changes for anti-A_1 IgM.

5 Discussion

The isoagglutinins play a central role in transfusion medicine. After their discovery alongside AB0 blood groups, the transfusion of whole blood could be performed in a safe manner. Their importance was recognized early on, and many studies have examined isoagglutinin titers in various populations using different manual methods, especially in relation with safe low-titer “universal donors”. (6) Advances in blood component manufacturing enabled the separation of whole blood into components, making AB0 compatible transfusion widely accessible. Thereafter, the isoagglutinins became somewhat less important until the transfusion of platelet units became common practice. Because platelet units have a short shelf life and are scarcely produced, they are not always available for all blood groups and therefore they are sometimes transfused minor incompatible. Additionally, the use of universal low-titer group 0 whole blood regained international interest in both military and civilian sectors (122), and with it the importance of isoagglutinin titration and titer changes in blood donors. Moreover, advances in transplant medicine made AB0 incompatible kidney transplantations possible, further increasing the importance of isoagglutinins for both patients and donors. (8) Furthermore, the isoagglutinin titer retains its clinical importance in obstetrics in AB0 incompatible pregnancies. (2)

Although the isoagglutinin titers have been analyzed extensively, it has been observed that the results obtained with different titration methods are difficult to compare. (6) Furthermore, the lack of consistency of manual titration results with inter- and intra-laboratory variation and inter- and intra-personal bias is well known (22, 23). Moreover, most current methods, whether manual or automated, cannot accurately differentiate antibody isotype (IgG from IgM) without laborious treatment of the sample. Therefore, evaluating titer results obtained in different studies with the goal of finding an internationally accepted critical titer on which to base treatment decisions is nearly impossible. This difficulty is reflected in the multitude of country- and institution-specific procedures that address AB0 minor incompatible transfusions and AB0 incompatible kidney transplantations. (7)

Isoagglutinin titers vary among individuals and the variance can be attributed only in part to genetics. (83) In the early literature, IgM isoagglutinins were described as naturally occurring antibodies and IgG as immune isoagglutinins produced after stimulation with AB0 antigens during pregnancy or after AB0 incompatible blood transfusion. (19) Although this view is still accepted, it is also known that environment dependent factors such as the microbiome, diet, seasonality, geographical location, personal and social environment play an important role in titer changes. (4, 20, 21, 87, 99) However, sometimes the increase in titer, usually discovered in blood donors due to transfusion reactions, has no apparent stimulation. (6)

Usually, titer changes over time have been analyzed in cross-sectional studies by comparing titers of different age groups. Longitudinal studies of isoagglutinin titer are scarce, and they investigate either small groups of individuals (5, 8) or selected blood groups reported at an arbitrarily set critical titer (4, 15).

The aim of this dissertation was to analyze the longitudinal changes of isoagglutinin titer in blood donors over an extended period of time. More specifically, the aim was to investigate whether longitudinal changes occur over a four-year period, the longest period analyzed to date, and whether these changes are significant. Furthermore, the changes were to be analyzed by blood group, isoagglutinin specificity, isotype, donation type and gender, as well as their correlation to age and gender. Moreover, the objective of the study was to identify and evaluate donors with large titer variations and the prevalence of donors with high titer values in relation to different critical titer cutoffs. Additionally, the opportunity was taken to analyze the prevalence of donors lacking IgG isoagglutinins.

For this purpose, sample pairs from whole blood and apheresis platelet donors were analyzed in a retrospective longitudinal study. Each donor donated during the same period of the year, with a maximum of 14 days between donation dates to avoid possible seasonal titer fluctuation. An important feature of the study, in addition to the longitudinal approach, the extensive timeline and the large sample size, was the titration method. An automated titration method was used to minimize fluctuations in results that could be misinterpreted as titer changes. Furthermore, this method was able to differentiate between the two clinically important immunoglobulin isotypes, IgG and IgM, and provide accurate results. The specification of isoagglutinin isotype is clinically relevant in neonatal, transfusion and transplant medicine. (2, 3, 70, 174)

5.1 Isoagglutinin Titer Changes by Blood Group

The present retrospective longitudinal study included 1073 blood donors who donated both in 2017 (April to July) and the same period in 2021 (April to June). Each donor donated during the same period of the year, with a maximum of 14 days between donation dates. The distribution by AB0 blood group was 43.3% group 0 donors, 40.9% group A, 11.6% group B and 4.2% group AB, which corresponds to the known distribution in the German population (49). Because blood group AB has no isoagglutinins, it was excluded from further analysis of titer changes. Therefore, the isoagglutinin titer results of the 1028 donors of blood groups 0, A and B were further analyzed. Slightly over two-thirds of these donors were male (68.5%) and the rest were female (31.5%). After stratification by donation type, just over three quarters were whole blood donors (76.7%) and the rest were apheresis platelet donors (23.3%). The age of the population ranged from 22 to 71 years in 2021, with a median of 49 years. Both isoagglutinin specificities, anti-A_1 and anti-B, and both clinical relevant isotypes, IgM and IgG, were investigated. The titration was performed with the NEO Iris® blood group analyzer, using agglutination for IgM titration and solid phase technology for IgG titration.

The present results showed that the isoagglutinin titer changes over the four-year period were statistically significant across all blood groups, isoagglutinin specificities and isotypes, with the exception of anti-A_1 IgM. The significance of the titer changes was analyzed using the Wilcoxon signed-rank test. The IgM *p*-values of anti-A_1 of blood groups 0 and B, and anti-B of blood groups 0 and A were .128 and .130, and 1.84E-5 and 3.52E-4, respectively. The IgG *p*-values of anti-A_1 of blood groups 0 and B, and anti-B of blood groups 0 and A were 5.26E-6 and 3.11E-5, and 6.78E-6 and 4.22E-7, respectively.

The mean titers in 2021 compared to 2017, across all isoagglutinins and study groups, indicate a slight increase in titer over the four-year period. The same increasing trend was seen in the percentage of positive TSD that was larger when compared with negative TSD, and in the mean TSD that had positive values across all study groups. The IgM mean TSD of anti-A_1 of blood groups 0 and B, and anti-B of blood groups 0 and A were 0.06 and 0.09, and 0.15 and 0.13, respectively. The IgG mean TSD of anti-A_1 of blood groups 0 and B, and anti-B of blood groups 0 and A were 0.22 and 0.39, and 0.26 and 0.22, respectively. The percentages of IgM positive TSD of anti-A_1 of blood groups 0 and B, and anti-B of blood groups 0 and A were 19.8% and 22.6%, and 26.5% and 27.2%, respectively. The percentages of IgG positive TSD of anti-A_1 of blood groups 0 and B, and anti-B of blood groups 0 and A were 37.8% and 43.6%, and 40.1% and 28%, respectively.

Furthermore, the data suggest that IgM isoagglutinins were more stable over time compared with IgG isoagglutinins. The titer variation estimated by mean TSD and by pooled *SD* was smaller for IgM isoagglutinins and larger for IgG isoagglutinins. The greatest variation was

observed for anti-B IgG of group 0. The IgM pooled *SD* of anti-A_1 of blood groups 0 and B, and anti-B of blood groups 0 and A were 0.37 and 0.33, and 0.39 and 0.39, respectively. The IgG pooled *SD* of anti-A_1 of blood groups 0 and B, and anti-B of blood groups 0 and A were 0.53 and 0.52, and 0.61 and 0.45, respectively. The observation that IgM isoagglutinins were more stable than IgG was also confirmed by the percentage of donors with unchanged titers, which was higher for IgM than IgG. Considering stratification by blood group, isoagglutinin and isotype, about 60% of IgM (from 57.1% to 63.2%) and about 40% of IgG isoagglutinins (from 36.4% to 40.3%) showed no changes in titer. The only exception was anti-B IgG of blood group A with 59.5% unchanged titers.

It is well known that automated titration methods have a good repeatability and reproducibility, which is important in titer comparisons, especially when analyzing changes over time. If a test method shows large variation in results, this variability could be misinterpreted as change in titer and affect the result analysis. The automated platform used in the present study is known to be consistent across laboratories, instruments, reagent lots and time periods, with the majority of the retests not exceeding one titer step difference. The method can measure the IgG isotype without additional sample preparation, such as DDT, a preparation that may also affect titration results. (26-30, 175). Isoagglutinin titration on this automated platform is also known to produce consistent results for samples frozen after the initial testing, even after multiple freeze-thaw cycles. (26)

Despite the known stability of the method, a titer difference of ±3 TSD and above was considered as significant in the analysis of large titer differences. This approach was also described in the AABB (Association for the Advancement of Blood & Biotherapies) technical manual (157), although it mainly refers to manual methods. The results show that 4.6% of all donors (47 of 1028) had indeed large titer changes, with the majority of 4.0% (41 of 1028) having an increase in titer. The percentages of IgM large TSD of anti-A_1 of blood groups 0 and B, and anti-B of blood groups 0 and A were 1.3% and 0%, and 0.9% and 0.9%, respectively. The percentage of IgG large TSD of anti-A_1 of blood groups 0 and B, and anti-B of blood groups 0 and A were 1.7% and 1.6%, and 4.3% and 1.6%, respectively. The highest prevalence and the largest TSD (+ 6 TSD) were observed for anti-B IgG of blood group 0.

As discussed in the Introduction section, the isoagglutinin baseline levels depend on several factors, of which the genetic constitution can account for only up to 30% (83) of the variation. Multiple factors that may lead to changes in isoagglutinin titers are closely related to the environment, such as age, gender, nutrition, geography, seasonality, and infectious diseases. Regarding the present results, it can be hypothesized that seasonal isoagglutinin titer fluctuations were minimized by collecting the samples during the same period of the year. Furthermore, due to the longitudinal design, where the same individuals are investigated over

a longer period of time, the genetic component, such as AB0 blood group and sex, and its influence on the titer changes was minimized but not completely eliminated, as the amplitude of the immune response to stimulation may vary depending on genetic makeup. It can also be hypothesized that the influence of geographic location was minimal, as the donors re-donated in the same geographic region after four years. However, no travel history was available in the present study, apart from the known travel restrictions associated with blood donation. There have been travel restrictions in place between 2020 and 2021, nonetheless, due to the COVID-s19 pandemic, which means that even if some individuals had elevated titers due to stimuli they were exposed to during travel, there was sufficient time for the titer changes to return to baseline by the time the second sample was collected.

Furthermore, the present titer changes, estimated as TSD, were investigated for a possible association with age and gender with multiple linear regression. The results showed no significant association between titer changes and age or gender over the four-year period across all blood groups and isoagglutinins, with one exception. The exception was anti-A_1 IgG of blood group 0, which showed a statistically significant association ($R^2 = .018$, $F(2, 462) = 4.136$, $p = .017$) with age ($B = -.008$, $p = .036$) but not with gender ($B = -.095$, $p = .061$). However, the regression model could only explain the changes of 8 donors of blood group 0 (1.8% of 465). Although statistically significant, this corresponds to 0.8% (8 of 1028) of the donors or to 0.3% (8 of 2986) of all analyzed isoagglutinins. Therefore, this association cannot be considered representative. Although early studies reported a decrease in titer with increasing age, that observation was not be confirmed in later studies (19, 176), which is consistent with the present results. The observations made in the early studies might be related to poorer health and nutritional status of the elderly population in the first decades of the 20th century, when biological age may have been more advanced than the chronological age. (76) Nutrition is known to influences the ageing process (177) and malnutrition impairs immune responses, including antibody production, particularly in the older population. (86)

In addition to its effects on the immune system, nutrition can also influence the gut microbiome (21) and thus isoagglutinin titers. (20) In recent years, triggered by awareness of the relationship between nutrition and good health, the use of dietary supplements and the popularity of probiotics have increased. (91) Probiotics contain live microorganisms with AB0-like antigen activity, and their consumption can stimulate isoagglutinin production. High anti-B IgM and IgG titers (16384) were observed in an apheresis platelet donor of blood group A, who started taking high potency probiotics three weeks prior to donation. Moreover, his anti-B isoagglutinin titers ranged from 8192 to 4096 over the previous three years when he also used probiotics of low-potency (18). A 2012 National Health Interview Survey in the United Stated found that probiotics and prebiotics (nondigestible food ingredient that stimulates the growth or activity of beneficial microorganisms) were the third popular dietary supplement after

vitamins and minerals (94). In Europe, probiotics sales grew by 9.1% between 2018 and 2021, and probiotic supplements accounted for 25% of global value, with Italy, Germany and France accounting for 64% of sales (178). In early 2020, the COVID-19 pandemic led to an increase in dietary supplement sales, and in the United States of America, for example, sales increased by 44% during the first wave of the pandemic compared with the same period in 2019 (179). Furthermore, the global probiotic supplement market shifted to electronic commerce during the pandemic, with growth of 81% in 2021 compared with 2018 (178), possibly as a result of the lockdown. Moreover, longitudinal studies on nutrition have shown that the diet quality improved slightly during the COVID-19 lockdown in some populations, as the individuals ate out less frequently and may have eaten home-cooked meals more often. (180, 181) Although no information was available on the dietary habits of the donors in the present study, it can be hypothesized that numerous individuals were motivated to improve their nutrition in the hope that it would improve immunity and protection against the SARS-CoV-2 virus. It can be hypothesized that a better nutrition, triggered by the COVID-19 pandemic and facilitated by the lockdown, in combined with the possible intake of probiotics may have played a role in the changes in isoagglutinin titers observed in the present study and explain, at least partially, the slight increase in titer.

The COVID-19 pandemic was an important event that took place during the present study. Several studies have examined possible associations between isoagglutinin levels and susceptibility to SARS-CoV-2 infection. It was observed that COVID-19 convalescents had lower levels of isoagglutinins compared with healthy controls, which persisted for up to five months after infection. (149, 151) The lower titers suggest that the virus does not stimulate isoagglutinin production, and furthermore, it could be hypothesized that the COVID-19 vaccine was also not responsible for the increase in titer observed in the present analysis. During the present study, 25.9% (182) to 50.8% (183) of the German population had received at least one dose of the COVID-19 vaccine. Furthermore, at the beginning of the present study on April 29, 2021, the incidence of confirmed COVID-19 cases in the German population was 3.97% (185). The incidence of large negative TSD observed in the present study was low, at 0.6% (5 donors), which when compared to the incidence of COVID-19 cases in the German population does not suggest a direct association. Nevertheless, in the present study, no information on SARS-CoV-2 infection or donor vaccination was available.

There is little to no information on intrapersonal changes in isoagglutinin titer over longer periods of time. Several longitudinal studies have examined changes over time periods ranging from one year to a maximum of 18 months. However, only one study reported a complete analysis of the isoagglutinin titers; the others examined changes reported at arbitrarily defined critical titers, making comparisons difficult. Moreover, the populations examined in these

studies were either small and had an atypical AB0 blood group distribution or were primarily represented by blood group 0 individuals. (4, 5, 8, 15, 16)

In the present analysis, the mean isoagglutinin titers showed that IgM and IgG values of blood group 0 donors were higher than those of groups A and B. Moreover, the IgG mean titers in group 0 were higher than IgM, whereas IgG mean titers were lower than IgM in blood groups A and B. These observations are consistent with the existing literature (88, 184) and applied to all subsequent study groups. The statistical significance of these observations was not tested because that was not the purpose of the present study. However, they are included to facilitate future study comparisons of populations and results. The overall IgM mean TS were 4.02 and 3.60, and 3.24 and 3.02 for anti-A_1 IgM of groups 0 and B, and anti-B IgM of groups 0 and A, respectively. The overall IgG mean TS were 5.53 and 1.44, and 4.35 and 0.27 for anti-A_1 IgG of groups 0 and B, and anti-B IgG of blood groups 0 and A, respectively.

Similar mean titers were published in 2017 in a small Danish population analyzed longitudinally using the same type of automated platform. The Danish population consisted of 56 healthy individuals (median age 55 years, 64.3% male, 78.6% blood donors), with a calculated blood group distribution of 33.9% blood group 0, 48.2% blood group A, 17.9% blood group B (blood group AB was not included). (5) Blood groups A and B were slightly overrepresented in this Danish study compared with the known Danish and German blood group distributions, which are very similar (49, 185). Compared with the present study, the Danish population was older by a median of 6 years, but with similar gender distribution. The Danish IgM mean TS were 4.7 and 4.0, and 4.3 and 3.5 for anti-A_1 of groups 0 and B, and for anti-B of groups 0 and A, respectively. The IgG mean TS were 4.9 and 0.3, and 3.5 and 0.1 for anti-A_1 of blood groups 0 and B, and for anti-B of blood groups 0 and A, respectively. (5) Similar to the present study, the Danish mean TS of IgM were higher than of IgG in blood groups A and B, and, in blood group 0, mean TS of IgG was higher than IgM, but only for anti-A. In contrast to the present results, Danish anti-B IgG of group 0 donors was lower than the IgM. The difference between the mean TS of the two populations ranged from +0.17 to +1.14 TS for IgG and from -1.06 to -0.46 TS for IgM. This detailed comparison was possible because the same automated method was used in both studies and the populations were similar, as both belong to highly developed European neighboring countries. Considering the wide range of mean titers published in the literature, the comparison was performed with the intention of validating the present mean titers obtained with an unbiased titration method, which was achieved due to the similarities observed. In the Danish study four samples per participant collected over a one-year period were analyzed and only minimal changes in titer were observed, which were estimated using pooled *SD*. The IgM pooled *SD* were 0.45 and 0.37, 0.50 and 0.41 for anti-A_1 of blood groups 0 and B, and anti-B of blood groups 0 and A, respectively. The pooled *SD* of IgG were 0.39 and 0.58, and 0.30 and 0.31 for anti-A_1 of blood groups 0 and B, and anti-B of blood groups 0

and A, respectively. (5) In contrast to the results of the present study, the Danish results show higher stability for IgG titers compared to IgM, with one IgG exception. The Danish anti-A IgG of blood group B showed the greatest titer variation. In the Danish study, the maximal variation was found in four different individuals, each of whom had only one antibody with a large variation. The highest variations were: 1.50 individual *SD* for anti-A IgG (equivalent to 3 TSD), 1.00 individual *SD* for anti-A IgM, anti-B IgM and IgG (equivalent to 2 TSD). (5) Danish titer variations were indeed minimal compared to the present results, with only 1.8% (1 in 56) calculated large titer variation, compared to 4.6% (47 in 1028) in the present study.

A second Danish study published in 2019 compared the population of healthy individuals from the first study with a population of hemodialysis patients (54 patients, median age 67 years, 61.1% male), and analyzed a total of 110 participants. Both Danish studies had similar prospective longitudinal study designs and used the same automated method. In the second Danish study, 88% of healthy volunteers and 80% of patients had a titer step difference of up to one titer step, while the rest of the study participants had variations of two (14, 12.7%) and three titer steps (4, 3.6%). It was also found that group 0 patients had significantly higher IgG TS compared with healthy participants. The patient IgM overall mean TS were 5.1 and 3.3, and 3.2 and 3.1 for anti-A_1 of blood groups 0 and B, and for anti-B of blood groups 0 and A, respectively. The patient IgG overall mean TS were 6.4 and 1.2, and 4.9 and 0.3 for anti-A_1 of blood groups 0 and B, and for anti-B of blood groups 0 and A, respectively. (8) Also, when compared with the second Danish, the present results showed a greater number of titer changes with a larger TSD.

Isoagglutinin changes have been examined in two other longitudinal studies, a Swedish study and a study from the United States of America, in active military personnel using manual methods. As mentioned previously, comparisons of isoagglutinin titers are difficult, especially when manual methods are used due to the low repeatability, reproducibility and consistency of manual methods. Furthermore, these studies did not include information on the titer values of the populations, other than titer changes with respect to various arbitrary critical titers used by the respective military programs to select low titer group 0 universal donors. Nevertheless, the results of these studies are considered here because they provide the only other longitudinal information on isoagglutinin titer changes available to date.

In the prospective longitudinal Swedish study, it was demonstrated that the standard pre-deployment vaccines used in 2013 had no boosting effect on isoagglutinin titers in military blood donors. The study population consisted of the crew of a military naval vessel of 120 individuals who were young (median age of 34 and 25 years for male and female crew, respectively) and majority male (81.7%). The blood group distribution in the Swedish donors was 35.8% blood group 0, 52.5% blood group A, 9.2% blood group B and 2.5% blood

group AB. From each of the 117 donors with isoagglutinins, two samples were collected, before and 8 to 9 months after vaccination. The samples were manually screened by column agglutination on gel cards with one dilution per isotype: 1:100 for IgM and 1:400 for IgG (with DDT), and only the positive samples were further titrated. Therefore, the Swedish results found only six high titer donors, two of them with titer changes, both decreases: anti-A IgG (from 800 to 400, 1 TS) and anti-A IgM (from 100 to < 100). (15) The titer changes reported in the Swedish study were minimal, but the titers were examined only in relation to one dilution corresponding to the cutoff used and did not provide information on changes occurring below the cutoff. Furthermore, as the authors of the Swedish study themselves acknowledged, transient increases in titer immediately after vaccination were not examined.

In the retrospective longitudinal study conducted in the United States of America, IgM isoagglutinin titers were analyzed in an active military blood donor population over an 18-month period, from May 2015 to January 2017. In that study, a decrease in titer was observed over time and with increasing age. However, the population was young (26.5 ± 6.5), male (98.2%) and of blood group 0 (94.8%). Of the 2237 soldiers included in the study, only 345 were tested at least twice. The isoagglutinins were titrated manually in tubes and the results were reported in relation to critical titer 256. It was observed that anti-A IgM titers were higher than anti-B IgM titers, and that in subsequent testing, 9.8% of the low titer donors changed to high titer and 73.3% of the high titer donors changed to low titer. In addition, the researchers reported seasonal changes in titer related to the winter months, with anti-A IgM titers higher in spring and lower in fall and anti-B IgM titers lower in spring and fall. Moreover, geographical differences in titer were also noted, with higher anti-A titers in individuals tested in California and lower in Washington than in Georgia. Anti-B titers were lower in individuals tested in Colorado and Washington compared with Georgia. (4) Compared with the study from the United States, the present IgM analysis of group 0 donors showed that only the changes in anti-B IgM titer were statistically significant, with both, anti-A_1 and anti-B IgM showing a slight increase in mean TS from 3.99 to 4.05 and from 3.16 to 3.31, respectively, between 2017 and 2021. Regarding seasonality and geography, the present study was design to minimize their influence on titer results by collecting the samples during the same period of the year from donors living in the same surrounding area.

5.2 Isoagglutinin Titer Changes by Gender

After stratification by gender, the present results show that the male study group behaved similarly to the overall study population having significant changes for all isoagglutinins, with the same exception for anti-A_1 IgM. It should be noted that the male study group represented the majority of the study population with 68.5%. The female study group showed significant changes only for some isoagglutinins, suggesting that the male study group carried the significance of the overall population. The female study group showed significant changes just for anti-B isoagglutinins, IgM and IgG of blood group 0, and IgG of blood group A. The IgM *p*-values of female donors were .166 and .439, and .003 and .449 for anti-A_1 IgM of blood groups 0 and B, and for anti-B IgM of blood groups 0 and A, respectively. The IgG *p*-values of female donors were .226 and .147, and .007 and 2.06E-4 and for anti-A_1 IgG of blood groups 0 and B, for anti-B IgG of blood groups 0 and A, respectively. The IgM *p*-values of male donors were .387 and .194, and .002 and 2.19E-4 for anti-A_1 IgM of blood groups 0 and B, and for anti-B IgM of blood groups 0 and A, respectively. The IgG *p*-values of male donors were 3.11E-6 and 6.47E-5, and 3.12E-4 and 2.88E-4 and for anti-A_1 IgG of blood groups 0 and B, for anti-B IgG of blood groups 0 and A, respectively.

It has been frequently observed that the isoagglutinin titers are generally higher in females compared to males. (88, 186) The same observation was made in the longitudinal study from the United States, but the percentage of female soldiers in that population was only 1.8% (40 females). (4) However, in the second Danish longitudinal study was observed that the differences between the IgG mean titers of males and females were not significant. (8) Although not statistically tested, as this was not the purpose of the present study, it was observed that the mean titers were slightly higher in females than in males, with one exception, anti-A_1 IgM of group B, which was higher in males than in females.

Furthermore, as previously discussed, the present multiple linear regression did not reveal any association between the present isoagglutinin changes and gender. The same observation was made in the second Danish study, in which no correlation was found between titer changes and gender and age. (8)

5.3 Isoagglutinin Titer Changes by Donation Type

After stratification by donation type, the present results showed that whole blood donors behaved similarly to the overall study population having significant changes for all isoagglutinins, with the same exception for anti-A_1 IgM. It should be noted that whole blood donors represented the majority of the study population with 76.7%. The platelet donors showed similar behavior to the overall population for anti-B, IgM and IgG. However, anti-A_1 of the platelet study group showed contrasting behavior: anti-A_1 IgG titer changes had no statistical significance, and anti-A_1 IgM of group B was the only anti-A_1 IgM isoagglutinin of the present study with significant titer changes. It should be noted that group B platelet donors represented a relatively small (21 male and 3 female) study group. The IgM *p*-values of whole blood donors were .267 and .647, and 3.57E-4 and .009 for anti-A_1 IgM of blood groups 0 and B, and for anti-B IgM of blood groups 0 and A, respectively. The IgG *p*-values of whole blood donors were 3.57E-7 and 5.15E-5, and 1.18E-4 and 3.86E-5 and for anti-A_1 IgG of blood groups 0 and B, and for anti-B IgG of blood groups 0 and A, respectively. The *p*-values of platelet donors were .266 and .016, and .017 and .012 for anti-A_1 IgM of blood groups 0 and B, and for anti-B IgM of blood groups 0 and A, respectively. The *p*-values of isotype IgG were .813 and .268 for anti-A_1 IgG of blood groups 0 and B, respectively, and .020 and .002 for anti-B IgG of blood groups 0 and A, respectively.

Although not statistically tested, it was observed that the majority of the present mean titers were slightly higher in platelet donors compared with whole blood donors, with two exceptions. Anti-A_1 IgG of group 0 (only in 2021) and anti-B IgG of group A showed slightly higher mean titers in whole blood compared with platelet donors.

The longitudinal studies discussed previously did not specify whether platelet donors were part of the study population, and therefore, a comparison with the present results was not possible.

5.4 Large Isoagglutinin Titer Changes

The analysis showed that the titer changes (estimated by TSD) of the present population had a considerable wide range, from a decrease by 4 titer steps to an increase by 6 titer steps. The distribution of large TSD donors (± 3 TSD and greater) was 4.6% for the overall study population (47 donors, 51 isoagglutinins) and was maintained in the stratification by gender and by donation type (4.7% ± 0.3%). However, the distribution by blood group differed, with higher prevalence in group 0 (7.3%) compared with the other blood groups (2.5% and 1.6% in group A and B donors, respectively). In comparison with the overall study population, the median age of these donors (*Md* = 51) showed an older population by 2 years.

There were only three donors with multiple isoagglutinins with large titer changes, two female and one male, all of blood group 0, with titer increases for anti-B IgG of +4 TSD and anti-B IgM of +3 TSD. The male donor had an additional increase in anti-A_1 IgM of +3 TSD.

As mentioned previously, the majority of large titer changes was represented by increases in titer, where 3.2% had +3 TSD, 0.7% had +4 TSD and 0.1% had +6 TSD. Large changes with titer decrease was found for 0.5% at -3 TSD and 0.1% at -4 TSD. IgG isoagglutinins had the most large variations (in 3.6% of all donors), of which 2.6% were anti-B IgG. Large IgM variation was detected in 1.3% of donors.

The largest titer increase was by 6 titer steps (+6 TSD, one whole blood donor, female, age 27, end titer value 32) and the largest decrease was observed for anti-B IgM by 4 titer steps (-4 TSD, one whole blood donor, male, age 38, end titer value 2).

Although most titer changes were increases, only 12 donors (1.2% of all donors) crossed over the 128 critical titer cutoff and 8 donors (0.8% of all donors) crossed over the 256 titer cutoff.

Of the longitudinal studies discussed previously, a detailed report of titer variation was provided only by the Danish studies. In the second Danish study, it was reported that the majority of variations were within one titer step. Only 12.7% (14 of 110) and 3.6% (4 of 110) of the participants had variations of 2 and 3 titer steps, respectively. Of the participants with titer variations, healthy individuals and hemodialysis patients, 17 belonged to blood group 0 and one was of group B. One patient was found to have multiple antibodies with large variations. (8) In the population of healthy individuals (56 participants), also presented in the first Danish study, the largest titer variation of three titer steps was observed just for one individual and involved anti-A IgG (1 of 56, 1.8%). (5) Compared with the two Danish studies that reported only minimal titer variation, the present results showed a higher prevalence of individuals with large titer changes. Moreover, the present results showed a wider variation of up to 6 titer steps compared with three titer steps in the Danish studies.

As previously discussed, only several studies have analyzed isoagglutinin changes longitudinally, however there is almost no information on the duration of high titers.

Several aspects could explain why the dynamics of titer changes remain unclear. One is that the rarity of large titer changes might make their discovery and analysis difficult due to insufficient data. Furthermore, the design of such a study must include frequent titer measurements in a very large population to yield conclusive information. Moreover, the behavior of study participants needs to be analyzed in correlation with the isoagglutinin titer to clarify which stimuli are involved and the magnitude of the booster effect.

Occasionally, donors with high isoagglutinin titers are discovered during investigations of minor incompatible transfusion reactions. The baseline isoagglutinin titers of these donors, however, are rarely known. In one case report published in 1954, large titer changes were reported for a blood donor of group 0. The unit of whole blood collected from this donor was transfused erroneously to a 78-year-old blood group A_1 woman. The unit of whole blood contained anti-A_1 IgM of titer 32768 (TS 15) and anti-B IgM of titer 16. Three and 21 months after the event, the titer of anti-A_1 was still very high at 8192 (TS 13) and 512 (TS 9), respectively. The cause for the high titer could not be determined. The donor had never received a blood transfusion, had not been vaccinated for 30 years prior to donation, and had no acute infections, no allergies or changes in his diet before donation. (6) During the 21-month observation period, the titer decreased by 6 titer steps to titer 512, which is still a high isoagglutinin titer. In another case report related to transfusion reactions to apheresis platelet units derived from a single donor, a sudden rise in titer of anti-A and anti-B IgM was observed within two months. Anti-A_1 IgM increased by 3 titer steps, from 128 to titer 1024. Anti-B IgM and IgG increase by 4 titer steps, from 8 to 128 and from 1 to 16, respectively. The titers increased between two donations, from October to December 2020. After the event, anti-A remained unchanged at titer 1024 over the reported six-month period, with minimal variation in the other isoagglutinins. Over a period of nearly four years prior to the event, the donor isoagglutinin titers showed stabile titers. (17) The titer changes described in these two case reports indicate that isoagglutinin titers can rise suddenly and can persist for extended periods, even years, until they return to baseline levels.

5.5 Isoagglutinin Titer Changes Around Various Critical Titers

In the longitudinal studies presented previously, isoagglutinin titer changes were examined to determine the frequency of repeated titrations and the incidence of titer change in low titer donors. The concept of low titer donor is applied in the manufacture of low titer blood products, which are primarily used in the resuscitation of trauma patients in civilian or military settings. The products can be either blood group A plasma, low-titer group 0 whole blood (LTOWB) or platelet units. Another approach that has been used on the battlefield in the treatment of hemorrhagic shock is the pre-identification of LTOWB donors among military personnel as a walking blood bank. Titer changes may later affect the composition of such a low-titer walking blood bank and disrupt troop deployment. (4, 5, 8, 15) Furthermore, the prevalence of low or high titer donors depends on the arbitrary defined critical titer, which varies by country and transfusion service, further complicating the comparison of populations and titer results. In the present study, the results were analyzed by two different critical titer cutoffs, 128 and 256, in order to be able to compare results with studies from different countries using various transfusion protocols.

Critical Titer of 128. Considering the critical titer of 128, the present analysis found that 22.5% of the donors had high titer at a given point in time. A similar prevalence was found in the study groups of male donors and whole blood donors, 19.0% and 22.7%, respectively, as these two groups constituted the majority of the participants. The highest prevalence of 40.6% was found in the platelet donor study group, followed by 29.9% in the female study group. After stratification by blood group, the highest prevalence of 47.7% was found in blood group 0 donors, followed by just 1.1% and 3.2% in blood groups A and B, respectively. Of the 22.5% overall high titer donors, 11.1% had consistently high titer isoagglutinins, 8.4% changed from low to high titer, and 3.0% changed from high to low titer. The most common high titer isoagglutinin found at the cutoff 128 was anti-A_1 IgG in 17.9% of all donors, followed by anti-B IgG in 8.2%, anti-A_1 IgM in 2.1%, and anti-B IgM in 1.0% of donors. In comparison to the overall study population, the median age of these donors (*Md* = 51) showed an older population by 2 years.

Critical Titer of 256. At a critical titer of 256, the prevalence decreased by half in all study groups except the platelet group, in which the prevalence decreased to one-quarter of the original value. The prevalence in the overall population decreased to 10.7% and was similar after stratification by gender and donation type, at 8.9%, 14.2%, 10.9%, 10.0% in male, female, whole blood, platelet donors, respectively. By blood group, the highest prevalence was observed in blood group 0 at 23.2%, followed by blood group A at 0.5%. In blood group B, there were no donors with a titer ≥ 256. Over the four-year study period, the titers changed around cutoff 256 as follows: 5.2% remained high titer, 3.4% changed from low to high titer,

and 2.1% changed from high to low titer. The most common high titer antibody remained anti-A_1 IgG, which was found in 8.6% of donors, followed by anti-B IgG at 3.2%, anti-B IgM at 0.3% and anti-A_1 IgM at 0.2%. Compared to the overall study population, the median age of these donors (*Md* = 53) showed an even older population, by 4 years.

In the second Danish study, where the critical titer employed was 256, the prevalence of high titer participants was calculated at 12.7% (14 of the 110). The prevalence in the Danish healthy volunteers was 8.9% (5 of 56) and in the patients was 17% (9 of 54). With the exception of one healthy volunteer of blood group B, all subjects with high titers belonged to blood group 0. The most common high titer isoagglutinin here was anti-A IgG at 10% (11 of 110), followed by anti-A IgM at 3.6% (4 of 110) and anti-B IgG at 2.7% (3 of 110). (8) In comparison, the distribution of high titers was similar in both studies, 12.7% Danish high titer participants and 10.7% in the present study. In both studies, the most common high titer isoagglutinin was anti-A IgG with similar distributions (8.6% and 10% in the German and Danish populations, respectively). Anti-B IgG, although comparable in percentage (3.2% and 2.7% in the German and Danish populations, respectively), was the second most common high titer isoagglutinin in the present study and only the third most common in the Danish study. As mentioned previously, Danish IgM mean titers were higher compared to the present study. This is also reflected in the prevalence of high titer anti-A IgM of 3.6% of the Danish population, compared with only 0.2% in the present study. Considering that the same automated testing platform was used in both studies, the difference in results could be generated by a difference in population characteristics. The discrepancy could be related either to the small Danish study population or to environmental differences, such as diet, geographic location, or seasonality, which may influence isoagglutinin titers, as discussed in the Introduction section. In addition, the absence of high titers in Danish group A individuals, compared with 1.1% high titer group A donors in the present study suggests that the small number of Danish participants may be one of the causes for the differences.

In the Swedish study, 5.1% of the military donors (6 of 117) were reported to have high titers at a critical titer of 100 for IgM and 400 for IgG. The highest prevalence of high titers was 11.6% in Swedish blood group 0 donors (5 of 43), followed by 1.6% in blood group A donors (one of 63, for anti-B IgM). The highest prevalence by antibody was 3.4% for anti-A IgM (4 of 117), followed by anti-B IgM at 2.6% (3 of 117), anti-A IgG at 2.6% (3 of 117), and anti-B IgG at 0.9% (1 of 117). Three of the donors had multiple high titer isoagglutinins. High IgM titers were detected in 4.3% of donors (5 of 117) and high IgG titer in 2.6% (3 of 117). Of these, two donors had high IgM and IgG titers in combination. There were no changes in titer for the majority of isoagglutinins, except for two decreases in titer, one for anti-A IgG from 800 to 400 and one for anti-A IgM from 100 to < 100. (15) Because of the difference in cutoff values, a comparison between the present high titer donors and the Swedish donors is possible

only for IgM at the critical titer 128. The present results show a prevalence of 3.0% IgM high titer donors, which is comparable with the 4.3% in the Swedish study population. The slightly higher Swedish prevalence might be related to the use of manual methods in combination with the small number of study participants. In contrast to the present results, where four group B donors had high IgM titers, there were no high titers group B donors in the Swedish study. This difference may also be attributable to the small Swedish study population of only 11 blood group B donors.

In the study from the United States of America, the results were reported to a critical titer of 256. The titer changes were mainly towards low titers, with the percentage of low titer donors increasing with each subsequent titration. The percentage of anti-A and anti-B IgM high titer decreased from 30.5% (682 of 2237 donors) at the first test to 16.5% (57 of 345 donors) at the second test. However, the majority of participants were tested only once, with 345 blood group 0 donors tested twice, 5.7 months (mean interval) later. The titer changes presented for these twice-tested donors showed that for anti-A IgM, 19.1% (66) changed to a low titer, 7.2% (25) changed to a high titer, and 7.0% (24) remained at a high titer. Titer changes calculated for anti-B IgM showed that 7.8% (27) changed from high to low titer, 3.5% (12) changed from low to high, and 1.5% (5) remaining at a high titer. (4) From these results, the prevalence of high titer donors who had high titer on at least one test can be calculated as 33.3% high anti-A IgM and 12.8% high anti-B IgM. Compared with these results, the present data show a lower percentage of high titers, with 0.5% of the study population (5 of 1028 donors) and 0.9% of blood group 0 donors (4 of 465) having high IgM at a critical titer of 256. The percentage of high titer donors in the United States study was notably higher, with 16.5% high titer donors observed in the second test. This significant difference between the German and the United States study populations could be related to both study variables, the participants and the testing method. There are geographic and social differences between the two populations that could influence exposure to different environmental factors due to location on different continents, but also considering military lifestyle with possible overseas service, vaccinations (4), diet and injuries. The two studies used different testing methods. In the United States study, multiple testing sites were involved in generating results using the manual tube method, whereas the present results were obtained with an automated method in a single laboratory. Manual methods are known to have inter-laboratory variability that causes large differences in titration results (22, 23). However, there are also similarities between the two studies, with both populations originating from highly developed countries with a diet based on processed foods and low contact to microorganisms. A Japanese study demonstrated a decline in isoagglutinin titers over time in the Japanese population, and hypothesized that a more Westernized lifestyle and a dietary shift from natural to processed foods may be responsible for the decline in isoagglutinin titers (20).

5.6 Isoagglutinin Critical Titer at International Level

Isoagglutinin titration is of utmost importance in defining a clinically relevant critical titer in transfusion medicine, especially for minor incompatible transfusions. Minor incompatibilities are commonly observed in the transfusion of platelet units, in the transfusion of LTOWB commonly practiced in emergency resuscitation in the military sector, or in the transfusion of group A plasma in the civilian trauma sector practiced in several countries. To date, there is no universally accepted critical isoagglutinin titer. The challenge in establishing a critical titer, given the wide variation in results obtained in different settings, is to correlate clinical outcome to isoagglutinin titers. (187) There are only a few countries that have defined a critical titer in their national transfusion guidelines. Usually, transfusion services have internal procedures that specify the acceptable threshold of isoagglutinin titer.

In **Germany**, the national Scientific Guideline for Hemotherapy recommends transfusion of AB0 identical platelet units, and if not possible, AB0 compatible (minor incompatible) transfusion is allowed, considering either plasma volume or isoagglutinin quantity without specifying a critical isoagglutinin titer. Furthermore, it is recommended to avoid the transfusion of minor incompatible platelet units in children under 25 kg, especially in the constellation of blood group 0 platelet units to blood group A patients. Plasma units must be transfused AB0 identical and only in exceptional situations AB0 minor compatible are allowed. (106)

In **the United Kingdom**, the Guidelines for the Blood Transfusion Services recommend the transfusion of group 0 units to non-group 0 patients only as a last resort. Here, low-titer units are defined as units with IgM isoagglutinins below 128 titer. (188) The four blood services in the United Kingdom implemented automated titration of IgM isoagglutinin at different critical titers: National Health Service Blood and Transplant titrates all donations at a dilution of 1:85 against A_2B cells; Northern Ireland Blood Transfusion Service titrates selected blood components at a dilution of 1:100 versus A_1 and B cells; Scottish National Blood Transfusion Service titrates all donations at a dilution of 1:100 (Glasgow) or 1:50 (Edinburgh) against A_1 and B cells; Welsh Blood Service titrates selected blood component at a dilution of 1:128 against A_1B cells. In the effort to regulate isoagglutinin titration, standardized positive (titer 128) and negative (titer 64) controls were developed and introduced into routine use in 2008. Blood components that tested negative are then labeled as low titer units. (187)

In **the United States of America**, the standards issued by the Association for the Advancement of Blood & Biotherapies, formerly known as the American Association of Blood Banks (AABB) are followed by institutions involved in blood banking and transfusion medicine. These standards allow the use of LTOWB in emergency situations for patients with unknown blood group and require the implementation of an internal procedure that specifies the titer

cutoff of low titer products, the maximum number of units to transfuse and the patients that are eligible to receive these units (189).

In **Australia** and **New Zealand**, the Guidelines for Transfusion and Immunhaematology Laboratory Practice issued by the Australian and New Zealand Society of Blood Transfusion allow the transfusion of low titer group A plasma in emergency to patients of unknown blood group as an alternative to group AB plasma. However, a critical titer value is not mentioned. (190) The Australian Red Cross implemented in March 2018 a national automated titer screening test for clinical plasma components and apheresis platelets, labeling them accordingly if they are low titer. (191) The Australian Red Cross manufactures platelet units suspended in additive solution, both pooled and apheresis (192). The production of plasma-suspended apheresis platelets ceased in 2019 (193).

The **Africa** Society for Blood Transfusion has issued a Guidance Document, which defines high titer isoagglutinins as a positive result to a saline dilution (IgM) of 1:64 versus A_1, B or A_1B cells. It is recommended that each facility has procedures in place for the transfusion of blood components with high isoagglutinin titers. These products may only be transfused to AB0 identical patients. The transfusion of non-AB0 specific units containing plasma is allowed only if the units have low titer isoagglutinins. (194)

Whole Blood Transfusion. The Trauma, Hemostasis and Oxygenation Research network and AABB Working Party have conducted several international surveys in recent years to determine the extent to which LTOWB is being used and what procedures are used in the hospitals. The number of survey participants increased from 16 in 2018, to 27 in 2019 and 37 in 2020. (121, 123, 195) In the 2020 survey, participants were from the United States of America, the United Kingdom, Israel and Norway. The majority (73%) of the hospitals reported using LTOWB only for patients with trauma and massive bleeding and just a few sites included non-trauma patients. Some of the sites (24%) used LTOWB also for pediatric patients. The average number of units allowed for transfusion by hospital protocol was 4 (28 sites) with a range of 2 to 8 unites. Various definitions of low titer were reported, with the majority (54%, 20 of 37) having a critical titer below 200, followed by critical titers below 50 (16%, 6), below 100 (14%, 5), below 256 (11%, 4), below 128 (3%, 1), and a combination of IgM titer below 250 and IgG titer below 500 for the one hospital in Norway. The majority of sites tested exclusively IgM isoagglutinins by the saline tube method, and just four hospitals used an automated method. (195) Sweden is other European country that uses LTOWB, where the threshold for low titer combines IgM below 100 with IgG below 400. (15) In the United States Armed Forces, LTOWB is defined by IgM titers below 256. (4) In Germany, the use of LTOWB is not currently practiced.

Platelet Transfusion. Although minor incompatible platelet transfusions are a daily occurrence, isoagglutinin titration is performed sporadically (except, of course, when regulated at a national level), and its use is often triggered by a severe hemolytic reaction that has occurred at the given center. (17, 196) An analysis of severe transfusion reactions reported up to 2007 showed that in the majority of cases (23 of 28) group 0 units were transfused to group A or AB patients. The isoagglutinin titers ranged from 128 to 16384 for IgM and from 1024 to 32000 for IgG in the transfused platelet units. The majority (82%, 23 of 28) of the products were apheresis and only four products were pooled units. It appears that the risk of hemolytic reactions is smaller for pooled platelet units compared to apheresis units because of the small amount of plasma per donor contained in the final pooled product. (116) However, in a 2008 study that examined 185 pooled group 0 plasma units was observed that the isoagglutinin titers were comparable to those reported for single donor group 0 platelet units. (120, 196) A survey conducted in 2005 that examined the practices of 3156 laboratories from North America regarding isoagglutinin titration for minor incompatible platelet transfusion found that only 53 (2.1%) laboratories screened incompatible products. (197) In another survey published in 2005 participated laboratories from Australia, the Czech Republic, Denmark, Finland, Germany (Marburg, Ulm), Italy, the Netherlands, Norway, Poland, Spain, Sweden, Switzerland, Japan, the United Kingdom, and the United States. These laboratories used various critical titers ranging from 64 to 100 for IgM and from 256 to 400 for IgG. (7) The cost of isoagglutinin titer screening was calculated at $1.20 per single donor platelet in 2004, with an annual cost of $6000 for screening more than 4500 group 0 platelet units. This cost can mitigate up to two hemolytic reactions and was considered modest compared with other transfusion safety measures implemented in the past. (196)

AB0 Incompatible Kidney Transplantation. Effective peritransplantation management protocols include immunosuppression, as well as plasmapheresis and immunoadsorption to lower the isoagglutinin titer before and after transplantation. (135, 198, 199) However, protocols vary with respect to the isoagglutinin isotype and critical titer used to monitor the outcome. Some centers that monitor IgM isoagglutinins observed a positive outcome at IgM titers of less than 4, despite concurrent IgG titers of 256. (70) Other transplant centers that monitor IgG isoagglutinins have reported antibody-mediated rejection already at IgG titers above 4. (138) Again, variations in titration results between different laboratories and test methods were noted and caution was advised in interpreting the results. This could be one of the reasons why the correlation between transplantation outcome and isoagglutinin titer is still unclear. (9-12)

5.7 Donors Lacking IgG Isoagglutinins

In early studies, it was considered that unimmunized individuals had only natural occurring IgM isoagglutinins produced in the absence of a stimulus, and that immune IgG isoagglutinins were primarily found in immunized individuals. AB0 incompatible pregnancies and erroneous blood transfusions were considered the main immunizing events. It was also hypothesized that the “immune” isoagglutinins disappear after one year. (71) Other studies have observed that unimmunized individuals may also have IgG isoagglutinins. (19) Nevertheless, little information is available on titer changes after immunization or on the percentage of individuals lacking IgG isoagglutinins.

Considering that pregnancy and blood transfusion (37) are recognized as the major contributors to IgG isoagglutinin production, the proportion of individuals without IgG isoagglutinins would have to be high, particularly in a group of healthy male blood donors.

The present data show that only 20.7% of the overall blood donor study population had no IgG isoagglutinins. The distribution remained similar after stratification by gender and was 21.5% and 19.1% in the male and female study groups, respectively. The prevalence of 19.1% female donors without IgG isoagglutinins can be plausibly explained considering that about 25% of women in Germany do not have children (200) and that not all pregnancies are AB0 incompatible. However, it is less plausible that the rest of the nearly 80% male healthy blood donors with measurable IgG isoagglutinins could be explained if transfusion is considered the sole IgG stimulus. The distribution within blood group showed that the proportion of donors lacking IgG isoagglutinins was low to very low in blood group B and 0 donors, 12.9% and 1.9%, respectively, whereas 42.8% of blood group A donors lacked IgG isoagglutinins. There were no donors of group 0 lacking both anti-A_1 and anti-B IgG antibodies.

The present data indicate that, in addition to stimulation with human AB0 antigens by pregnancy and blood transfusion, other important stimuli must be involved in the production of IgG isoagglutinins. Furthermore, the present data indicate that the production of IgG isoagglutinins depends on AB0 blood group and antigen specificity.

5.8 Strengths and Limitations

The main strengths of the present study are the robust sample size, the long duration and the test method used. The test method featured several important attributes that provided strength to the study. First, the tests were performed and interpreted in a standardized manner due to the automated platform, ensuring the accuracy of the results. Second, quantification of IgM and IgG isotypes was possible without additional sample treatment. Another important strength of the study was that the effects of seasonality on titer changes were minimized by collecting samples during the same period of the year in both years.

The present study also had some limitations. Although isoagglutinins were studied over a four-year period, the dynamics of titer changes could not be analyzed due to unavailability of intermediate samples. In addition, the study was limited by the absence of information on donor environment, diet, infection and vaccination, information that could have been analyzed in relation to the titer changes.

6 Conclusion

The current analysis showed that the isoagglutinin titers of blood donors changed significantly over the four-year period and that these changes were not associated with age and gender. In the study population, 4.6% of blood donors had large titer variations of three or more titer steps, the majority of which were IgG isoagglutinins. In addition, the prevalence of high titer donors was just over 20% and 10%, depending on the critical titer cutoff applied, 128 and 256, respectively. Furthermore, only 20% of the donors lacked IgG isoagglutinins.

In the present retrospective longitudinal study, the isoagglutinin titer changes of 1028 blood donors were analyzed over a four-year period using an automated method. The study design allowed the longitudinal analysis of titer changes in the same individuals and provided information on actual changes compared with previously available cross-sectional data. Considering the robust sample size, it was possible to find large titer changes, which are relatively rare. In addition, the automated platform ensured the accuracy of the results and of the isotype identification.

Due of the retrospective nature of the study, the intermediate titer variations and the stimuli responsible for the titer changes could not be investigated. To date, neither all stimuli nor the extent of their influence have been fully elucidated. Future longitudinal research could further investigate the rate at which the titer changes occur and their relationship to potential stimuli by analyzing the behavior and the environment of the population studied. In addition, further studies should be performed using accurate titration methods for the results obtained to be comparable to the present data. From a clinical perspective, given that large titer changes have been identified in 4.6% of the study population, it should be considered either to titrate all blood components that might be transfused AB0 minor incompatible or to test the blood donors prior to each donation.

The current research provides accurate information on IgM and IgG isoagglutinin titers and their changes in the current blood donor population in Germany. Furthermore, as part of evidence-based knowledge, the present study provides high quality evidence that is essential for the development of guidelines, such as guidelines for AB0 minor incompatible blood transfusion and organ transplantation, thus facilitating life-saving medical decisions.

7 Summary

The isoagglutinins were discovered over one hundred years ago (1) and represent an important pillar of transfusion medicine as an integral part of the AB0 blood group system. Over the years, their importance has expanded to other medical disciplines, concerning AB0 minor incompatible pregnancies, bone marrow, stem cell or solid organ transplantation. (2, 3) Although they have been intensely studied, the analysis has almost exclusively been performed in cross-sectional studies without fully elucidating whether or how the titers change over time. (4)

The main objective of this dissertation was to analyze longitudinal changes of isoagglutinin titers in blood donors over a four-year period by blood group, isoagglutinin specificity, isotype and donation type and their correlation with age and gender. In addition, the aim of the study was to identify and evaluate donors with large titer changes and to determine the prevalence of high titer donors in relation to various critical titer cutoffs. Furthermore, the prevalence of donors lacking IgG isoagglutinins should be analyzed. For this purpose, sample pairs from 1028 whole blood and apheresis platelet donors were analyzed in a retrospective longitudinal study using an automated titration method known for minimal variation in results and the ability to distinguish between the two clinically important immunoglobulin isotypes, IgG and IgM. In the present study, it was demonstrated that the isoagglutinin titer changes were statistically significant across all blood groups and study groups except anti-A_1 IgM. Furthermore, the changes could not be associated with either age or gender. Moreover, large titer changes of three or more titer steps were observed in 4.6% of the population, with IgG isoagglutinins accounting for the majority (3.6%). The prevalence of high titers was slightly over 20% and 10%, depending on the critical titer cutoff applied, 128 and 256, respectively. In addition, it was observed that 20% of the population lacked IgG isoagglutinins.

The recent interest in isoagglutinin titer changes involves defining a safe critical titer cutoff for platelet units or group 0 whole blood units to be transfused to AB0 minor incompatible patients without the risk of hemolytic transfusion reactions. The titration frequency of low titer donors depends on the probability of titer change. (4, 5) Furthermore, defining a critical titer cutoff is challenging because of the known variation in titration results, especially with the manual method commonly used for isoagglutinin titration. From this perspective, it is difficult to compare titration results obtained in different studies and correlate them with clinical data. (6) Nowadays, it is common for transfusion services to use internal protocols for AB0 minor incompatible transfusion (7), with only a few countries having nationally recognized critical titers. In addition to transfusion medicine, reliable information on isoagglutinin titer changes is essential in AB0 incompatible kidney transplantation. (8) Here as well, there is no internationally accepted protocol regarding a critical titer or which isoagglutinin isotype may

better predict organ engraftment. This may be attributed at least partially, to method result variation. (9-12)

The design of the present research aimed to minimize method and seasonal variation. In addition, large titer changes were considered as differences of three and more titer steps in order to exclude random fluctuations. To date, little to no information is available on intrapersonal changes in isoagglutinin titer over time, particularly those obtained using automated methods. The present study is the largest and longest longitudinal study to date. Due to the robust sample size, it was possible to observe large titer changes and examine the prevalence of high titer donors in relation to various critical titer cutoffs. The present data could only be compared with one small Danish longitudinal study in which the same test method was used. In contrast to the present data, only minimal titer variation was observed in the Danish study, with only one of the 56 healthy subjects having a large titer variation (three titer steps). (5) However consistent with the Danish results, the present data showed no associated between titer changes and age or gender. Furthermore, the present analysis of the IgG isotype showed that only 20% of the population lacked IgG isoagglutinins, independent of gender or donation type. This finding cannot be explained solely by considering pregnancy or transfusion as responsible stimuli, suggesting further involvement of stimulating factors in the production of IgG isoagglutinins. Other factors such as diet, probiotics, and environmental factors may be responsible for isoagglutinin titer changes.

The present results represent a reliable source of data on isoagglutinin titer and titer changes that can be used when establishing guidelines for AB0 minor incompatibility in both transfusion medicine and organ transplantation. In addition, the results provide a reliable basis for isoagglutinin titer comparisons with future studies of various populations, particularly for further investigations of titer variation dynamics and of cause-effect relationships with various stimuli.

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9 Appendix

Table 28 *Donor Distribution and Age Parameters Within Blood Groups*

	Age in 2021					
	n	*Min*	*Max*	*Md*	*M*	*SD*
Blood Group 0						
Male	305	22	71	49.0	46.34	13.40
Female	160	22	69	50.0	47.42	13.49
Whole Blood	366	22	71	51.0	47.27	13.84
Platelets	99	22	65	46.0	44.66	11.59
Blood Group A						
Male	310	22	70	48.0	46.60	13.15
Female	129	22	70	50.0	46.16	13.66
Whole Blood	323	22	70	51.0	47.62	13.53
Platelets	116	24	64	42.5	43.28	12.05
Blood Group B						
Male	89	23	70	46.0	45.88	12.84
Female	35	28	68	59.0	53.77	11.58
Whole Blood	100	23	70	53.0	48.71	13.12
Platelets	24	24	66	48.5	45.58	12.17

Note. N = 1028

Table 29 *Titer Step and Titer Step Difference Within Blood Groups*

	TS 2017					TS 2021					TSD 2021-2017				
	Min	*Max*	*Md*	*M*	*SD*	*Min*	*Max*	*Md*	*M*	*SD*	*Min*	*Max*	*Md*	*M*	*SD*
	Anti-A$_1$ IgM														
Blood Group 0															
Male	0	7	4	3.91	1.18	0	7	4	3.95	1.23	-2	3	0	0.04	0.74
Female	1	8	4	4.15	1.19	1	8	4	4.24	1.36	-1	3	0	0.09	0.76
Whole Blood	0	8	4	3.97	1.21	1	8	4	4.02	1.26	-2	3	0	0.05	0.73
Platelets	1	7	4	4.06	1.12	0	8	4	4.16	1.35	-2	3	0	0.10	0.80
Blood Group B															
Male	1	7	3	3.66	1.20	1	7	4	3.75	1.25	-1	2	0	0.09	0.65
Female	2	5	3	3.29	1.05	1	6	3	3.37	1.19	-1	2	0	0.09	0.66
Whole Blood	1	7	3	3.51	1.16	1	7	3.5	3.54	1.12	-1	2	0	0.03	0.66
Platelets	2	6	4	3.75	1.19	2	7	4	4.08	1.25	0	2	0	0.33	0.57
	Anti-A$_1$ IgG														
Blood Group 0															
Male	-1	11	5	5.28	2.17	-1	10	6	5.57	1.93	-3	3	0	0.29	1.05
Female	-1	11	6	5.69	2.22	-1	10	6	5.78	2.08	-3	3	0	0.09	1.01
Whole Blood	-1	11	6	5.39	2.16	-1	10	6	5.66	1.96	-3	3	0	0.28	1.01
Platelets	-1	11	6	5.54	2.33	-1	10	6	5.56	2.06	-3	3	0	0.02	1.14
Blood Group B															
Male	-1	6	1	1.24	1.85	-1	5	2	1.66	1.84	-2	2	0	0.43	0.92
Female	-1	4	1	1.29	1.43	-1	5	1	1.57	1.70	-2	3	0	0.29	1.07
Whole Blood	-1	6	1	1.15	1.75	-1	5	2	1.58	1.88	-2	3	0	0.43	0.97
Platelets	-1	5	2	1.67	1.61	-1	5	2	1.88	1.42	-1	2	0	0.21	0.93
	Anti-B IgM														
Blood Group 0															
Male	0	7	3	2.90	1.31	0	6	3	3.04	1.20	-2	3	0	0.14	0.74
Female	0	7	4	3.64	1.32	1	8	4	3.83	1.30	-1	3	0	0.19	0.79
Whole Blood	0	7	3	3.15	1.33	0	8	3	3.30	1.25	-2	3	0	0.14	0.74
Platelets	0	7	3	3.18	1.49	0	8	3	3.38	1.45	-2	3	0	0.20	0.82
Blood Group A															
Male	0	6	3	2.70	1.34	0	6	3	2.85	1.25	-4	3	0	0.15	0.79
Female	0	7	4	3.60	1.36	0	8	4	3.65	1.40	-2	3	0	0.05	0.73
Whole Blood	0	7	3	2.94	1.44	0	8	3	3.04	1.38	-4	3	0	0.10	0.76
Platelets	0	6	3	3.03	1.30	1	6	3	3.22	1.22	-2	3	0	0.20	0.82
	Anti-B IgG														
Blood Group 0															
Male	-1	11	4	4.08	1.97	-1	11	4	4.33	2.01	-3	4	0	0.26	1.18
Female	-1	9	5	4.48	2.11	0	11	5	4.76	2.04	-2	6	0	0.28	1.21
Whole Blood	-1	11	4	4.19	1.99	-1	11	5	4.45	1.96	-2	6	0	0.26	1.17
Platelets	-1	9	3	4.31	2.19	-1	11	3	4.61	2.26	-3	4	0	0.29	1.26
Blood Group A															
Male	-1	9	-1	0.16	1.57	-1	9	0	0.35	1.67	-2	4	0	0.19	0.88
Female	-1	5	-1	0.15	1.50	-1	5	0	0.44	1.73	-2	3	0	0.29	0.83
Whole Blood	-1	9	-1	0.18	1.54	-1	9	0	0.40	1.69	-2	4	0	0.22	0.91
Platelets	-1	6	-1	0.09	1.58	-1	6	-1	0.31	1.70	-2	2	0	0.22	0.72

Note. TS represents titer steps. TSD represents titer step difference. TS 0 encodes titer 1.

Table 30 *Wilcoxon Signed-Rank Test Results Within Blood Groups*

	n	*Z*	*p*-value	effect size	*n*	*Z*	*p*-value	effect size
	Blood Group 0				**Blood Group B**			
				Anti-A_1 IgM				
All	465	-1.522	.128	0.050	124	-1.514	.130	0.096
Male	305	-0.864	.387	0.035	89	-1.298	.194	0.097
Female	160	-1.385	.166	0.077	35	-0.775	.439	0.093
Whole blood	366	-1.110	.267	0.041	100	-0.457	.647	0.032
Platelet	99	-1.113	.266	0.079	24	-2.530	**.011**	0.365
				Anti-A_1 IgG				
All	465	-4.554	**5.26E-6**	0.149	124	-4.165	**3.11E-5**	0.264
Male	305	-4.663	**3.11E-6**	0.189	89	-3.995	**6.47E-5**	0.299
Female	160	-1.211	.226	0.068	35	-1.452	.147	0.174
Whole blood	366	-5.091	**3.57E-7**	0.188	100	-4.049	**5.15E-5**	0.286
Platelet	99	-0.236	.813	0.017	24	-1.107	.268	0.160
	Blood Group 0				**Blood Group A**			
				Anti-B IgM				
All	465	-4.284	**1.84E-5**	0.140	439	-3.574	**3.52E-4**	0.121
Male	305	-3.150	**.002**	0.128	310	-3.697	**2.19E-4**	0.148
Female	160	-2.936	**.003**	0.164	129	-0.757	.449	0.047
Whole blood	366	-3.570	**3.57E-4**	0.132	323	-2.616	**.009**	0.103
Platelet	99	-2.378	**.017**	0.169	116	-2.520	**.012**	0.165
				Anti-B IgG				
All	465	-4.501	**6.78E-6**	0.148	439	-5.059	**4.22E-7**	0.171
Male	305	-3.605	**3.12E-4**	0.146	310	-3.626	**2.88E-4**	0.146
Female	160	-2.693	**.007**	0.151	129	-3.711	**2.06E-4**	0.231
Whole blood	366	-3.851	**1.18E-4**	0.142	323	-4.116	**3.86E-5**	0.162
Platelet	99	-2.324	**.020**	0.165	116	-3.091	**.002**	0.203

Table 31 *Multiple Linear Regression Results*

Coefficients	*B*	*SE*	*β*	*t*	*p*-Value	95% *CI* *LL*	95% *CI* *UL*
	Blood Group 0						
			Anti-A_1 IgM				
Constant	.068	0.036		1.863	.063	-0.004	0.139
Gender	.024	0.036	.030	0.648	.517	-0.048	0.095
Age	.004	0.003	.068	1.469	.143	-0.001	0.009
	N = 465; R^2 = .006; R^2adjusted = .001; F(2, 462) = 1.328; p = .266						
			Anti-A_1 IgG				
Constant	.194	0.050		3.845	**1,38E-4**	0.095	0.293
Gender	-.095	0.051	-.087	-1.880	.061	-0.194	0.004
Age	-.008	0.004	-.097	-2.103	**.036**	-0.015	-4,92E-4
	N = 465; R^2 = .018; R^2adjusted = .013; F(2, 462) = 4.136; $\mathbf{p = .017}$						
			Anti-B IgM				
Constant	.163	0.037		4.400	**1,35E-5**	0.090	0.235
Gender	.025	0.037	.031	0.667	.505	-0.048	0.097
Age	4,82E-4	0.003	.009	0.184	.854	-0.005	0.006
	N = 465; R^2 = .001; R^2adjusted = -.003; F(2, 462) = 0.244; p = .783						
			Anti-B IgG				
Constant	.269	0.058		4.642	**4,50E-6**	0.155	0.383
Gender	.015	0.058	.012	0.264	.792	-0.099	0.129
Age	-.005	0.004	-.054	-1.153	.249	-0.013	0.003
	N = 465; R^2 = .003; R^2adjusted = -.001; F(2, 462) = 0.689; p = .502						
	Blood Group A						
			Anti-B IgM				
Constant	.105	0.041		2.583	**.010**	0.025	0.184
Gender	-.050	0.041	-.059	-1.232	.219	-0.130	0.030
Age	.001	0.003	.026	0.534	.593	-0.004	0.007
	N = 439; R^2 = .004; R^2adjusted = -4.00E4; F(2, 436) = 0.912; p = .402						
			Anti-B IgG				
Constant	.242	0.045		5.346	**1,46E-7**	0.153	0.331
Gender	.052	0.045	.055	1.143	.254	-0.037	0.141
Age	-.001	0.003	-.021	-0.443	.658	-0.007	0.005
	N = 439; R^2 = .003; R^2adjusted = -.001; F(2, 436) = 0.759; p = .469						
	Blood Group B						
			Anti-A_1 IgM				
Constant	.080	0.066		1.218	.226	-0.050	0.210
Gender	-.020	0.068	-.028	-0.295	.768	-0.154	0.114
Age	.005	0.005	.091	0.961	.339	-0.005	0.014
	N = 124; R^2 = .008; R^2adjusted = -.009; F(2, 121) = 0.462; p = .631						
			Anti-A_1 IgG				
Constant	.352	0.097		3.623	**4.27E-4**	0.160	0.544
Gender	-.081	0.100	-.077	-0.812	.418	-0.280	0.117
Age	.003	0.007	.037	0.390	.697	-0.011	0.017
	N = 124; R^2 = .006; R^2adjusted = -.011; F(2, 121) = 0.345; p = .709						

Table 32 *Titer Step Difference Distribution Within Blood Groups*

	Titer Step Difference 2021-2017									
	-4	-3	-2	-1	0	+1	+2	+3	+4	+6
Blood Group 0					**Anti-A_1 IgM**					
All			5 (1.1)	74 (15.9)	294 (63.2)	78 (16.8)	8 (1.7)	6 (1.3)		
Male			5 (1.6)	46 (15.1)	197 (64.6)	48 (15.7)	6 (2.0)	3 (1.0)		
Female				28 (17.5)	97 (60.6)	30 (18.8)	2 (1.3)	3 (1.9)		
Whole Blood			2 (0.5)	64 (17.5)	228 (62.3)	92 (16.9)	6 (1.6)	4 (1.1)		
Platelets			3 (3)	10 (10.1)	66 (66.7)	16 (16.2)	2 (2.0)	2 (2.0)		
Blood Group B										
All				19 (15.3)	77 (62.1)	26 (21.0)	2 (1.6)			
Male				14 (15.7)	54 (60.7)	20 (22.5)	1 (1.1)			
Female				5 (14.3)	23 (65.7)	6 (17.1)	1 (2.9)			
Whole Blood				19 (19.0)	60 (60.0)	20 (20.0)	1 (1.0)			
Platelets					17 (70.8)	6 (25.0)	1 (4.2)			
Blood Group 0					**Anti-A_1 IgG**					
All		2 (0.4)	17 (3.7)	87 (18.7)	183 (39.4)	127 (27.3)	43 (9.2)	6 (1.3)		
Male		1 (0.3)	11 (3.6)	50 (16.4)	122 (40.0)	83 (27.2)	33 (10.8)	5 (1.6)		
Female		1 (0.6)	6 (3.8)	37 (23.1)	61 (38.1)	44 (27.5)	10 (6.3)	1 (0.6)		
Whole Blood		1 (0.3)	12 (3.3)	60 (16.4)	146 (39.9)	109 (29.8)	34 (9.3)	4 (1.1)		
Platelets		1 (1.0)	5 (5.1)	27 (27.3)	37 (37.4)	18 (18.2)	9 (9.1)	2 (2.0)		
Blood Group B										
All			2 (1.6)	18 (14.5)	50 (40.3)	40 (32.3)	12 (9.7)	2 (1.6)		
Male			1 (1.1)	12 (13.5)	35 (39.3)	30 (33.7)	11 (12.4)			
Female			1 (2.9)	6 (17.1)	15 (42.9)	10 (28.6)	1 (2.9)	2 (5.7)		
Whole Blood			2 (2.0)	12 (12.0)	41 (41.0)	33 (33.0)	10 (10.0)	2 (2.0)		
Platelets				6 (25.0)	9 (37.5)	7 (29.2)	2 (8.3)			

	Titer Step Difference 2021-2017									
	-4	-3	-2	-1	**0**	+1	+2	+3	+4	+6
Blood Group 0					**Anti-B IgM**					
All			3 (0.6)	67 (14.4)	272 (58.5)	105 (22.6)	14 (3.0)	4 (0.9)		
Male			3 (1.0)	43 (14.1)	179 (58.7)	71 (23.3)	7 (2.3)	2 (0.7)		
Female				24 (15.0)	93 (58.1)	34 (21.3)	7 (4.4)	2 (1.3)		
Whole Blood			2 (0.5)	53 (14.5)	217 (59.3)	82 (22.4)	9 (2.5)	3 (0.8)		
Platelets			1 (1.0)	14 (14.1)	55 (55.6)	23 (23.2)	5 (5.1)	1 (1.0)		
Blood Group A										
All	1 (0.2)	1 (0.2)	4 (0.9)	63 (14.4)	251 (57.1)	107 (24.4)	10 (2.3)	2 (0.5)		
Male	1 (0.3)	1 (0.3)	3 (1.0)	39 (12.6)	177 (57.1)	79 (25.5)	9 (2.9)	1 (0.3)		
Female			1 (0.8)	24 (18.6)	74 (57.3)	28 (21.7)	1 (0.8)	1 (0.8)		
Whole Blood	1 (0.3)	1 (0.3)	2 (0.6)	48 (14.9)	187 (58.0)	78 (24.1)	5 (1.5)	1 (0.3)		
Platelets			2 (1.7)	15 (12.9)	64 (55.2)	29 (25.0)	5 (4.3)	1 (0.9)		
Blood Group 0					**Anti-B IgG**					
All		2 (0.4)	16 (3.4)	100 (21.5)	169 (36.4)	122 (26.2)	38 (8.2)	11 (2.4)	6 (1.3)	1 (0.2)
Male		2 (0.7)	10 (3.3)	63 (20.7)	118 (38.5)	74 (24.3)	25 (8.2)	9 (3.0)	4 (1.3)	
Female			6 (3.8)	37 (23.1)	51 (31.8)	48 (30.0)	13 (8.1)	2 (1.3)	2 (1.3)	1 (0.6)
Whole Blood			14 (3.8)	79 (21.6)	135 (36.8)	96 (26.2)	28 (7.7)	9 (2.5)	4 (1.1)	1 (0.3)
Platelets		2 (2.0)	2 (2.0)	21 (21.2)	34 (34.4)	26 (26.3)	10 (10.1)	2 (2.0)	2 (2.0)	
Blood Group A										
All			11 (2.5)	44 (10.0)	261 (59.5)	91 (20.7)	25 (5.7)	6 (1.4)	1 (0.2)	
Male			8 (2.6)	36 (11.6)	183 (59.0)	61 (19.7)	17 (5.5)	4 (1.3)	1 (0.3)	
Female			3 (2.3)	8 (6.2)	78 (60.4)	30 (23.3)	8 (6.2)	2 (1.6)		
Whole Blood			10 (3.1)	33 (10.2)	189 (58.5)	65 (20.1)	19 (5.9)	6 (1.9)	1 (0.3)	
Platelets			1 (0.9)	11 (9.5)	72 (62.0)	26 (22.4)	6 (5.2)			

Note. Data are reported as number (%).TSD = TS of 2021 – TS of 2017. There are no samples with 5 TSD

10 Acknowledgements

11 Curriculum vitae