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Atypical Carbohydrate Blood Groups and Their Removal: Implications in Transfusion Medicine

Ricci Hagman, Jennifer

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JENNIFER RICCI HAGMAN

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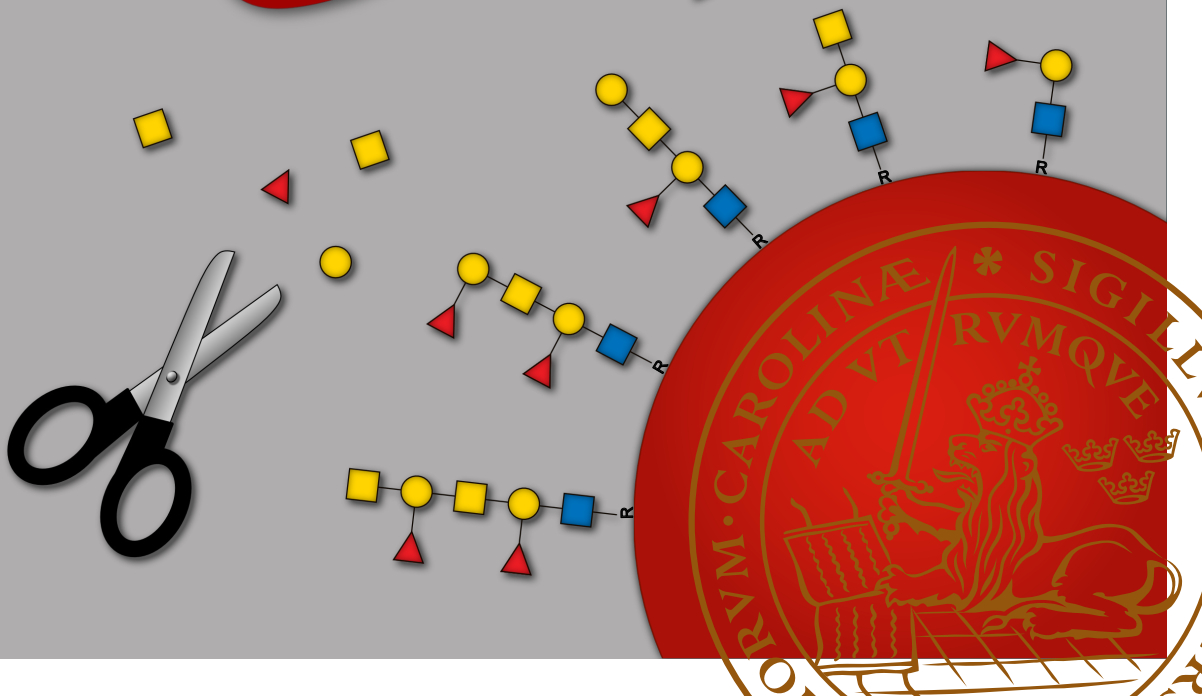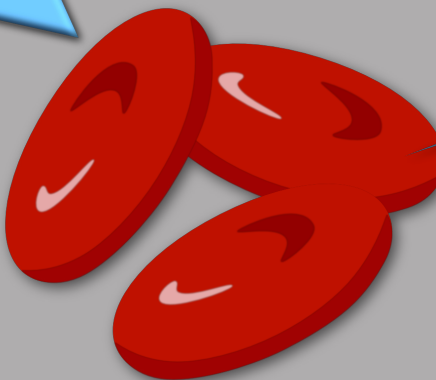
This is me. I am a biomedical analysist by trade. My speciality is Transfusion Medicine and I have an "old fashioned" passion for serology. By night I work at the Department of Clinical Immunology and Transfusion Medicine in Lund (Sweden), where I get to help improve the quality of life for the great number of patients in need of blood transfusions. For the past years, my days have been spent as a PhD student at the Division of Hematology and Transfusion Medicine with the Department of Laboratory Medicine, also in Lund. Here I get a chance to immerse myself in the wonderful world of carbohydrate-based blood groups, the ABO blood group system in particular. The conclusion of the body of work in this thesis certainly is a milestone in my career. Yet, I am both marvelled and humbled by the increasing complexity that this seemingly straightforward blood group system, discovered more than a century ago, has to offer.

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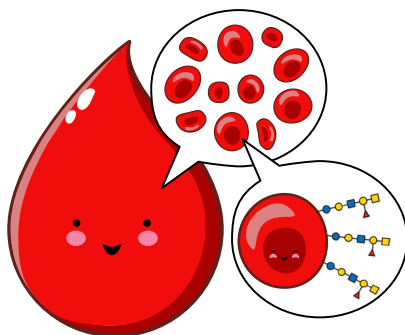
Jennifer Ricci Hagman

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Removal of A and B determinants has been achieved by treating RBCs with bacterial exoglycosidases. Although these blood units type as group O with monoclonal antisera, unexpected positive crossmatch reactions were observed between recipient plasma and converted donor RBCs. This is hypothesised to depend on antibodies against conversion-resistant, atypical ABO antigens, related to, but not identical to A and B.

The aim of my PhD project was to characterise the biochemical structure of these antigens and investigate the genetic background of carbohydrate-based antigens/phenotypes and GTs involved in their synthesis. From a clinical standpoint, determining if antibodies to these antigens are present in human plasma is of great interest.

We describe a previously unreported extension of the blood group B glycolipid (ExtB), ending with a β3-linked *N*-acetylgalactosamine. Antibodies to this antigen were affinity-purified from human plasma. Co-expression experiments demonstrated that 3-β-*N*-acetylgalactosaminyltransferase (P synthase) acts in concert with glycosyltransferase B to synthesise ExtB. Furthermore, anti-ExtB extracted from plasma of globoside-deficient group AB or B individuals binds to enzymatically converted group B RBCs, indicating the presence of ExtB on such cells.

As part of these studies, a novel genetic basis of globoside-deficiency in two Thai sisters was described. Also, several principally new null alleles of the ABO system were investigated. Finally, bacterially-derived exoglycosidases were explored to create ABO-universal RBCs by targeting both traditional and extended A and B antigens. This promising approach improved crossmatch compatibility of such RBCs with group O plasmas.

Key words: blood group, ABO, glycosyltransferase, red blood cell, transfusion medicine, ECO

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In memory of Ingela Åkesson

"I stand on the sacrifices of a million women before me
thinking what I can do to make this mountain taller
so the women after me can see farther"
– Rupi Kaur

Table of Contents

Abstract

ABO blood group compatibility is essential in transfusion medicine. This is due to the presence of naturally-occurring antibodies in the plasma directed against the A and B antigens on red blood cells (RBCs) and other cells. These antigens are found on glycoproteins and glycolipids and their synthesis is catalysed by glycosyltransferases (GTs) encoded by the *ABO* gene. Due to inactivating mutations blood group O lacks the A and B determinants. Group O can be transfused to recipients regardless of ABO group and is commonly called "universal blood".

Removal of A and B determinants has been achieved by treating RBCs with bacterial exoglycosidases. Although these blood units type as group O with monoclonal antisera, unexpected positive crossmatch reactions were observed between recipient plasma and converted donor RBCs. This is hypothesised to depend on antibodies against conversion-resistant, atypical ABO antigens, related to, but not identical to A and B.

The aim of my PhD project was to characterise the biochemical structure of these antigens and investigate the genetic background of carbohydrate-based antigens/phenotypes and GTs involved in their synthesis. From a clinical standpoint, determining if antibodies to these antigens are present in human plasma is of great interest.

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Populärvetenskaplig sammanfattning
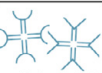
Ett stick i fingret och en droppe blod kan väcka olustkänslor hos många, men om vi skulle undersöka den där droppen lite noggrannare så skulle den avslöja att den innehåller över 200 miljoner röda blodkroppar eller erytrocyter som de också kallas. Vi skulle också upptäcka att den röda färgen inte alls har något med passion att göra utan helt enkel är till följd av det syrebärande proteinet, hemoglobin, som röda blodkroppar är fulla av.

Om man tittar på en röd blodkropp i förstoring så ser den inte så märkvärdig ut. Den är förhållandevis liten, sju tusendels millimeter i diameter, jämfört med andra celler och den har ingen cellkärna så den är lite ihopsjunken (bikonkav) på mitten. Just denna form gör förvisso den speciellt välanpassad för sin uppgift i kroppen nämligen att cirkulera runt överallt och syresätta vävnader.

Skulle man däremot skruva upp förstoringen rejält så kan man se att ytan omkring en erytrocyt är alldeles "luden" och det är precis här, i luddet, som det riktigt spännande händer. Det är nämligen här vi hittar våra blodgrupper.

Blod från olika individer må vid första anblick se likadant ut, men Karl Landsteiner, en österrikisk och sedermera Nobelprisbelönt läkare, visade år 1900 att så inte är fallet. Han blandade blod och serum från olika människor och observerade att i vissa kombinationer klumpade blodet ihop sig (bildade agglutinat). Han fastslog att det var substanser (antikroppar) i serum som reagerade med strukturer (antigen) på erytrocyterna.

Till följd av sina experiment delade han in folk i tre olika blodgrupper: A, B och O. Strax därpå identifierades ytterligare en blodgrupp, AB och tillsammans utgjorde de det första blodgruppssystemet: ABO. "Landsteiners regel" säger att i serum har man de antikroppar vars antigener man själv saknar. Eftersom O-blod inte har några antigen så kan det transfunderas oberoende av mottagarens ABO-grupp – så kallat "universalblod".

Landsteiners regel			
A	**B**	**AB**	**O**
antigener (erytrocyter) A	B	A and B	inga
antikroppar (serum) anti-B	anti-A	inga	anti-A, anti-B

Det här var dock bara början, i skrivande stund känner vi till 360 blodgruppsantigen som är organiserade i 45 olika blodgruppssystem. Däröver känner vi till ytterligare 30 antigen som ännu inte passar in i ett system. Vi vet också att Landsteiners regel i huvudsak bara omfattar blodgrupper som är baserade på kolhydratstrukturer.

Vad skiljer då kolhydratbaserade blodgrupper från protein-blodgrupper? Jo, proteiner är vad man kallar primära genprodukter vilket innebär att de syntetiseras med en gen som "mall". Kolhydrater (glykaner) syntetiseras istället genom att flera olika enzymer så kallade glykosyltransferas, oberoende av varandra,

förlänger kolhydratkedjan genom att sätta fast ett specifikt socker. Varje enzym (som ju är ett sorts protein) kodas i ett tidigare steg av en separat gen. Således byggs kolhydrater utan någon egentlig "mall" men baserar sig istället på enzymernas preferens för vissa sockerstrukturer och att de kan känna igen mottagarkedjan som dessa sockerbitar ska sättas på.

Om ett av enzymen i "produktionskedjan" saknas, på grund av ett fel i just det enzymets gen, så kan kolhydraten inte förlängas ytterligare. Denna fundamentala skillnad mellan proteiner och kolhydrater ligger till grund för att förstå hur genetiska variationer påverkar uttrycket av kolhydratbaserade blodgrupper. Ett exempel på detta är just det som händer i blodgrupp O.

ABO-genen har tre principiella alleler (varianter). Dessa nedärvs co-dominant det vill säga att alla har två varianter, en från vardera föräldern, vilket ger upphov till följande möjliga genkombinationer i en individ: *A/A*, *A/O*, *B/B*, *B/O*, *A/B* och slutligen *O/O*. *A*- och *B*-allelerna kodar för var sitt enzym: glykosyltransferas A (förkortas ofta GTA eller ibland α1,3GalNAc-T) kontra glykosyltransferas B (GTB, α1,3Gal-T) som båda verkar på en gemensam mottagarmolekyl (H-antigenet, som i sin tur faktiskt är en annan blodgrupp men det är en egen historia som vi kan hoppa över just nu). Den del av ABO genen som ger upphov till ett enzym är över tusen baser lång. Baser, egentligen kvävebaser, är molekylära byggstenar som utgör DNA och ordningen på baserna avgör den genetiska koden. Utomordentligt intressant är att endast sju punktmutationer (förändring som bara påverkar en kvävebas) skiljer dessa allelerna åt, varav fyra ändrar en beståndsdel (aminosyra) i enzymet men bara två bestämmer vilket socker enzymet kan koppla till och förlänga H-antigenet med. Dessa socker heter *N*-acetylgalaktosamin för GTA och galaktos för GTB.

Den allra vanligaste *O*-allelen har en mutation där en bas är borta (c.261delG), vilket gör att det inte blir något funktionellt enzym. Utan funktionellt enzym så kan inte kan inte H-antigent förlängas och därför saknas både A och B. Det är detta vi kallar blodgrupp O.

Idag känner vi till mer än 200 varianter av *ABO*-genen, med olika stor inverkan på enzymerna de kodar för. Det är den huvudsakliga orsaken till att vi (som jobbar inom transfusionsmedicin) emellanåt har svårt att bestämma vilken ABO blodgrupp en blodgivare eller patient har. I det som i denna avhandling kallas Paper II undersöker jag en rad sådana konstiga blodgrupperingsresultat. Efter att den genetiska bakgrunden fastställts fördjupar jag mig i hur dessa nya varianter påverkar enzymernas förmåga att syntetisera A- och B-antigen genom att uttrycka dem på konstgjord väg i en cellinje.

Blodtransfusioner har idag blivit en viktig och integrerad del i modern sjukvård. Enligt WHO så transfunderas årligen 118 miljoner enheter erytrocyter i hela världen, motsvarande siffra i Sverige är cirka 358 000 enheter. En enhet röda blodkroppar tillverkas från blodgivarens gåva av cirka 4½ deciliter blod. Dessa

enheter används vid större kirurgiska ingrepp och avancerad cancervård men även olyckstrauma och graviditetsrelaterade komplikationer. En stor andel används också till patienter med olika typer av blodcancer och efter stamcellstransplantation. Eftersom blodgrupp O kan transfunderas till alla patienter oavsett deras blodgrupp så används dessa O-enheter flitigt i situationer när patientens blodgrupp är okänd, till exempel vid svåra akuta tillstånd, efter att patienten redan blivit transfunderad så att man inte vet vems blod man blodgrupperar, eller när den inte definitivt kan bestämmas på grund av svagt uttryck av A och/eller B antigen (som i Paper II). Således finns det periodvis en viss överkonsumtion av dessa O-enheter blod, som i sin tur kan leda till brist i våra blodlager på sjukhusen. Det är också en stor logistikutmaning att ha rätt blod av rätt blodgrupp på rätt plats på alla sjukhusen i hela Skåne, eller var man nu befinner sig. Slutligen är det förenad med stor risk för patienten om fel ABO-grupp transfunderas av misstag och detta har i många studier visats vara en alltför vanlig orsak till biverkningar vid transfusion.

I korthet skulle tillgången på mer blod som fungerar som blodgrupp O alltså kunna lösa många problem inom sjukvården.

Precis som det finns enzymer som kan lägga till socker på kolhydrater så finns det enzymer som kan ta bort socker, dessa kallas för glykosylhydrolaser eller glykosidaser. En idé som är speciellt fängslande är att med hjälp av dessa enzymer "klippa" bort det sista sockret som avgör om blodgivaren är blodgrupp A eller B. Därmed exponeras H-antigenet som ju är synonymt med blodgrupp O.

Redan under 1980-talet lyckades man med denna episka uppgift, ett koncept som kom att kallas Enzyme-Converted to group O, eller på svenska enzymkonverterad blodgrupp O (ECO/EKO). Genom att använda enzymer utvunna ur gröna kaffebönor kunde man på detta påhittiga sätt omvandla blodgrupp B till blodgrupp O. Att omvandla blodgrupp A visade sig vare lite mer komplicerat (orsaker och lösningar presenteras i Paper V). Dessa B-ECO erytrocyter testades rigoröst, men trots att de såg ut som "nollor" hur man än vände och vred på dem så upptäckte man att de reagerade med antikroppar i viss plasma (en möjlig anledning till detta presenteras i Paper III). Nästa genombrott kom 2007 när min handledare och andra forskare letade efter lämpliga enzymkandidater bland bakterier. Denna gång lyckades man omvända även grupp A till O. De enzymer man identifierade var dessutom betydligt effektivare än de som använts tidigare och man lyckades till och med omvandla hela blodenheter, men även här så hittade man oförklarliga reaktioner när ECO-blodet testades med plasma i det som i vardagligt tal kallas korstester som används för att förutspå om en transfusion kommer att gå bra.

Denna oförklarliga reaktivitet är ett återkommande fenomen i ECO-sagan och har till nu inte fått någon förklaring. I mina studier har vi haft idén att de besvärliga korstesterna beror på att ABO-antigener kan förlängas ytterligare, något som varit känt länge för A men som visas för första gången för B i Paper III.

Eftersom bakterier i vår tarmflora kan bryta ner de A- och B-antigen som även återfinns på slemhinnorna i våra tarmar så skulle man kunna säga att nästa framsteg hade en riktig skitig start. Denna gång presenterades en två-enzymlösning av kanadensiska forskare för att konvertera blodgrupp A. Det första enzymet gjorde om det A-specifika sockret till en intermediär struktur som sedan kunde klyvas bort av enzym nummer två. Dessa A-ECO erytrocyter testades enbart med kommersiella antisera och några försök att korstesta med plasma visades inte ens. I Paper IV utvärderar jag möjligheten att använda det första enzymet från "tvåstegslösningen" för att återskapa röda blodkroppar som uppvisar en tillfällig blodgrupp som kallas "förvärvad B", varvid A görs om till något som liknar B om patienten har bakterier i blodet. Detta fenomen uppstår just vid bakteriella infektioner mag-tarmkanalen och det är av stor vikt att kommersiella reagens inte feltolkar detta som en riktig blodgrupp. De celler vi producerar i Paper IV kan komma att användas för att se till att sådana feltolkningar inte sker.

I Paper V presenteras ett mastodontprojekt i samarbete med en forskargrupp vid det Tekniska Universitet i Danmark (DTU). Här beskrivs 21 olika enzymer från en slemälskande bakterie från tjocktarmen, *Akkermansia muciniphilia*. Dessa enzymer framställdes syntetiskt av danskarna och kunde sedan utvärderas och användas i Lund för att konvertera blodgrupp A och B, inklusive de A- och B-specifika (Paper III) förlängda strukturerna som misstänks ligga bakom de oförklarliga korstestreaktionerna. Den goda nyheten var då att vi såg att problemen med korstesterna minskade betydligt. Den något sämre nyheten var att de inte försvann helt. Det finns alltså all anledning att fortsätta forska för att komma fram till hur ABO-universellt blod för transfusion ska framställas. Jag kan dock stolt att konstatera att mina studier lagt ännu några pusselbitar som hjälper oss att se hela bilden och så småningom förhoppningsvis lägga färdigt det komplicerade tusenbitarspusslet så att ECO-blod kan bli verklighet någon gång i framtiden.

List of Papers

Paper I

Ricci Hagman J, Hult AK, Westman JS, Hosseini-Maaf B, Jongruamklang P, Saipin J, Bejrachandra S, Olsson ML. Multiple miscarriages in two sisters of Thai origin with the rare P^k phenotype caused by a novel nonsense mutation in the *B3GALNT1* locus

Transfusion Medicine, 2019;29(3):202-208 [doi: 10.1111/tme.12544]

Paper II

Ricci Hagman J, Hult AK, Hellberg Å, Olsson ML. Truncated glycosyltransferase coding regions in novel ABO alleles give rise to weak A or B blood group expression and discrepant typing results

Transfusion, published online ahead of print Sept 2023 [doi: 10.1111/trf.17534]

Paper III

Ricci Hagman J, Barone A, Westman JS, Storry JR, Chunsheng J, Hult AK, Clausen H, Teneberg S, Olsson ML. β1,3GalNAc-T1-dependent extension of the human blood group B antigen results in a novel ABO-related glycolipid on erythrocytes

Manuscript (incl. supplement), not yet submitted for publication

Paper IV

Ricci Hagman J, Rahfeld P, Withers SG, Kizhakkedathu JN, Olsson ML, Storry JR. Use of a recombinant deacetylase to convert A_1 red blood cells to the acquired B phenotype for quality control purposes

Blood Transfusion, published online ahead of print Sept 2023
[doi: 10.2450/BloodTransfus.584]

Paper V

Jensen M*, Stenfelt L*, **Ricci Hagman J**, Pichler JM, Nielsen TS, Hult A, Morth JP, Olsson ML**, Hachem MA**. Targeting extended blood group antigens by *Akkermansia muciniphilia* exoglycosidases unveils a missing link for generating ABO-universal donor blood

Manuscript incl. supplements submitted to Nature Microbiology on July 26, 2023 (under review)

* = These authors contributed equally.
** = Corresponding authors (these authors jointly supervised this work)

Author's contribution to the papers

Paper I

Jennifer was substantially involved in the design of this study and performed all the experimental work, independently collected and analysed data as well as interpreted the results under the guidance of her supervisors. Jennifer also drafted the manuscript, including figures and tables, and was actively involved in the publication process including revision of the manuscript.

Paper II

Jennifer was substantially involved in the design of this study and performed a sizeable amount of the experimental work specifically the transient expression studies, including flow cytometric evaluation and interpreting the output. Jennifer also independently drafted the manuscript, including all the figures and tables and was significantly involved in the revision of the accepted manuscript as well as drafting the revision letter to the editor.

Paper III

This complex manuscript is the result of an extensive collaboration together with experts in the field of glycobiology initiated more than a decade ago. Jennifer has affinity purified and characterised the crucial anti-ExtB reagent from plasma used in flow cytometric immune staining. She also performed confirmatory transfection experiments. All figures in the manuscript and supplement which do not pertain mass spectrometry data were finalised by Jennifer who also made substantial contributions regarding collating all the data and drafting the manuscript.

Paper IV

Jennifer was substantially involved, demonstrating independence, in all processes from start to finish regarding this accepted manuscript.

Paper V

This manuscript is another result of an impressive collaboration. Jennifer performed all the experimental immunohaematological work including enzymatic conversion of ABO groups on red blood cells during the start-up of this project and demonstrated the feasibility in sequential removal of all known traditional and extended ABO-related blood group structures from group A and B red blood cells (RBCs). Jennifer has continuously shown great interest in this project and has critically reviewed the final draft of this manuscript.

Abbreviations

bp	base pairs
CAZy	Carbohydrate Active enZyme database
Cer	ceramide
CMP-	cytitine monophosphate
DTU	Danish Technology University
ECO	Enzyme Conversion to group O
ER	endoplasmic reticulum
ExtB	Extended B
fs	frame shift
Fuc	fucose
Gal	galactose
GalN	galactosamine
GalNAc	*N*-acetylgalactosamine
GDP-	guanosine diphosphate
Glc	glucose
GlcNAc	*N*-acetylglucosamine
GSL(s)	glycosphingolipid(s)
GT(s)	glycosyltransferase(s)
GTA	glycosyltransferase A
GTB	glycosyltransferase B
HBOC	Hemoglobin-Based Oxygen Carriers
IgG	Immunoglobulin G
IgM	Immunoglobulin M
ISBT	International Society of Blood Transfusions
kbp	kilo base pairs
Km	Michaelis-Menten constant
mf	mixed field
NRLGBT	Nordic Reference Laboratory for Genomic Blood Group Typing
nt	nucleotide
O_h	Bombay phenotype
ORF	open reading frame
PCR-ASP	polymerase chain reaction with allele specific primer assay
PEG	polyethylene glycol
RBC(s)	Red Blood Cell(s)
Se	secretor gene (*FUT2*)
SNFG	Symbol Nomenclature for Glycans
SNP	Single Nucleotide Polymorphism
SNV	Single Nucleotide Variant
TLC	thin layer chromatography
UDP-	uridine diphosphate
WHO	World Health Organization

"It must therefore be concluded that the blood in the animal body moves around in a circle continuously and that the action or function of the heart is to accomplish this by pumping. This is the only reason for the motion and beat of the heart."

Though the statement [1] above might seem apparent to you and me, the association was first described by the English physician William Harvey in 1628 [1]. As it contradicted the prevailing teachings of Galen claiming that blood was consumed by the extremities and continuously replenished by the liver, it caused quite a stir at the time. Nevertheless, it did instigate an era of questionable interspecies blood transfusions leaving a trail of deaths to the extent that the practice was promptly banned and lay dormant for almost two centuries.

The first successful transfusion between humans was documented in 1818, when obstetrician James Blundell prolonged the life of a terminally ill man suffering from "cirrhosis of the pylorus" [2, 3], although he might be more known for the treatment of a woman suffering from postpartum haemorrhaging in 1825 [4]. Not only did he employ a strict scientific approach to the transfusion of blood, he also invented gadgets like the "impellor" and the "gravitator" to facilitate transfusions. Much later it was understood that his, in fact, rather limited success was purely up to chance since there was no knowledge of blood group antigens or antibodies back in those days– or bacteria for that matter.

A major breakthrough in transfusion medicine came in 1900 when Dr. Karl Landsteiner showed that individuals could be divided into three groups depending on how their red blood cells (RBCs) agglutinated (stuck together in clumps), when mixed with serum from other individuals [5, 6]. He named the groups: A, B and C, the latter later renamed O from the German "ohne", meaning "without" agglutination.

The Nobel Prize winning discovery of the ABO blood group system, along with implementation of pre-transfusion cross matching paved the way for safer transfusion practices [7, 8]. In addition, the development of anticoagulants and use of nutrient solutions allowed for longer storage of blood [9, 10], and thus the modern blood bank was born.

It was working in one of these blood banks that my personal interest in blood groups was sparked. Little did I know that my curiosity would eventually take me on this journey of scientific investigations.

Blood groups

If you were to ask a group of people about their blood groups, some might proudly announce that they are "universal" donors and there might even be some talk about whether they are "positive" or "negative" (recalling something about pregnancy) but that is usually where common knowledge ends (and why I still have to explain to friends and family what I do for a living).

Blood groups, like people, come in all shapes and forms. A person's blood group is their unique combination of a multitude of antigens on the RBC surface. An antigen is a substance capable of stimulating an immune response. Blood group antigens are characterised as inherited variations (often polymorphisms) found on proteins or carbohydrates attached to the RBC membrane. They are defined by antibodies found in human sera or plasma. These antibodies can be naturally-occurring (present throughout most of a person's lifespan without prior external stimulation) or can result from alloimmunisation after exposure to non-self-cells, which can occur in the case of pregnancy, blood transfusion or transplantation.

A blood group system is a set of one or more antigen(s) expressed by a single gene locus i.e., a specific and fixed position on a chromosome. The definition of a blood group system also encompasses antigens that are expressed by very closely linked homologous genes. Currently, there are 390 blood group antigens recognised by the International Society of Blood Transfusion (ISBT), of which 360 are organised into 45 blood group systems as seen in Table 1 [11].

The remaining 30 antigens do not yet fit the criteria required to belong to a new or existing blood group system and are sometimes referred to as "orphans", but maybe "foundlings" has a more modern ring. Eleven are currently placed in "Collections" since they are biochemically, genetically or serologically similar but the genetic background has yet to be discovered. That leaves three high-prevalence (more than 90% of the population carry them) antigens in the 901 series and 16 low-prevalence (less than 1% of the population carry them) antigens in the 700 series.

Table 1. Blood group systems recognised by the ISBT (as of July 2023)

No	Blood group system	Symbol	Antigen(s)	Gene(s)
001	ABO	ABO	4	*ABO*
002	MNS	MNS	50	*GYPA, GYPB, (GYPE)*
003	P1PK	P1PK	3	*A4GALNT*
004	Rh	RH	56	*RHC, RHD*
005	Lutheran	LU	26	*BCAM*
006	Kell	KEL	37	*KEL*
007	Lewis	LE	6	*FUT3*
008	Duffy	FY	5	*ACKR1*
009	Kidd	JK	3	*SLC14A1*
010	Diego	DI	23	*SLC4A1*
011	Yt	YT	6	*ACHE*
012	Xg	XG	2	*XG, MIC2*
013	Scianna	SC	9	*ERMAP*
014	Dombrock	DO	10	*ART4*
015	Colton	CO	4	*AQP1*
016	Landsteiner-Wiener	LW	4	*ICAM4*
017	Chido/Rogers	CH/RG	9	*C4A, C4B*
018	H	H	1	*FUT1/FUT2*
019	Kx	XK	1	*XK*
020	Gerbich	GE	13	*GYPC*
021	Cromer	CROM	20	*CD55*
022	Knops	KN	12	*CR1*
023	Indian	IN	6	*CD44*
024	Ok	OK	3	*BSG*
025	Raph	RAPH	1	*CD151*
026	John-Milton Hagen	JMH	8	*SEMA7A*
027	I	I	1	*GCNT2*
028	Globoside	GLOB	3	*B3GALNT1*
029	Gill	GIL	1	*AQP3*
030	Rh-associated glycoprotein	RHAG	5	*RHAG*
031	FORS	FORS	1	*GBGT1*
032	JR	JR	1	*ABCG2*
033	LAN	LAN	1	*ABCB6*
034	Vel	VEL	1	*SMIM1*
035	CD59	CD59	1	*CD59*
036	Augustine	AUG	4	*SLC29A1*
037	KANNO	KANNO	1	*PRNP*
038	Sid	SID	1	*B4GALNT1*
039	CTL2	CTL2	2	*SLC44A2*
040	PEL	PEL	1	*ABCC4*
041	MAM	MAM	1	*EMP3*
042	EMM	EMM	1	*PIGG*
043	ABCC1	ABCC1	1	*ABCC1*
044	ER	ER	5	*PIEZO1*
045	CD36	CD36	1	*CD36*

Carbohydrate-based blood group systems are highlighted in red.

In the early days of discovery, blood groups were quite arbitrarily named, for instance after the individual who made the antigen-defining antibody or after the geographic location where the antibody was discovered and sometimes even the investigator's name was added to the mix. The need for clear and consistent vocabulary eventually became apparent. This monumental task of being the guardian of blood group nomenclature and also the gatekeeper, approving new antigens and systems (or not), was undertaken by the ISBT in the 1980s and each blood group system was given an official name, symbol and three-digit number by the ISBT Working Party for Red Cell Immunogenetics and Blood Group Terminology. Blood group antigens were allocated a six-digit number, the first three representing the system and the last three representing the antigen. Subscripts, superscripts, and italics are used to differentiate the traditional nomenclature, still used colloquially. For instance, in the ABO system A1, A_1 and A^1 refer to the antigen, phenotype and allele, respectively.

Blood group antigens can be carried on proteins, glycoproteins or glycolipids and although all are present on the RBCs membrane, some are also found on other tissues and referred to as histo-blood groups. Under certain conditions the soluble form of some antigens can be detected in secretions and can be adsorbed from plasma onto the RBC surface. Molecules carrying blood group antigens have been associated with several different functions such as transmembrane transporters, adhesion, enzymatic activity and complement regulation [12]. Antigens on glycoproteins and glycolipids also constitute the glycocalyx surrounding the RBC contributing to the overall negative charge of the RBCs, which decreases the risk of spontaneous agglutination. Many antigens are also used as receptors by pathogens and toxins, making blood groups involved in host defence as well as innate immunity [13].

Carbohydrate-based blood groups

Glycosylation, glycosyltransferases and some general glycobiology concepts

Carbohydrates as opposed to proteins

There is a key difference when it comes to carbohydrate-based blood groups compared to protein-based blood groups that needs to be kept in mind. Proteins are the primary gene products. They are synthesised as identical copies through the translation of a messenger RNA template transcribed from the genome. The activity or function of a protein is primarily dependent on it having a correctly folded three-dimensional structure. Carbohydrates, or glycans, on the other hand are secondary gene products. They are instead assembled, without a template, through the actions of enzymes i.e., glycosyltransferases (GTs). These independently acting GTs catalyse the stepwise addition of monosaccharides to an elongated carbohydrate chain [14]. Several GTs are usually involved in glycan formation and each is encoded by a separate gene. Importantly, the lack of a GT or reduced capacity of a GT upstream affects all further synthesis of the glycans downstream as schematically depicted in Figure 1. This fundamental principle is necessary for the understanding of how genetic variation affects carbohydrate phenotypes.
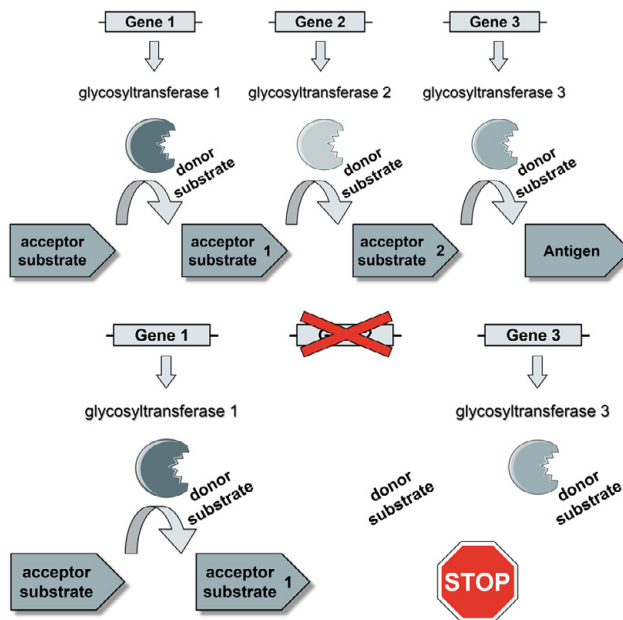
Figure 1 Schematic figure demonstrating the elongation of the growing glycan due to the independent actions of separate GTs. In the presence of inactivation mutations in a gene encoding a GT the extension of the glycan is interrupted and cannot carry on beyond this step.

Glycosylation

Glycosylation, a form of post-translational modification, is the biochemical process in which a glycan is covalently attached to another molecule forming a glycoconjugate. The glycan can be a single monosaccharaide or a long carbohydrate chain. The molecule can be a protein or a lipid and the product is either a glycoprotein or a glycolipid. Glycoproteins tend to be highly branched structures whereas glycolipids have a more linear structure, making them easier to study, or at least depict.

Glycans can be either N-linked or O-linked to proteins. N-linked glycans are attached to the nitrogen (N) of the amino acid (aa) Asparagine (Asn) when this appears in a specific sequence: Asn-X-Serine (Ser) or Asn-X-Thr (Threonine), where X denotes any aa except Proline (Pro). O-linked glycans are attached to the oxygen (O) of a Ser or Thr residue but can also occur on Tyrosine (Tyr) [14].

The majority of ABH antigens on RBCs are in the form of N-linked glycans [15], mainly associated with the anion transporter Band 3 and the glucose transporter Band 4.5. Glycophorins have also been shown to carry some ABH antigens [16]. However, a substantial fraction, around 10%, are present as GSLs.

When glycans are attached to lipids they form glycolipids. In humans the major class of glycolipids are glycosphingolipids (GSL) [14]. The lipid part is composed of a sphingosine, a structure that was so difficult to characterise that it was named after the enigmatic Egyptian Sphinx. The sphingosine is linked to a fatty acid and together they make up the lipid or ceramide portion of the GSL (Figure 2).

Throughout this thesis monosaccharides are depicted and abbreviated according to the Symbol Nomenclature for Glycans (SNFG) [17-19]
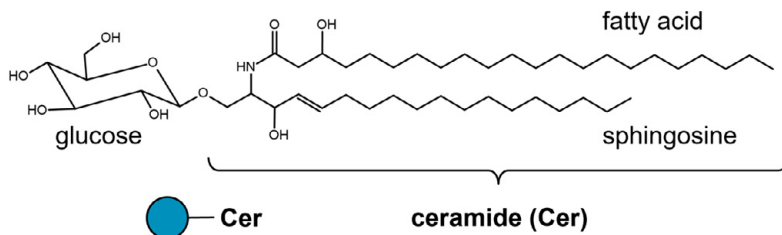
Figure 2 The GSLs in this thesis carrying ABO-, GLOB- or P1PK system determinants all originate from a glycosylceramide consisting of a glucose ● and a ceramide (Cer), comprised of a fatty acid linked to a shingosine.

Based on which neutral core structure they share, GSLs can be organised into seven groups or series [14]. At least three of them can feature ABO/GLOB/P1PK blood group determinants and are therefore of particular relevance for this thesis. These are the globo-, neolacto-, and lacto-series [20]. The specific linkages and defining monosaccharides are highlighted in Table 2, as well as the thesis-relevant blood group antigens they are known to carry.

Table 2 . Globo-, neolacto- and lacto- series neutral GSL core structures and associated blood groups.

GSL Series	Core Structure	Selected blood group epitopes
Globo-		P^k, P, Globo-H, Globo-A, Globo-B
Neolacto-		A, B, H, P1, PX2, ExtB, Gal-A, H type 3, A type 3 (repetitive A)
Lacto-		A, B, H

The carbohydrate sequence defining the different core structures are highlighted with **thick lines** and **bold letters**. Monosaccharides are as follows: ●Glucose (Glc), ●Galactose (Gal), and ■*N*-acetylglucosamine (GlcNAc). according to SNFG

Both globo- and neolacto- series GSLs are found on RBCs. Globo-series GSLs are the most abundant on RBCs, neolacto- series GSLs are present on haematopoietic cells, including RBCs, whereas lacto-GSLs are mainly associated with secretory organs [14].

A more comprehensive overview of the relationship between GSLs in the neolacto- and globo-series can be seen in Figure 3

28
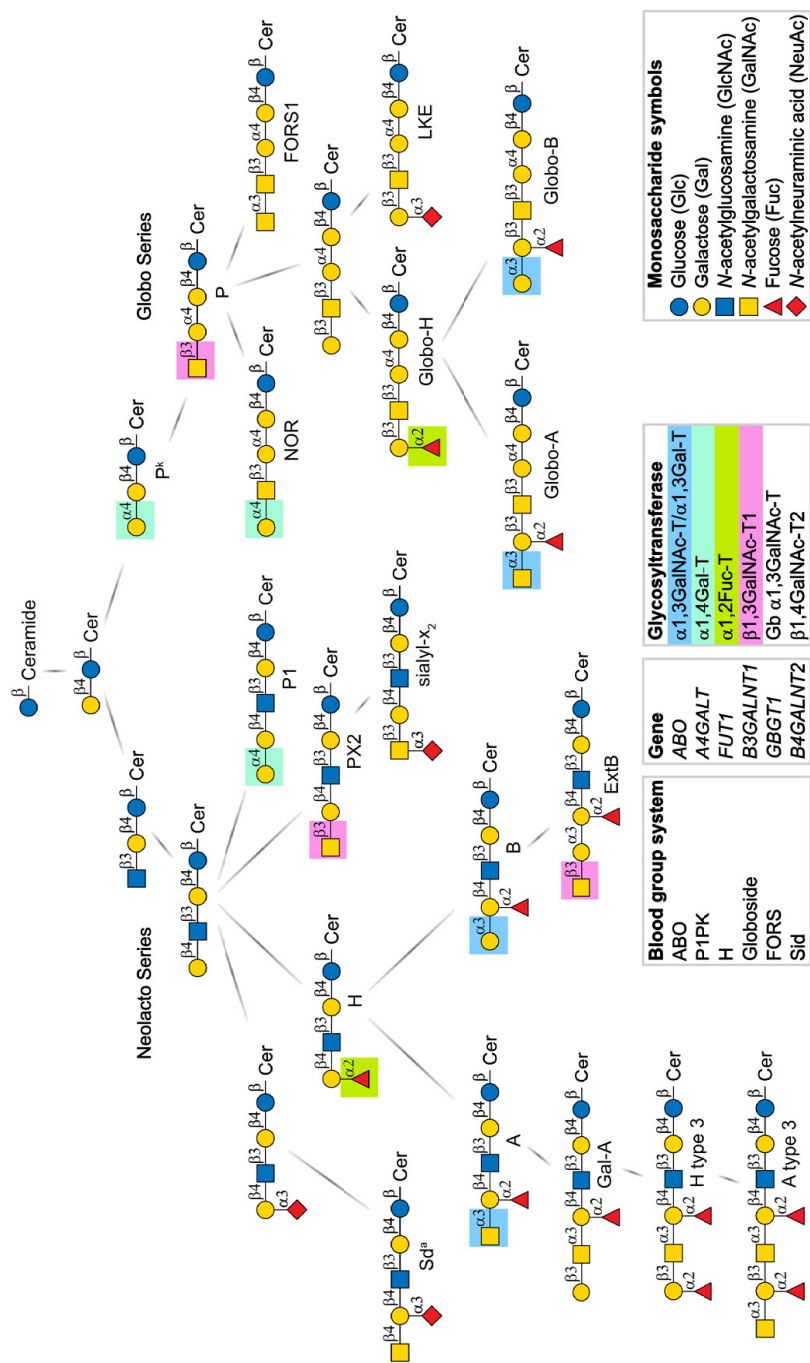
Figure 3 Schematic representation of selected carbohydrate blood group antigens and their precursors in the neolacto-and globo- series of GSLs. Although six blood group systems are represented in the table, only GTs involved in the synthesis of ABO and Globoside and their respective precursors are highlighted.

Glycosyltransferases

GTs catalyse the transfer of monosaccharides from an "activated" sugar phosphate, which acts like a glycosyl donor to a glycosyl acceptor substrate molecule. The most common "donor" in vertebrates is uridine diphosphate (UDP-) but others like guanosine diphosphate (GDP-) and cytidine monophosphate (CMP-) are also relevant in the glycan blood group context.

There are two main types of glycosidic bonds catalysed by GTs, α and β [14]. Combined with several possible carbon links (1-2, 1-3, 1-4, 1-6 etc.) and a considerable number of monosaccharide permutations, the result is virtually a cornucopia of possible glycan combinations. As many GTs act in sequence to synthesise glycans, they have been shown to form complexes in the Golgi [21, 22], thereby facilitating the addition of monosaccharides.

GTs involved in the synthesis of carbohydrate blood group antigens are mainly present as type II transmembrane proteins in the Golgi apparatus [23, 24]. They typically have a short N-terminal domain projecting out of the Golgi, a hydrophobic transmembrane region, a stem region (often containing a proteolytic site) and a large intralumenal, globular C-terminal domain, which contains the catalytically active site (Figure 4).
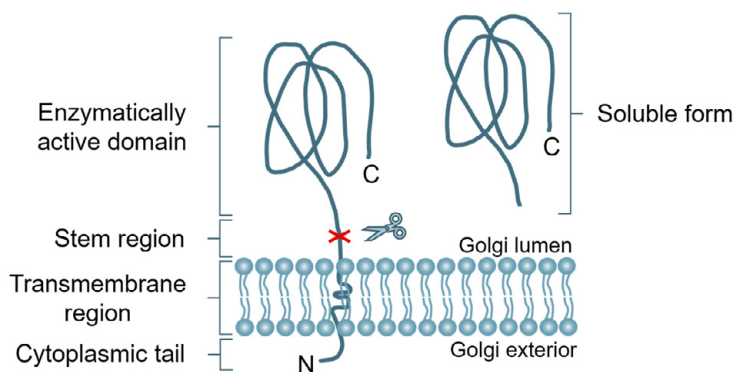
Figure 4 Overview of the organisation of a GT type II membrane protein. The N-terminal extends out of the Golgi and the C-terminal domain resides inside the Golgi lumen.

GTs are classified into families based on similarities in amino acid sequence and function. The Carbohydrate-Active enZYme Database (CAZy) [25] provides information on genomic, structural and biochemical properties of carbohydrate-acting enzymes. Enzymes within a family typically have structurally-related catalytic domains

ER to Cell membrane via Golgi

The endoplasmic reticulum together with the Golgi complex acts as the cell's protein factory so to speak. Most proteins in mammalian cells, not destined for the cytosol, are transferred to the lumen of the rough endoplasmic reticulum (ER) while they are being translated by ribosomes bound to the ER. This network of membrane-enclosed vesicles called cisternae plays a crucial role in early protein processing and is the site where the initial N-linkage (described above) occurs in N-linked glycoproteins before they are transported to the Golgi apparatus for further processing. The O-linkage, described earlier, instead occurs in the Golgi which is an organised complex of flattened cisternae. Proteins enter the convex *cis* side of the Golgi from the ER via transport vesicles. Regarding the GSLs, the initial monosaccharide is linked to the ceramide (Figure 2) from outside the Golgi. The GSL then "flips" in the Golgi membrane and additional monosaccharides are added within the luminal compartment(s) [26-28]. Glycoproteins and GSL move through the Golgi complex as they are being glycosylated and sorted for their final destination before leaving the concave *trans* side of the Golgi via transport vesicles. The exact mechanisms by which glycoconjugates move through the Golgi has not been established yet although different theories have been suggested. The pathway by which glycoconjugates are moved from the ER trough the Golgi and to the cell's membrane is referred to as the secretory pathway (Figure 5).
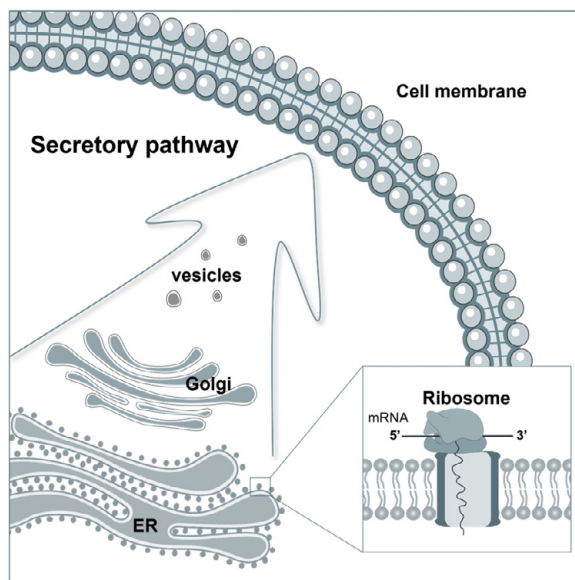
Figure 5 Basic overview of the secretory pathway. ER bound ribosomes translate mRNA. Post translational modifications begin in ER and continues throughout Golgi. Glycoconjugates, such as carbohydrate based blood groups move through the secretory pathway to their final destination i.e., the cell membrane.

The proteins, i.e. GTs, that function within the Golgi must of course somehow be retained within the Golgi. Amino acid sequences within the transmembrane domain of GTs have been shown to be critical for Golgi retention [29], but aa sequences close to the transmembrane domain on both sides of the Golgi membrane have also been implicated in keeping the GTs in place. Interestingly, there has been a series of studies investigating how GTs work together in functional complexes, as dimers or oligomers [30, 31]. This also seems to be of relevance for blood group-related GTs [32].

ABH and GLOB

Eight blood group systems are classified as carbohydrates.

For the purpose of giving a relevant background to the studies presented in this Ph.D. thesis, a detailed description of two blood group systems will dominate this section, ABO and GLOB, whilst most of the other carbohydrate-based blood groups will just be mentioned in passing and shown in some of the overview tables. However, since many of the antigens in the carbohydrate-based blood group systems are dependent on the expression of their respective precursor (Table 3) it is practically impossible to discuss the ABO system and the GLOB system without at least mentioning the "immediate precursor blood group systems", which precede them in the synthetic pathways, namely the H and P1PK systems.

The H blood group system

The H blood group system contains only one antigen, namely H. It is predominantly found on group O RBCs but to some extent also on group A, B and AB. Two homologous genes, *FUT1* and *FUT2,* each encode a fucosyltransferase. *FUT1* gives rise to α1,2Fuc-T1 which catalyses the addition of fucose (Fuc) to Galβ1,4GlcNAc-R (type 2 precursor chain in the neolacto-series and on glycoproteins, where R represents the rest of the molecule) on RBCs and in tissues [33]. *FUT2*, also called the *Secretor* (*Se*) gene, instead adds Fuc to Galβ1,3GlcNAc-R (the type 1 precursor chain in the lacto-series GSLs (Table 2), in secretions [34].

The implications of the *FUT2* on the expression of other blood groups such as Le[a] and Le[b] occurring on type 1 chains, however interesting, will not be explored further here but have been extensively reviewed [35].

Table 3. Overview of the eight carbohydrate blood group systems. ABO and GLOB are highlighted in red.

System	Chromosome	Gene(s)	GT(s)	Antigen(s)	Precursor(s)	EC number(s)	CAZy family
ABO	9q34.2	ABO	α1,3GalNAc-T (GTA) α1,3Gal-T (GTB)	A B	H	2.4.1.40 2.4.1.37	GT6
P1PK	22q13.2	A4GALT	α1,4Gal-T	P1 P^k NOR	Galβ1,4GlcNAcβ-R Galβ1,4Glc-R P (Gb4)	2.4.1.228	GT32
LE	19p13.3	FUT3	α1,3/4Fuc-T	Le^a Le^{b*}	Galβ1,3GlcNAcβ-R H type 1	2.4.1.65	GT10
H	19q13.33	FUT1 FUT2	α1,2Fuc-T1 α1,2Fuc-T2	H	Galβ1,4GlcNAcβ-R Galβ1,3GlcNAcβ-R	2.4.1.69	GT11
I	6p24.2	GCNT2	β1,6GlcNAc-T2	I	N-acetyllactosamine	2.4.1.150	GT14
GLOB	3q25	B3GALNT1	β1,3GalNAc-T1 (P-synthase)	P PX2 ExtB	P^k (Gb3) Galβ1,4GlcNAcβ-R B	2.4.1.79	GT31
FORS	9q34.13-q34.3	GBGT1	globoside α1,3GalNAc-T	FORS1	P (Gb4)	2.4.1.88	GT6
SID	17q21.32	B4GALNT2	β1,4GalNAc-T2	Sd^a	NeuAcα1,3Galβ-R	2.4.1.165	GT12

The trivial name for the ABO GTs and the GLOB GT are given in parentheses

* in presence of the secretor gene (FUT2) H type 1 synthesis is favoured over Le^a. As a result FUT3 mostly elongates H typ 1, resulting in Le^b [15].

Exceedingly rare H-deficient phenotypes

The Bombay phenotype (O_h or *hh*) was, as the name implies, first discovered in India [36]. Individuals with this phenotype do not express the H antigen at all since they lack functional products of both *FUT1* and *FUT2*. In addition, they do not express A nor B antigens since there is no precursor for functional ABO transferase to build on. The Para-Bombay ($H+^w$) phenotype comes in two different variants: one cannot synthesise the H antigen on RBCs due to an inactive *FUT1* product, however due to functional *FUT2*, H and consequently A and B can be present in secretions. These secreted antigens can be adsorbed in small quantities to the RBCs and therefore give rise to the so-called A_h and B_h phenotypes if *A* and *B* alleles are present, respectively. The other type of para-Bombay phenotype has weakening amino acid substitutions in the *FUT1* product so that it only synthesizes small amounts of H type 2. This type can either be a secretor or a non-secretor and the phenotype is very similar to the first para-Bombay type described above. A German study found the frequency of *FUT1* null (*h*) alleles to be 1 in 347 [37].

Individuals of the Bombay phenotype produce highly reactive anti-H and can only be transfused with blood of the same phenotype. Para-Bombay individuals typically produce anti-H but of lower clinical significance. Before H-negative units are used for this group of patients it is advised to investigate the strength (titre) of antibody as well as its temperature optimum, which can range from 4°C to 37°C [15]. Antibodies which react below body temperature are usually of less clinical significance.

The ABO blood group system

Despite being discovered twelve decades ago [5, 6], the knowledge of the ABO blood group system is far from comprehensive and this seemingly straightforward system continues to grow in complexity over time. Although I have certainly added my piece of the puzzle, we are still a long way away from seeing the whole picture.

Yet, the fundamental doctrine remains true: Landsteiner's law stating that, under normal conditions, antibodies to the A and/or B antigen(s) lacking on the RBCs are always present in the plasma. Accordingly, to this day, ABO blood typing consists of two steps. (1) Determine the presence or absence of A and/or B antigens on the RBCs. This is known as *forward* typing and is achieved using standardised serological methods [15, 38] and validated and highly regulated test reagents such as monoclonal antibodies or various lectins (carbohydrate-binding proteins) able to differentiate between immunogenic epitopes/phenotypes. (2) Evaluate the presence or absence of anti-A and/or anti-B in the plasma, known as

reverse typing. In order to definitively interpret of the blood group as A, B, AB or O, forward and reverse blood typing must correlate.

ABO antigens

A person's ABO blood group is determined by which AB(H) antigens are present on the RBCs and what antibodies are present in the plasma. The presence of AB(H) antigens is dependent on the GT(s) encoded by the *ABO* (and *FUT1/FUT2*) gene(s) [39-41]. Somewhat surprisingly the ISBT currently recognises four antigens in this system: A, B, A1 and A,B [11]. The A and B antigens correspond with the A and B blood group phenotypes. A1 was given antigen status based on the presence of an "A1" reactive antibody found in some A individuals whose RBCs show weaker (but still acceptably strong in routine tests) reactivity with anti-A, i.e. the A_2 phenotype, but the exact nature of the A1 antigen has not been demonstrated. Some clarification to this matter is offered further below. The A,B antigen is also defined by an antibody specificity. Interestingly, group O plasma contains antibodies which cannot be separated into anti-A and anti-B. This A,B-antibody reacts equally well with the terminal trisaccharide of A and B antigen but is non-reactive with the shared fucose element. It has therefore been suggested that A and B have one more epitope in common and that this is situated maximally distant from the A and B distinguishing epitope [42]. Table 4 below offers a brief overview regarding the different ABO phenotypes, RBC antigens, their corresponding antibodies and possible genotypes.

Table 4. The ABO blood group system, a brief overview

Phenotype		ABO antigens on RBCs	ABO antibodies in plasma	ABO genotype[†]
A:	A_1	A , A1*	Anti-B	A/A or A/O
	A_2	A	Anti-B Anti-A1**	
	B	B	Anti-A	B/B or B/O
AB:	A_1B	A, (A1) and B	None	A/B
	A_2B	A and B	Anti-A1**	
	O	None***	Anti-A Anti-A1 Anti-B Anti-A,B****	O/O

[†]Zygosity for *A*, *B* or *O* alleles is given without consideration of A^1 and A^2 alleles or all different *B* and *O* alleles known to exist.

*The exact nature of the A1 antigen has not been demonstrated.

**Not always detectable under routine conditions

***The H antigen, belongs to the H blood group system. The amount of H antigen in different ABO phenotypes is as follows, in decreasing order: O→A_2→A_2B→B→A_1→A_1B→para-Bombay and O_h [33]

****The anti-A,B specificity found in group O plasma can not be separated into anti-A and anti-B.

ABO phenotypes

The ABO phenotype is synonymous with what is often designated the individual's ABO blood group. The four main phenotypes in the ABO system are: A, B, AB and O. Soon after its discovery the A phenotype was subdivided into A_1 and A_2. The division was made upon the observation that not all group A reacted equally strong with polyclonal anti-A [43]. Similarly, the AB phenotype is also be divided into A_1B and A_2B. It would have been less obvious to make the same discovery today, given that the monoclonal anti-A reagents used for routing typing nowadays all given very strong (4+ or at least 3+) reactions in tests independent of A_1 or A_2 phenotype. Table 5 shows the occurrence of these phenotypes in different populations.

Table 5 Blood group phenotypes in different populations (% occurrence) [33].

	Caucasians*	Blacks*	Asian*	Hispanic* (Mexican)
A_1/A_2	33/10	19/8	27/rare	22/6
B	9	20	25	13
O	44	49	43	55
A_1B/A_2B	3/1	3/1	5/rare	4/rare

* Within these main populations, large variation occurs between ethnic subgroups.

A_1 and A_2, is the differences merely quantitative?
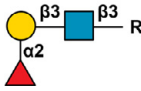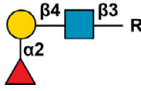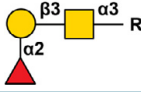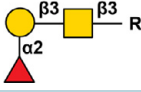
The short answer is no. The early division between A_1 and A_2 was based on reactivity with polyclonal antibodies. However, as alluded to above, modern monoclonal anti-A are not diluted in a way to optimise differentiation between the two sub-phenotypes in routine blood typing as this is usually of little clinical relevance for blood transfusions. A distinction can be made using anti-A1 lectin extracted from *Dolichos biflorus* which will, at a certain dilution, agglutinate A_1 but not A_2. There are also monoclonal reagents claiming to do the same. A notable example when it can be of clinical importance to subtype for A_1 vs. A_2 is in solid organ transplantation, particularly in the setting of ABO-incompatible kidneys. A recent study found serological typing to be inferior to ABO genotyping and thus a potentially safer method to confirm ABO-incompatible A_2-to-O transplantations [44].

The two sub-phenotypes are so common they account for more than 99 % of all group A individuals and in Caucasians the frequencies are ~80% vs. ~20% in favour of A_1 [33]. Estimating the number of A antigen epitopes per RBC is quite challenging but it has been approximated to be in the range of 1 million on the A_1 phenotype [45], other estimations claim at least 2-3 times more [46].

The obvious quantitative difference is that the A_2 phenotype expresses four to five times less A antigen on the RBCs [47]. However, several other differences of a more qualitative nature have been shown. For instance, different GTAs are involved in synthesis of the A phenotypes. The *A²* allele [*ABO*A2.01*] [48] carries a c.1061delC SNP [49] [33], which abolishes the normal stop codon resulting in a GTA with an additional 21 aa. This GTA is less efficient at elongating the H precursor, having a pH optimum of 7-8 (compared to 5.6 for A1-GTA) and the GTA has a higher K_m (Michaelis-Menten constant) i.e. it has lower affinity for its acceptor substrates [50]. The iso-electric point also differs between A_1 and A_2 GTs [51]. Thus, there is no doubt that there are also qualitative differences underlying the two variants of A when it comes to genes and enzymes.

Furthermore, there is some controversy regarding the difference in the ability to use the different peripheral core structures (Table 6) between the two enzyme variants. Clausen et al. demonstrated the presence of A type 3 and Globo-A (Figure 3) on A_1 RBCs with little or no amounts on A_2 [52, 53] and argued that this is a qualitative difference. However, Svensson et al., after having accounted for the overall decreased quantity of A antigens in the A_2 phenotype (i.e. 5x less), found equal amounts of A type 3 in both phenotypes but no Globo-A (A type 4) in A_2 [54]. This could suggest that the anti-A1 reactivity might be directed towards and as of yet uncharacterised conformational epitope on Globo-A, since A type 2, A type 3 and Globo-A have the same immunogenic GalNAcα1,3(Fucα1,2)Gal-terminal trisaccharide configuration as A type 4.

Table 6 . Blood group A and B peripheral precursor structures [20].

Peripheral core type	Structure	Predominantly found
H type 1 (glycoproteins and lacto-series GSL)	Gal—β3—GlcNAc—β3—R, Gal α2 Fuc	Secretions, plasma, endodermal tissue Small amounts can be adsorbed onto RBCs in the presence of the secretor (functional *FUT2*, *Se*) gene
H type 2 (glycoproteins and neolacto-series GSL)	Gal—β4—GlcNAc—β3—R, Gal α2 Fuc	Ecto- and mesodermal tissue Main type on RBCs
H type 3 (neolacto-series GSL)	Gal—β3—GalNAc—α3—R, Gal α2 Fuc	O-linked mucin type Also on RBCs, the precursor to A type 3 (repetitive A)
H type 4 (globo-series GSL)	Gal—β3—GalNAc—β3—R, Gal α2 Fuc	Kidneys Also on RBCs, the precursor to Globo-A

Only the terminal trisaccharide is shown, "R" represents the rest of the glycan. Monosaccharides are as follows: ●Glucose (Glc), ●Galactose (Gal), ■*N*-acetylglucosamine (GlcNAc), ▪ *N*-acetylgalactosamine (GalNAc) and ▲ Fucose (Fuc) in accordance with SNGF.

Other weak phenotypes

There are several other less frequent phenotypes associated with the ABO system. They all show weakened expression of A and/or B antigen and are collectively referred to as: A_{weak} or B_{weak}. Each group can be unfolded into several subgroups (e.g., A_3, A_x, A_{el}, A_m and B_3, B_{el} and B_m) based on their serological traits (and later also shown to have different genetic backgrounds). A few select weak subtypes are shown below as examples in Table 7, modified from the AABB Technical Manual 20th ed [15].

Table 7 Serological reactions in selected A weak and B weak phenotypes.

Phenotype	Forward typing				Reverse typing		Saliva (secretors)
	Anti-A	Anti-B	Anti-A,B	Anti-H	A1 RBCs	B RBCs	
A_1	4+	–	4+	–	–	4+	A, H
A_2	4+	–	4+	2+	–/2+	4+	A, H
A_3	mf*	–	mf*	3+	–/2+	4+	A, H
A_x	–/+	–	1+/2+	4+	–/2+	4+	H
A_{el}	–**	–	–	4+	–/2+	4+	H
A_m	–/w	–	–/w	4+	–	4+	A, H
B	–	4+	4+	–	4+	–	B, H
B_3	–	mf*	mf*	4+	4+	–	B, H
B_{el}	–	–	–	4+	4+	–	H
B_m	–	–/w	–/w	4+	4+	–	H

Agglutination reactions are graded –, w (weak), 1+, 2+, 3+, 4+, and mf (mixed field). Results before and after "/" sign should be interpreted as variable reaction strength.
* these reactions are characterised by one or two large agglutinates in a field of free cells.
** positive reactions following adsorbtion with anti-A

Other phenotypes that fall into the A_{weak} and B_{weak} categories are the B(A) phenotype and the cis-AB phenotype. B(A) is characterised as very weak A expression on B RBCs [15, 55] wheras the cis-AB phenotype involves an ABO-GT that can utilise both A- and B-specific nucleotide sugars in a more equal fashion [56]. The underlying genetic cause for these phenotypes will be briefly discussed below but is not a primary focus of this thesis.

Altered or acquired phenotypes

Pregnancies and malignancies can alter the ABO phenotype in some cases resulting in discrepancies between phenotype and genotype [12, 57, 58]. This can be due to loss or gain of expression, as well as altered expression. One case of altered expression is seen in the acquired B phenotype or phenomenon. This was first described when a weakly reactive B antigen was observed in a patient

previously typed as group A_1 [59]. Acquired B has also been reported in a case of A_2 [58] Bacterial enzymes have been shown to cause conversion of the immunodominant terminal blood group A sugar *N*-acetylgalactosamine, into galactosamine, which highly resembles the blood group B sugar galactose, through deacetylation [60, 61]. This phenotype is often associated with patients suffering from diseases where the integrity of the gastrointestinal wall has been compromised [62]. Serologically these patients' RBCs show strong agglutination with anti-A and weak reactions with certain (no longer approved/licensed) monoclonal and most polyclonal anti-B. In an emergency situation they could be mistyped as group AB, a mistake that can be fatal [63]. Reverse typing would however reveal strong anti-B in the plasma unless the patient is immunocompromised. Clearing of the underlying bacterial infection reverses the acquired B phenotype.

Unravelling ambiguous typing results

Nowadays genetic approaches are frequently used to predict phenotypes within other blood group systems. This is not routinely the case regarding the ABO system where we still heavily rely on basic serological methods for all routine blood typing. Not only are these methods fast and cheap, but they are also remarkably accurate at picking up discrepancies in forward and reverse typing as seen in Paper II and Table 7. Because of the highly polymorphic nature of the *ABO* locus, with more than 200 acknowledged *ABO* alleles [64] and many more reported, there is a real risk of misinterpreting genetic data to predict a common A or B phenotype if crucial variants are not picked up by the genotyping method used. Conversely, another type of risk occurs due to nonsense mutations that appear to stop the reading frame prematurely. In such a case predicting the resulting seems logical if the affected allele is combined with a common *O* allele. This could easily have been the case with the single nucleotide variants (SNVs) discussed in Paper II, if *only* genetic data were available [65]. If blood donors were mistakenly assigned blood group O based on genotype it could have dire consequences as their group "O" RBC units would be used in an emergency scenario to patients, regardless of ABO blood group, without further testing.

It is rarely necessary to determine a patient's exact subgroup beyond A_{weak} or B_{weak}, in a clinical setting. However, ABO discrepancies regarding blood donors should be thoroughly investigated. Here, it is for instance important to be able to distinguish whether a mixed field reaction is from a chimera (double population) or from an A_3 subgroup. In fact, any ABO typing discrepancy in blood donors should be investigated thoroughly, including *ABO* genotyping or *ABO* gene sequencing, combined with sensitive flow cytometry [57] to detect small subpopulations of cells expressing A or B that might have been missed by routine serology but could explain the lack of anti-A or -B, respectively.

When it comes to unravelling ambiguous typing results due to hereditary weak phenotypes, transfusion-related mixed field (mf) reactions or chimeras after transplantation, genotyping together with flow cytometric analysis have become valued tools [66]. Flow cytometric evaluation of RBCs has demonstrated very precise flow patterns, in some cases quite specific for a certain subgroup, thus being able to predict genotype [57, 65].

The polymorphic *ABO* locus

The three-allele model

In 1910, a mere decade after Landsteiner's seminal discovery, Dungern and Hirszfeld showed without a doubt that the ABO blood groups were inherited traits after having performed extensive family studies [43]. They also showed that the traits were co-dominantly inherited and proposed the following genetic explanation: two independent pairs of genes *A* and the recessive *a* as well as *B* and its recessive counter *b*, likely located on different chromosomes, were responsible for the expression. Fourteen years passed before a German mathematician came up with the three-allele model after having re-examined the original family data [67]. Three *ABO* alleles at the same locus resulted in six genotypes: *AA*, *AO*, *BB*, *BO*, *AB* and *OO*. This multiple allele model has been extensively reviewed [68, 69] and has later had to be expanded significantly.

Common and not so common ABO alleles

During the 1950's the biochemical characteristics of the ABO blood group antigens were thoroughly investigated and in fact realised to be of glycan nature. Based on collective efforts by several groups, the structure of the H antigen on group O RBCs was established as the precursor for A and B antigens and the terminal immunodominant trisaccharide sequence for the A and B antigens were shown to be GalNAcα1,3(Fucα1,2)Gal- and Galα1,3(Fucα1,2)Gal-, respectively [12, 70, 71]. Yet, the identity of the *ABO* locus remained unknown.

Not until sufficient amounts of a possible candidate A transferase-protein were purified from lung tissue [72] could the unravelling of the *ABO* locus and its alleles begin. This massive task was undertaken by Yamamoto et al., who in 1990 reported the successful cloning of cDNA corresponding to the *A* allele [73].

As the *ABO* alleles have been extensively reviewed [74] [75] and there are several excellent resources available online [64, 76, 77] as well as an upcoming ISBT data base (not yet official), the following segment is far from compete and the purpose is solely to highlight a few fundamental concepts.

The *ABO* gene spans more than 18,000 base pairs (bp) and consists of at least seven exons [78, 79] which encompasses the entire coding region. The total length

of exon 1-7 is 1062 bp which translates to a 354 aa long protein sequence (Figure 7). Most of the globular C-terminal domain, which contains the catalytically active site (Figure 4) is encoded by exons 6 and 7 (which are the two largest 135 and 688 bp, respectively).

As stated, there are three main alleles: *A*, *B* and *O* encoded by the *ABO* gene. The *A* allele or more precisely the *A¹* allele [*ABO*A1.01*] [73] is considered the consensus allele, meaning it is the allele that all other variations of *ABO* alleles are compared to. It encodes glycosyltransferase A (GTA), 1,3-α-*N*-acetylgalactosaminyl-transferase (α1,3GalNAc-T), which catalyses the transfer of GalNAc from the donor sugar uridine diphosphate- (UDP-) GalNAc to the terminal Gal of the H antigen precursor in an alpha 1-3 linkage (Figure 6). The *B* allele [*ABO*B.01*] encodes glycosyltransferase B (GTB) 1,3-α-galactosyltransferase (α1,3Gal-T). Which enables the transfer of Gal from UDP-Gal to the terminal Gal of the H antigen precursor in an alpha 1-3 linkage. This specific linkage is independent of which H core structure is available (Table 6).
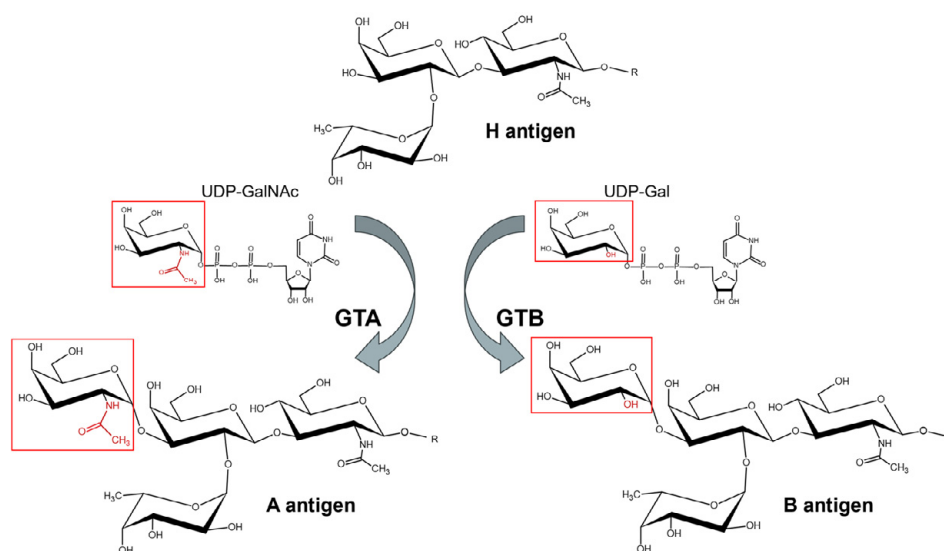
Figure 6 Illustration of the biosynthetic actions of the GTA and GTB on the H antigen precursor

It is remarkable that the difference between the common *A¹* and *B* alleles is just seven single nucleotide polymorphisms (SNPs). Three are silent c.297A>G, c.657C>T and c.930G>A i.e., they do not alter the aa sequence of the product. Only four (!) SNPs are missense mutations (causing aa substitutions) [39]. Two of them, c.796C>A (p.Leu266Met), and c.803G>C (p.Gly268Ala), are crucial in defining donor substrate specificity of the GTs [80], while the other two, c.526C>G and c.703G>A, have been implicated in turnover rate and acceptor

binding [81]. However, c.703G>A is also involved in A vs. B-recognition as shown in the B(A) phenotype that expresses a little more A antigen than expected due to a *B* allele with the A-specific c.703A variant [82]. An *ABO* allele which encodes a GT that does not discriminate between UDP-GalNAc and UDP-Gal is a *cisAB* allele. When inherited together with a O allele the expression of both A and B antigens is low. The most common *cisAB* variant is *ABO*cisAB.01*, where an A^1 allele has the *B*-specific c.803G>C polymorphism.
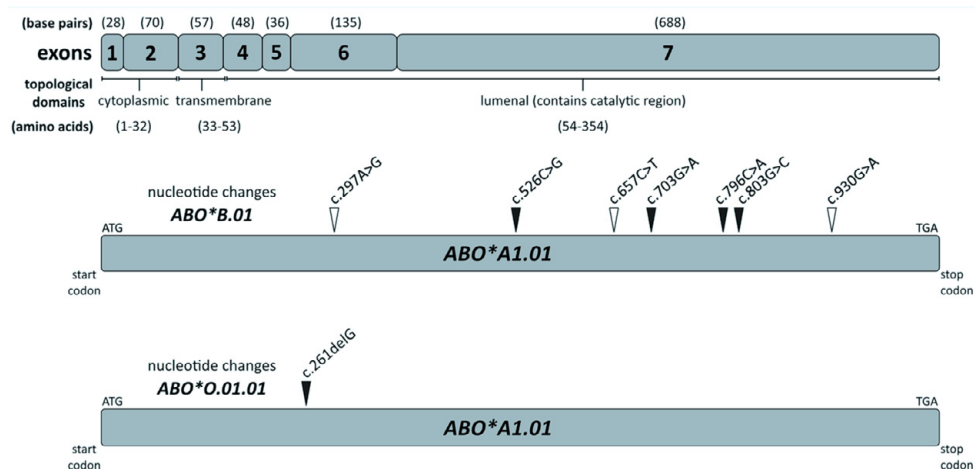
Figure 7 Schematic overview of the coding region of the *ABO* allele (exons 1-7). Regions of the gene encoding different protein domains are emphasised. Arrowheads mark the nucleotide (nt) changes between the consensus *ABO*A1.01* and **B.01* (black being crucial in defining the donor substrate-specificity). The crippling c.261delG variant of the **O.01.01* allele is signified by the black arrowhead on the A^1 back bone.

Currently, there are more than 200 *ABO* alleles included in the official ISBT allele tables [64]. This is however, a gross underestimation and a full inventory is underway. Several of these allelic variants exhibit normal expression of A and B antigens suggesting that SNPs that have only minor impact on GT activity are not uncommon at the *ABO* locus. On the other hand, it has been noted that the aa affected in A weak or B weak phenotypes are often invariant or evolutionarily conserved in the GT6 family of enzymes, to which GTA/GTB belong [83].

The *O* allele does not yield a gene product with enzymatic activity. Hence, the H antigen precursor is left unmodified. *O* alleles can be deletional or non-deletional [69]. The vast majority are deletional, meaning they carry c.261delG, which causes a frameshift (fs) in the ORF and results in a premature stop codon (p.Thr88Profs*31) and a severely truncated protein (if any gene product is expressed at all) [39]. The two most common ones are *ABO*O.01.01* (previously known as O^1 or *O.01*) and *ABO*O.01.02* (O^{1v} or *O.02*) [80]. The ISBT so far

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recognises more than 40 deletional alleles, all based on c.261delG [64]. There are some other principally interesting "deletional" *O* alleles, the least uncommon of which is *ABO*O.16*. It does not carry the signature c.261delG variant but instead, exons 5-7 are missing due to a 5821-bp deletion [75]. It appears as if this latter deletional O allele is the third most common among individuals of African descent. Yet another different deletional O allele has a 2169-bp deletion from intron 1 to intron 3 but has not received an official ISBT name [84]. Finally, a 24-bp deletion was reported to affect exon 5 and the adjacent splice-site in a 30-member family, 16 members of which inherited the variant allele. However, even if the authors dub this an O allele, they also note inconclusive adsorption/elution results and trace expression of A following transfection experiments in a cell line [85].

The largest group of non-deletional *O* alleles (keeping in mind that they only account for less than 3-5% of the O alleles in some population groups) belong to *ABO*O.02.01-04* (previously O^2 or *O.03*). They share c.802G>A (p.Gly268Arg) which abolishes GT activity [86, 87] by altering the donor substrate binding site [88]. Interestingly, also about this group of *O* alleles has there been a longstanding debate whether they actually express small amounts of A or not [89] *ABO*O.02.04* has even been given an alternative name when weak A expression was detected in one case, *ABO*AW.08* [90]. To complicate things further, it has been conclusively found that the RBCs from individuals with c.802G>A do not express A but have a lower titre of anti-A [89].

Apart from the well characterised *ABO*O.01.xx* and *ABO*O.02.xx* alleles there are seven other principally different *O* alleles acknowledged by the ISBT: *ABO*O.03/O.04.01-02/O.05/O.06/O.08/O.10* [89, 91, 92]. Another two (*ABO*O.09.01-02*) share the same sequence as the *ABO*AW.31* cluster of A^x hybrid alleles and are therefore questionable. As discussed in Paper II, they have all received *O* allele status despite sharing two common serological features: (1) none the RBCs gave positive reactions with anti-A, (2) strong plasma reactions were absent with A_1 test RBCs. Since this implies that there may be very weak expression of A in these cases (please note that these O alleles are all based on the A allelic backbone). This does prompt a discussion regarding a unified definition of an *O* allele and what serological criteria should be met in order before novel variants are considered *O* alleles.

The GLOB blood group system and its close relative P1PK

The P antigen, in some circles better known as globoside or Gb4, represents the most abundant neutral GSL on the RBC [93]. Together with the PX2 antigen [94] and the recently added ExtB [64, 95] they make up the GLOB blood group system.

Apart from erythroid cells, the P antigen is also present on other cell types and tissues, characterising it as a histo blood group. The antigen is well developed at birth [33] and highly expressed on the foetal portion of the placenta [96]. The PX2 antigen has been shown to be present on RBCs and in a wide variety of normal tissues as well as tumorous tissue [97]. The presence of ExtB has so far only been demonstrated on GSL isolated from RBC membranes (as reported in Paper III).

The gene responsible for P, PX2 and ExtB (the latter only present in group B and AB individuals) expression, *B3GALNT1*, is located on 3q26.1 and encodes a 331 aa long 1,3-β-*N*-acetylgalactosaminyltransferase, (β1,3GalNAc-T1, EC 2.4.1.79), shown to catalyse the addition of GalNAc in the β1-3 linkage to their respective precursors, P^k (also known as Gb3), paragloboside (nLc-4) and the B antigen, respectively (Table 3). The gene consists of at least 5 exons and spans more than 21 kilo base pairs (kbp). The entire open reading frame (ORF) is found in exon 5 (Figure 8). Inactivating or nonsense mutations in the ORF, like the one described in Paper I, leads to the absence of functional P-synthase which prevents the GSL to elongate beyond the P^k antigen [98] in the globo series of GSLs but also prevents PX2 and ExtB from being synthesised in the neolacto series (Figure 3).
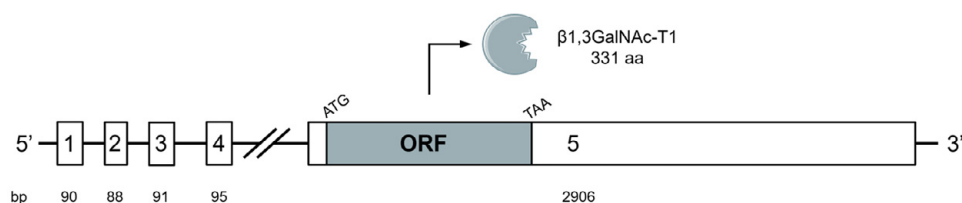
Figure 8 Schematic representation of *B3GALNT1*. The gene spans 21,467 bp and consists of at least five exons. The entire ORF is in exon 5 and codes for the 331 aa long P-synthase (β1,3GalNAc-T1).

Globoside-deficient RBCs become strongly positive for P^k since the precursor of P is left unaltered. Although such P-negative, strongly P^k-positive individuals are very rare, present in less than 0.01% of the population [33], as patients they present great challenges in the field of transfusion medicine due to naturally-occurring antibodies. The P^k phenotype is further divided into P_1^k and P_2^k based on the presence or absence of the P1 antigen, which is independent of *B3GALNT1* but instead governed by *A4GALT* (see below).

The most potent specificity is anti-P, given its ability to bind and activate complement, potentially causing serious intravascular haemolytic transfusion reactions. Furthermore, women with the rare Pk phenotypes have a higher incidence of early and recurrent spontaneous abortions due to cytotoxic attack by anti-P on the globoside-rich placental tissue [96, 99, 100]. While anti-PX2 is present in plasma from both P_1^k and P_2^k individuals [94], the clinical significance is still unclear. However, it is recommended to avoid transfusing PP1Pk-negative

blood of the p phenotype to these patients if blood of the P^k phenotype is available, since p RBCs have the highest expression of PX2 [94]. The possible clinical implications of anti-ExtB (Paper III) will be discussed further down.

Whilst the P^k antigen is structurally related to the P antigen it actually belongs to a different blood group system altogether, namely the P1PK system. Two other antigens, P1 and NOR, also belong to this blood group system but since they are not the focus of this thesis they will not be discussed in much. The P^k antigen is synthesised by the addition of Galactose (Gal) in the α1-4 linkage to the Gal residue of lactosylceramide. This reaction is catalysed by 1,4-α-galactosyltransferase (α1,4Gal-T, EC 2.4.1.228) encoded by the *A4GALT* gene located on chromosome 22q.13.2 [101].

Nonsense mutations in *A4GALT* result in the p phenotype (formerly known as the Tj(a-) phenotype), in which the P, P^k and P1 antigens are all lacking and the corresponding antibodies are found naturally-occurring in plasma [33].

Use of blood in modern medicine

225 units transfused every minute worldwide

Transfusion of red blood cells has become a relatively common procedure. According to the World Health Organisation (WHO) 118 million units are transfused worldwide every year [102], equivalent of 225 units every minute. The corresponding number in Sweden is around 358,000 units per year [103]. There are several reasons for receiving blood transfusions, e.g., surgery and trauma, aggressive chemotherapy and pregnancy-related complications as well as several haematological malignancies and haemoglobinopathies. Patient blood management is an essential part of health care systems around the world to optimise the use of blood, nevertheless donor and patient blood types must be carefully matched before transfusion to avoid unfavourable outcomes.

The minimal change in terminal sugar between blood group A and B, *N*-acetylgalactosamine versus galactose, has a great impact on antigenicity (Figure 9) and downstream clinical consequences of transfusion against the ABO barrier.
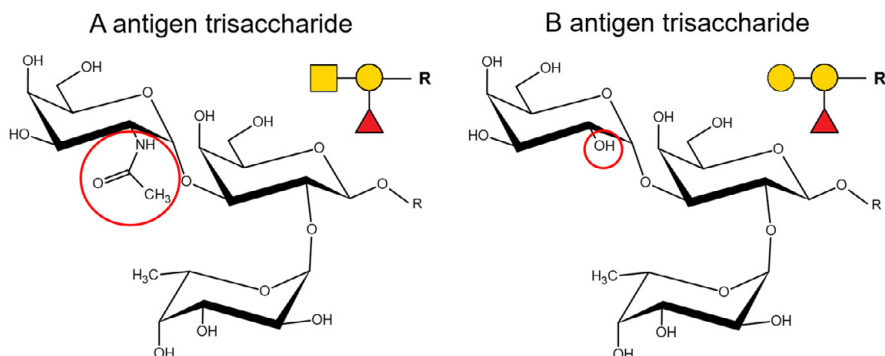
Figure 9 Structure of the terminal immunogenic trisaccharides in blood group A vs. blood group B. The different functional groups in GalNAc and Gal are highlighted in red circles.

Naturally-occurring antibodies directed towards the epitopes highlighted in Figure 9 develop spontaneously within the first few months of life and reach adult titres within 5-10 years. Although no conclusive evidence of their regulation has been put forth, they are speculated to arise from exposure to microbes [104]. They are predominantly immunoglobulin M (IgM) in group A and group B individuals even if small quantities IgG antibodies can be detected. Interestingly, IgG antibodies are the major isoform in many group O individuals [105], although IgM is virtually always present. The AB(H) antibodies are highly reactive and transfusion of ABO-incompatible blood can be associated with acute intravascular haemolysis and can

be fatal [106, 107]. Similarly, transplanted ABO-incompatible solid organs can undergo hyperacute humoral rejection, mainly due to ABO antigens displayed on endothelial cells These severe reactions are primarily due to antigen copy number on the RBCs, large natural variations in AB(H) titres and the AB(H) antibodies' ability to activate complement. According to haemovigilance monitoring systems, complications due to ABO incompatibility are among the most common adverse effects of transfusion [108-110].

Since blood group O RBCs can readily be transfused to all patients regardless of their ABO group, without risking an ABO-dependent haemolytic transfusion reaction, it is commonly referred to as "universal" blood (but should perhaps rather be called ABO-universal) (Figure 10).
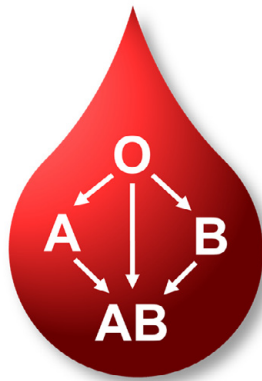
Figure 10 Transfusion compatibility within the ABO blood group system. Devoid of A and B antigens, blood group O RBCs and platelets can be transfused to patients of all other ABO blood groups regardless of their ABO antibody status.

In a clinical setting these blood units (also preferably negative for RhD, RhC, RhE and Kell/KEL1 if applicable to emergency situations) are frequently used in situations where the patient's blood group is unknown in the case of time-sensitive traumas or ambiguous blood grouping results, e.g. due to weakly expressing ABO subgroup phenotypes. However, due to this overconsumption of group O and also discrepancies in frequency between the recipient and blood donor population this leads to a risk for shortages of group O RBC units in many parts of the world. Furthermore, handling four main ABO groups, in addition to the two main RhD groups, results in a considerable fragmentation of the blood inventory. This gives rise to a major logistic challenge, especially since blood component production is often centralised nowadays and hospitals rely on transports delivering appropriate quantities of units of the different groups. Simultaneously, some units of certain blood group may risk outdating at a smaller hospital and may have to be sent back to the main hub etc.

In essence, access to more group O blood would solve many problems and the prospect of man-made group O-like blood is therefore intriguing to say the least.

There are several strategies in play to try to achieve this, including masking the RBC antigens using polyethylene glycol (PEG) [111] or creating haemoglobin

carrying nano vesicles [112, 113]. There have also been attempts to use haemoglobin from outdated units or even animal blood to realise haemoglobin-based oxygen carrier (HBOC) solutions for (mainly) substitution of acute blood loss, which has resulted in several clinical trials with polymerised and pegylated haemoglobin [113, 114]. All these approaches have encountered more or less unexpected challenges. In the case of PEG stealth RBCs, different molecular structures of PEG have been tried and hiding most blood group molecules seems feasible but a sizeable fraction of healthy individuals appear to have naturally-occurring antibodies against PEG [111, 115-117]. Infusing free haemoglobin results in adverse effects like changes in blood pressure and kidney damage. Polymerization and pegylation of the haemoglobin molecules have increased the safety profile but clinical trials have still suffered problems with adverse events [118]. A promising approach involves differentiating and culturing haematopoietic stem cells in bioreactors [119-123], yet the yield is still insufficient for this to be a practical option. for more than a limited number of units, e.g. rare blood or for chronically transfused, immunised patients to increase the transfusion interval. In addition to these innovative prospects, there is yet another intriguing possibility, involving enzymatic modulation of the RBC surface.

Enzyme Conversion to group O (ECO)

Proteolytic enzymes such as papain, bromelain, and trypsin are widely used by serological reference laboratories to aid in routine antibody screening and antibody identification [38], by altering the protein structures on the RBCs surface. ABO antigens on the other hand are synthesised by the stepwise addition of specific monosaccharides to growing glycan chains, facilitated by GT enzymes. What if another class of enzymes e.g., glycosidases could be used to remove the terminal antigenic structure from the ABO antigens, thereby reversing the job done by the ABO-GTs?

Enzymatic removal of the terminal GalNAc and Gal from A and B respectively would reveal the underlying universally accepted H antigen. Early work indicated that enzymes with such capabilities existed in nature [124].

Pioneering work was done by Goldstein et al. in the 1980s , successfully converting group B RBCs to group O using an α-galactosidase extracted from green coffee beans , serving as an exoglycosidases, i.e. cleaving the terminal sugar in the glycan chain [125]. This was the starting point for the "Enzyme Conversion to group O" (ECO)-project. The conversion required large amounts of enzyme and because the enzyme had a low pH optimum (pH 5.7), the conversion conditions were certainly suboptimal for RBCs. However, RBC structure and viability was maintained and phase I clinical trials showed that they were well tolerated and exhibited normal half-life [126-128], albeit a small increase in anti-B titre in blood group O volunteers was observed, suggesting remaining B antigens could trigger the immune system. Later, a phase II clinical trial came to the same conclusions, increased anti-B titre but no ill effects due to infusion of B-ECO (RBCs converted from blood group B) were recorded. Even though B-ECO RBCs could not be agglutinated by monoclonal anti-A and anti-B, crossmatch studies surprisingly revealed weak agglutination reactions when mixed with group A and group O serum (observed in 20% and 40% of cases, respectively) [129]. Regardless, it was concluded that group B RBCs could be converted to B-ECO RBCs in a way compatible enough for transfusion, but such conversion practices would not become widespread until efficient A-cleaving enzymes were identified.

The coffee bean α-galactosidase belonged to the CAZy family GH27 and researchers, spurred on by the B-ECO success, began exploring the closely related GH36 family in search of a suitable α-*N*-acetylgalactosaminidase for group A conversion [130]. Disappointingly, none of the investigated α-*N*-acetylgalactosaminidases could achieve complete conversion of group A RBCs.

The next leap in the "ECO-project" was realised by Liu et al. by using a novel approach in their search for glycosidases. New groups of α-*N*-acetylgalactosaminidases and α-galactosidases, which could function at pH 7, were found buy tediously screening a library of around 2500 fungal and bacterial

lysates for hydrolysing activity on A and B substrates [131] substrates longer (and thereby more biochemically similar to the RBC glycans) than those used in the past. Strikingly, this impressive effort resulted in the selection of one candidate exoglycosidases from a new bacterial exoglycosidase from *Elizabethkinga meningosepticum*, representing a novel enzyme family, GH109, with ability to cleave the α1,3GalNAc off A antigens. In parallel, another bacterial exoglycosidases from *Bacteroides fragilis* was discovered to represent yet another enzyme family, GH110, and have the ability to cleave α1,3Gal from the terminal end of the B antigen. These enzyme candidates were investigated in great detail and were designated A-zyme (EC 3.2.1.49) and B-zyme (EC 3.2.1.22). In combination with a low-ionic strength conversion buffer (reducing the net charge between the enzyme and the RBC surface [132]) these enzymes could convert whole units of blood group A, B and AB to group O [131] at pH 7 using a fraction of the enzyme quantities previously reported (300 μg/mL RBCs or ~60mg/unit for group A and 10 μg/mL of <2 mg/unit for group B). The ECO RBCs were thoroughly evaluated using an array of increasingly sensitive methods [131] reviewed in [133]. Only trace amounts of A and B antigens could be detected with the most sensitive methodology. Yet, crossmatch results obtained with this new generation of A-ECO and B-ECO RBCs, mainly with group O plasma were still in the range of those previously reported for B-ECO [129] and worse for A-ECO. Later efforts by other groups have either resulted in similarly poor crossmatching results [134] or not reported any crossmatch data at all [135]. This enigmatic reactivity, in the absence of detectable A or B antigens, remains unexplained and while we offer one plausible explanation in Paper III for B-ECO, the situation regarding A-ECO is more complex and crossmatch results are stronger on average for A-ECO than B-ECO and in a larger proportion of random healthy donors.

Thus, converting group A RBCs has proven more challenging, most likely due to the increased biochemical complexity of this antigen. Extended structures based on the A antigens (H type 3 and A type 3) were described already during the mid-1980s by Clausen and Hakomori [52, 53] but have never achieved formal blood group status by ISBT. Yet another structure, Gal-A is the intermediate step between the traditional A antigen and H type 3 (Figure 3). In Paper V we investigate the consequences of converting also these extended structures, which have in effect protected the underlying A antigens from being fully converted since the extended structures are resistant to treatment with the previously tested A-zyme(s).

The possibility of utilising endo-glycosidases and by so removing the entire antigenic trisaccharide from A and B antigen(s) in the hope that a single enzyme could be used for conversion of all blood groups (A, B and AB) has also been investigated [136-138] but abandoned due to concerns regarding the impact of the exposed GlcNAc (Figure 3).

The launch of metagenomic analyses [139-141] constitutes a new landmark regarding further exploration of possible glycosylhydrolases (often abbreviated GH when different families are categorised but otherwise also known as glycoside hydrolases or simply glycosidases) for the removal of antigenic blood group epitopes. Metagenomics is defined as the genetic analysis of microbial genomes contained within an environmental sample. It can provide information regarding genomic linkages between function and phylogeny (evolutionary history) for uncultured organisms. Bacteria residing in the gut are known to digest the AB(H) antigens on glycans in the mucin lining the gut, as reviewed by Tailford et al. [142]. It has also been shown that there is a correlation between human microbiome content and blood type [143]. In light of this Rahfeld et al. set out to apply metagenomic screening on microbes in the human gut in search of A- and B-degrading organisms [135]. By screening a metagenomic library generated from a donor of AB blood type they could identify a particularly promising pair of enzymes capable of specifically cleaving the A antigen [135]. These enzymes work in concert where the first enzyme, a GalNAc deacetylase, specifically targets the A antigen and generates an intermediate terminal galactosamine (GalN) and the second enzyme, a GH36 family α-galactosaminidase, acts on the GalN, resulting in the H antigen. The conversion from group A to group O is so efficient that when benchmarked it requires 15-30 fold less enzyme and can be performed in standard buffers (i.e. phosphate buffered saline, PBS). As little as 1 mg is predicted to convert one unit of group A RBCs. There are however no indications of any crossmatch reactions performed between these "two-step"-enzymatically converted A-ECO RBCs and plasma of any ABO group. In later papers, the same investigators both reviewed the ECO topic [144] and also attempted conversion of lung tissue with the goal to facilitate transplantation across the ABO barrier [145].

Present investigation

Paper I

Aim

The aim of this project was to identify and isolate naturally-occurring antibodies from blood samples of Thai origin to use as a reagent for the project defined in paper III. Amplify and sequence the open reading frame of *B3GALNT1* to investigate and understand the sample's genotype in correlation to the reported phenotype.

Background

The blood sample in question came from a woman with the rare P_k phenotype, suffering from multiple miscarriages due to naturally-occurring anti-P in her plasma. This cytotoxic antibody attacks the P-rich foetal portion of the placenta [96, 99], often terminating pregnancy already within the first trimester. The P antigen, carried on globoside [100], belongs to the GLOB blood group system and is synthesised by 3-β-N-acetylgalactosaminyltransferase (β1,3GalNAc-T1 or P synthase) this GT catalyses the addition of GalNAc in the β1–3 linkage to the terminal Gal of the Pk precursor. Inactivating mutations in *B3GALNT1* are known to lead to the absence of functional P synthase [146].

Main findings

The sample's genotype was evaluated by amplifying and sequencing the *B3GALNT1* coding region. This revealed homozygosity for a previously unreported nucleotide substitution at c.420T>G, introducing a premature stop codon, p.Tyr140Ter [147], which is predicted to severely truncate the P synthase rendering it non-functional. The proposita's sister, sharing the same tragic medical history of multiple miscarriages, was also found to be homozygous for this nonsense mutation. Inactivating, rare mutations are often found to be regional in distribution. Thus, a cohort of healthy Thai blood donors (n=384) was screened for the novel substitution using a PCR allele-specific primer assay (PCR-ASP) developed by me. One donor was screen positive and, following confirmatory amplification and sequencing, shown to be heterozygous for the unreported variant. The novel null allele of the GLOB blood group system was given the official name *GLOB*01N.13* and added to the 12 already acknowledged by the ISBT, all reported by the Olsson group at the Division of Hematology and Transfusion Medicine at Lund University.

Paper II

Aim

The aim of this project was to investigate the impact of seven nonsense mutations identified in novel *ABO* alleles found in blood samples showing ambiguous ABO serological results.

Background

Despite being discovered over a century ago the ABO blood group system continues to pose challenges, due to its highly polymorphic locus [68, 69], and it remains one of the most complex blood group systems to this day. Polymorphisms (SNPs and SNVs) at the *ABO* locus can underly weak A and/or weak B phenotypes. Variants resulting in nonsense mutations i.e., direct stops codons and fs mutations resulting in early termination of the ORF, are expected to result in the O phenotype. That is however not always the case. Thirteen blood group samples were referred to the Nordic Reference Laboratory for Genomic Blood Group Typing (NRLGBT) for further investigation due to ambiguous blood typing result. The initial routine and in some cases extended serology performed by the referring centres showed unexplained discrepancies between forward and reverse typing i.e. antigen detection on the RBCs did not match antibody reactivity in the plasma. Flow cytometric evaluation of weak phenotypes has been shown to correlate remarkably well with genotype. Hult and Olsson have shown that many inherited weak ABO phenotypes have very characteristic histogram patterns that can predict the underlying molecular basis of the A subgroup in question with surprising precision [57]. Furthermore, the percentage of antigen-positive RBCs in a sample can also indicate the type of genetic alteration. Hence, a combination of genetic analysis and flow cytometry is increasingly often used in routine practice at reference laboratories to determine the reasons underlying samples with A_{weak} and B_{weak} phenotypes.

Main findings

Our investigation into the aberrant blood typing results observed in 13 samples revealed seven novel *ABO* alleles. All carried nonsense mutations which were predicted to severely truncate the encoded ABO glycosyltransferases. Based on genomic data alone, these variants could all have been thought to represent *O* alleles. However, of the four *ABO*A1.01*-related alleles, flow cytometric analysis revealed expression of A antigen on 10-32% of the RBCs. In line with previous observations, samples from individuals with the same variant displayed remarkably characteristic percentages of A-positive cells: c.102C>A (31-32%, n=2), c.106dup (16-17%, n=3) and c.181_182ins (12-13%, n=2). One variant found even earlier in the coding region c.42C>A showed 10% positive cells (n=1).

The flow cytometric histogram patterns did not resemble or overlap with any of the previously reported A subgroup patterns [57]. Instead, this group of A_{weak} samples all displayed heterogenous A expression, with one large negative or almost negative population along with a fraction of cells (10-32%) with varied expression of A. The expression patterns of B antigen on the X samples with alleles based on *ABO*B.01* was not so striking, however very low levels of B antigen could be observed by flow cytometry (<0.5%), and this was supported by the absence of anti-B in these samples. In general, it was more difficult to differentiate the B expression in these samples from the negative (group O) control RBCs than it was for the A-expressing samples described above.

Our observations with these clinical samples were reiterated by transient over-expression studies in HeLa cells. Once again, A antigen expression could clearly be detected in HeLa cells expressing any one of the four novel variants, and the further from the start codon the mutation was situated, the weaker the A antigen expression observed. These results were mirrored by the variant *B* alleles however, the level of B antigen expressed by the cells carrying the c.496del-insert was not significantly different from mock-transfected HeLa cells.

Together, these results raise perhaps at least two potential mechanisms underlying the observed ABO antigen expression observed despite the ORF-truncating nonsense mutations: one is that there is a low level of translation from a full-length transcript that has "overlooked" the mutation (e.g. due to so-called RNA slippage [148-150]). This mechanism has been proposed for phenotypes such as A_{finn} in which the underlying variant affects a donor splice site, c.374 +4a>g and causes approximately 1-2% of all RBCs to stain positive for A antigen [151]. A second potential mechanism is the use of downstream initiation codons, such that an N-truncated glycosyltransferase with an active enzyme domain is produced despite the nonsense mutation. This has hitherto been shown *in vitro* by Seltsam et al. following the investigation of a similar sample in which they identified a missense SNV in the initiation codon [152]. Similarly, other investigators have shown that N-truncated ABO-GTs can be enzymatically active if the alternative start codon used is located too far downstream. It should be noted though that the presence of N-truncated GTA or GTB have not yet been shown to occur *in vivo*.

Paper III

Aim

The aim of this project was to identify and characterise the biochemistry and serological behaviour of an atypical blood group B-related antigen of suspected GSL nature and the corresponding antibody in human plasma. Identify the gene and glycosyltransferase responsible for the synthesis of said antigen.

Background

Group O was early recognised as a universal donor group, meaning it can be safely transfused regardless of the recipient's ABO type. The aim of the Enzyme Converted to group O - (ECO-) project was to remove the immunogenic terminal structures from the A and B antigen respectively, by enzymatic digestion as a way of making more of this precious blood group.

The α-galactosidase from the GH110 family of exoglycosidases (B-zyme) efficiently removes the terminal Gal from the B antigen and the resulting B-ECO RBCs will type as group O with all monoclonal antisera tested [131]. Yet, when mixed with group O plasma, positive, often weak but sometimes stronger, crossmatch reactions are observed [129]. We hypothesised that this unexplained reactivity was due to the presence of an unknown elongated B structure, resistant to degradation by Bzyme. We also had an interesting patient case supporting our hypothesis [94]. This individual had the exceedingly rare A_1B P_1^k phenotype and was expected only to have naturally-occurring anti-P in plasma according to traditional textbooks. However, following the acknowledgement of x2 glycolipid as a GLOB blood group system antigen, PX2, in 2015, based on work performed in our research group in collaboration with Prof. Susann Teneberg at the University of Gothenburg [153], we also expected anti-PX2 to be present in this plasma. This indeed turned out to be the case, as judged by reactions against various RBC samples of the P-negative p phenotype. Surprisingly though, the plasma reacted stronger with B p RBCs compared to A p and O p. We therefore speculated that this apparently ABO-dependent difference could be related to the hypothesised elongation of the B antigen and that such an extension, in analogy with P and PX2, would depend on the presence of functional P-synthase.

Main findings

In order to investigate the structure on the RBC surface we again collaborated with the Teneberg group in Gothenburg, who had a very large amount of GSLs from blood group B RBC units available for purification. To complement this, we asked for permission to receive outdated, anonymized group B RBC units and collected a second cohort of B-positive RBC membranes also from Lund in order to be able to validate and confirm results in two independent preparations from different cohorts. Mass spectrometric analysis revealed a blood group B hexasaccharide elongated with a terminal HexNAc. After β-*N*-acetylhexosaminidase treatment the peak disappeared, demonstrating that the terminal HexNAc was β-linked. Purified fractions were separated by thin layer chromatography (TLC) and probing with the GalNAc recognising Wisteria floribunda lectin, showed that the terminal β-linked HexNAc was a GalNAc. The novel structure is referred to as Extended B (ExtB). The hypothesised antibody in the patient's plasma was enriched and isolated by a series of adsorptions steps using specific donor RBCs, followed by a final elution step. The specificity of the eluted antibody was assessed by flow cytometry. Group

B p RBCs were positive with the eluate whereas B P_1^k and O p were negative, excluding the presence of both anti-P and anti-PX2 (regular anti-B had been ruled out in an earlier step). B-ECO cells showed weakly positive reactivity. The eluted antibody bound to the ExtB fractions separated by TLC, thus immunoblotting the hypothesized culprit underlying B-ECO crossmatch positivity.

Since individuals with the p phenotype have fully functional P synthase and their RBCs were positive with anti-ExtB, we considered this enzyme as the prime candidate for ExtB synthesis, given that the A_1B P_1^k case above appeared to lack ExtB and presented with anti-ExtB. Co-transfection experiments with P synthase and glycosyltransferase B (GTB) revealed the enzymatic and genetic origin of ExtB, namely that functional versions of both enzymes are required to create ExtB in a group O cell line model. Altogether, we have described a previously unreported extension of the B antigen, resulting in a novel ABO-related glycolipid structure, isolated antibodies from human plasma and demonstrated the enzymatic and genetic origin of this new antigen. Based on the presented evidence, ExtB was accepted by the ISBT Working Party for Red Cell Immunogenetics and Blood Group Terminology as the third blood group antigen (028005) in the GLOB blood group system.

Furthermore, anti-ExtB reactivity with B-ECO RBCs reveals the presence of ExtB on these cells, offering a possible explanation for the crossmatch positive reactions observed between group O plasma and B-ECO RBCs.

Paper IV

Aim

Correct ABO blood group typing is the corner stone of transfusion medicine. Hence, it is crucial that commercial monoclonal anti-B is nonreactive with acquired B antigen on RBCs. This is indeed a requirement, both according to European and American regulatory rules. However, it has been increasingly difficult for reference laboratories and commercial companies to gain access to RBCs from patients who have been verified to type positive for the acquired B antigen. Our objective here was therefore to explore the possibility of creating RBCs of the acquired B phenotype by enzymatic conversion for quality control purposes.

Background

A full ABO blood group assay involves determining the ABO antigens on the RBCs as well as identifying the corresponding antibodies in the plasma. This is achieved using validated and highly regulated antisera as well as blood group specific test RBCs from donors of known ABO type. Interpretation of typing results can be complicated by both inherited and acquired factors. Genetic variants

weakening the expression of A and B antigens are typically identified by routine typing complemented by genomic confirmation but acquired phenotypes may easily go undetected. The acquired B phenomenon was first described in 1959 after a weakly reactive B antigen was observed in patients previously typed as group A_1 [59].

This phenomenon is often associated with patients suffering from malignancies or infections where the integrity of the gastrointestinal wall is compromised. Bacterial enzymes have been shown to cause conversion of the immunodominant terminal blood group A sugar N-acetylgalactosamine (GalNAc), into galactosamine (GalN) which highly resembles the blood group B sugar galactose [60]. Although clearing of the underlying bacterial infection reverses the acquired B phenotype, it is vital that these patients are not falsely typed as AB, especially in emergency situations, as shown by a case of fatal haemolytic transfusion reaction in an elderly patient [63]. By exploiting the first step in a novel approach using a two-step enzymatic digestion system to convert A_1 RBCs to universal group O [135], we realised that the acquired B intermediate could be used for quality control purposes when procuring monoclonal anti-B. Accordingly, the hypothesis underlying Paper IV was that a bacterially-derived, recombinantly produced deacetylase taken from a two-step conversion of A antigen to H, would create GalN-positive RBCs mimicking the otherwise hard-to-obtain acquired B phenotype. This study was the fruitful result of a collaboration with Peter Rahfeld and Steve Withers, who discovered and characterised this enzyme in 2019.

Main findings

Enzymatically modified A1 RBCs reacted 3+ and 1+ with the ES-4 clone (known to react with GalN as well as regular Gal-terminating B antigen) from two different manufacturers, but not with an anti-B reagent from other clones (B84+B97). Loss of reactivity with anti-A was also noted after treatment. Currently four monoclonal anti-B are in use in our local reference laboratory: two are used for rapid typing (tube) and two are in ABO routine typing (gel column agglutination). None detected the exposed acquired B antigen. Group B RBCs retained reactivity with anti-B upon enzyme treatment and group O RBCs remained non-reactive with both anti-A and anti-B reagents. Hence, specific deacetylation of the terminal N-acetylgalactosamine on A antigen of the A_1 RBCs was achieved whilst no signs of other unexpected modifications interfering with ABO typing could be shown. The acquired B phenotype remained stable upon freezing, thawing and subsequent storage in ID-Cellstab, up to a period of four months at $4^{\circ}C$. Crossmatch tests showed 1+ to 3+ reactions in 17 of 26 group A plasmas with the modified acquired B RBCs, but not with their untreated controls. Weak reactions were seen in 2 of 6 group B plasmas. Interestingly, 2 of 3 group AB plasmas reacted strongly with the modified RBCs, underscoring the fact that acquired B is considered to be a polyagglutinable state of RBCs, like NOR-

positivity, Cad and numerous other inherited or acquired variants [154]. Overall, these results suggest that antibodies to the acquired B epitope are prevalent and naturally-occurring.

In summary. By using a recently discovered recombinant, bacterially-derived deacetylase, *Fp*GalNAcDeAc, we demonstrate the feasibility of producing acquired B test RBCs from readily available group A_1 donor cells, enabling a reagent for quality assurance of commercially available anti-B clones. These enzymatically modified RBCs can be frozen and thawed with maintained reactivity, thus providing a practical and reliable source of this unusual but clinically important phenotype.

Paper V

Aim

The primary goal of this study was to identify and characterise bacterial enzymes that could cleave the terminal sugar off not only the two traditional A and B antigens but also target all known extended ABO-related structures, i.e. Gal-A, H type 3, A type 3 and the newly acknowledged ExtB (discovered in Paper III). Two secondary goals were to characterise the resulting ECO-RBCs following treatment with the new enzymes and finally to evaluate if targeting also the unorthodox, extended antigens would affect crossmatching results. The hypothesis for the whole study was that a dedicated mucin-degrading colonic bacterial species like *Akkermansia muciniphila* would have all the exoglycosidases required and that utilising these enzymes would improve the ECO-RBCs produced.

Background

The ECO field has developed slowly over the past 40 years since the seminal paper in Science by Jack Goldstein and colleagues in 1982 [125]. After a flurry of activities up until the year 2000, it took some time for the next leap when two new exoglycosidases families were discovered and reported in 2007 [131]. At that point, it was clear that positive crossmatching results were to be expected even if all detectable A and B was converted to H. There was some activity among Chinese researchers [134] also confirming positive crossmatches but it took until 2019 and the two-step enzyme approach of conversion reported by Rahfeld et al. until it took off again [135] No crossmatch results were published at that point but there was no reason to believe it would be different from the GH109/GH110 approach reported in 2007-2008 [133]. Thus, it took a long time for the field to recover after each shower of optimism that followed upon each discovery during the past four decades. Based on our discovery in Paper III, we first asked the question if there would be any enzymes that could convert ExtB to regular B and if this would improve the outcome of crossmatching. We also knew that the

crossmatches were much more problematic for A-ECO than for B-ECO RBCs. Therefore, we were keen to collaborate with the Prof. Maher Abou Hachem's group at the Danish Technology University (DTU), once we realized that they were as interested as we are to select enzyme candidates to test in the RBC setting.

Main findings

Since the Paper V manuscript is so large and reports such a lot of work, particularly if one includes the massive Supplement with 34 Figures and 16 Tables, it is difficult to summarize it here in a meaningful way. A total of 21 enzymes from *A. muciniphila* were explored and evaluated as candidates, first by biochemical screening in Denmark and later by conversion of RBCs in Lund for the most promising candidates. The manuscript describes how each enzyme was cloned, recombinantly expressed and thereafter characterised biochemically and enzymically. Factors like pH and temperature optimum as well as specificity for and activity with different glycoconjugates was assessed before an enzyme could be appointed an RBC-worthy candidate. In brief, enzyme candidates were selected for each of the six antigens we were concerned about. Conversion buffers and other parameters were optimized. Flow cytometric evaluations of remaining antigens after incubation were performed for each enzyme and each physical condition tested. Once all antigens could be converted into their precursor structures in an efficient manner by sequentially adding one enzyme after the other, it was also tested if it would be possible to perform a so-called one-pot digestion, which would be the most practical way to go about it. Crossmatch testing was performed with cohorts of healthy donor plasmas of group O but also other ABO groups including AB plasmas to assess if there were signs of crypt- or neoantigens having been formed. Our hypothesis that extended antigens contribute significantly to the crossmatch problem was confirmed. By cutting both traditional and extended antigens off, crossmatching results were improved for both A-ECO and B-ECO RBCs. However, even if many donor plasmas became negative and most improved, remaining crossmatch positivity was seen in a significant number of A-ECO crossmatches and a few B-ECO. Whilst B-ECO RBCs resulted in only one plasma reacting stronger than 1+ out of 100 reactions (91% of which were negative), results were worse for A-ECO. Only half of the plasmas gave negative results and some reactions were very strongly positive. Thus, our approach helped improve the results and show that antibodies against extended ABO structures is a significant problem for the ECO concept. Our study identified numerous enzymes, and even structurally characterised some of them, but was unable to solve the principal problem why crossmatches occur in the absence of known antigens.

Future prospects

The presented studies contribute novel knowledge to the field of transfusion medicine in general and more specifically to the in-depth area of carbohydrate blood groups, both when it comes to molecular genetics, biochemistry and immunohematology. Even so, the results obtained trigger more questions than those I started out with and did my best to answer. Below is a potential list of new questions that a post-doctoral me, or a future Ph.D. student, may sink their scientific teeth into:

- Should we be stricter when we designate *O* allele status to a certain sequence. Our studies as well as previously named *O* alleles, demonstrate that what appears to be an *O* allele may in fact express some A antigen. The mechanisms underlying this phenomenon are not clear and deserve further studies.

- It is interesting to note that most so-called *O* alleles are based on *A* allelic backbones. During the course of my studies, we encountered a *B* allele with a certain aa substitution, which in one individual resulted in complete lack of B antigen (including a negative adsorption/elution test) and a strongly reactive anti-B in plasma. This sounds like it may qualify as a *B*-based *O* allele. However, the other individual with the same genotype had very weakly expressing B antigen in combination with a very weak anti-B in plasma. Even if this continues to feed the terminological nightmare of naming *ABO* alleles properly, it will be interesting to study how this missense mutation causes complete or partial loss of function in the resulting GTB.

- The most obvious question that awaits an answer is why it is so difficult to create a crossmatch-compatible ECO RBC product? Even if we were able to find enzymes to tackle each of the known extensions of the traditional A and B antigens, crossmatches improved but did not go away in some cases. The old hypothesis that these reactions are due to remaining A or B antigen can now be refuted. Instead, other hypotheses spring to mind:

- Is it simply so that healthy individuals have antibodies to the bacterial enzymes used and that sufficiently high levels of exoglycosidases remain on the RBCs post-treatment?

- Or could it be that we have just scraped the surface when it comes to revealing the unexpected, unorthodox ABO-related antigens which were not in the textbooks? It seems quite unlikely that we would have happen to discover all A- or B-related antigens and not too surprising if there are others waiting to be discovered.

- A more troublesome scenario would be that our exoglycosidases are not as specific as we would like to think that they are. If so, they may well be good at removing an extended structure (to which we have unknown antibodies as part of what we have hitherto called anti-A, -B and -A,B in group O plasma) and thereby decreasing the crossmatch problem, while at the same time creating a neo- or cryptantigen that reacts with yet other antibodies, thereby increasing the problem.

- A piece of information lacking when it comes to the known extensions of A (mostly relevant for Gal-A and H type 3), is to determine which GT is responsible for their synthesis and consequently which gene governs their expression. A type 3 is thought to originate from GTA activity, particularly A1 transferase is therefore linked to the traditional ABO locus. H type 3 is likely to be governed by *FUT1* since no variation dependent on secretor status has been reported but in principle, it could probably be another fucosyltransferase (gene) so this might be worth following up. The most exciting among these three extensions is Gal-A. If it would gain status as a new blood group antigen, it is very likely to form a separate blood group system of its own since the only galactosyltransferase known in the blood group world appears to be very $\alpha 1,4$-specific (*A4GALT*-encoded) and Gal-A is based on an $\alpha 1,3$-specific linkage.

No matter what, carbohydrate blood groups constitute a great model to gain insight into complicated matters like the fine-tuned complexity of glycobiology, enzymology, biochemistry and molecular biology. The reason is that the fields of transfusion medicine and immunohematology have spent decades gathering a rich flora of phenotypic information which is seldom the case in other fields. Thus, small changes in blood group phenotype will continue to allow for identification of underlying molecular principles, sometimes important beyond the field of blood groups.

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