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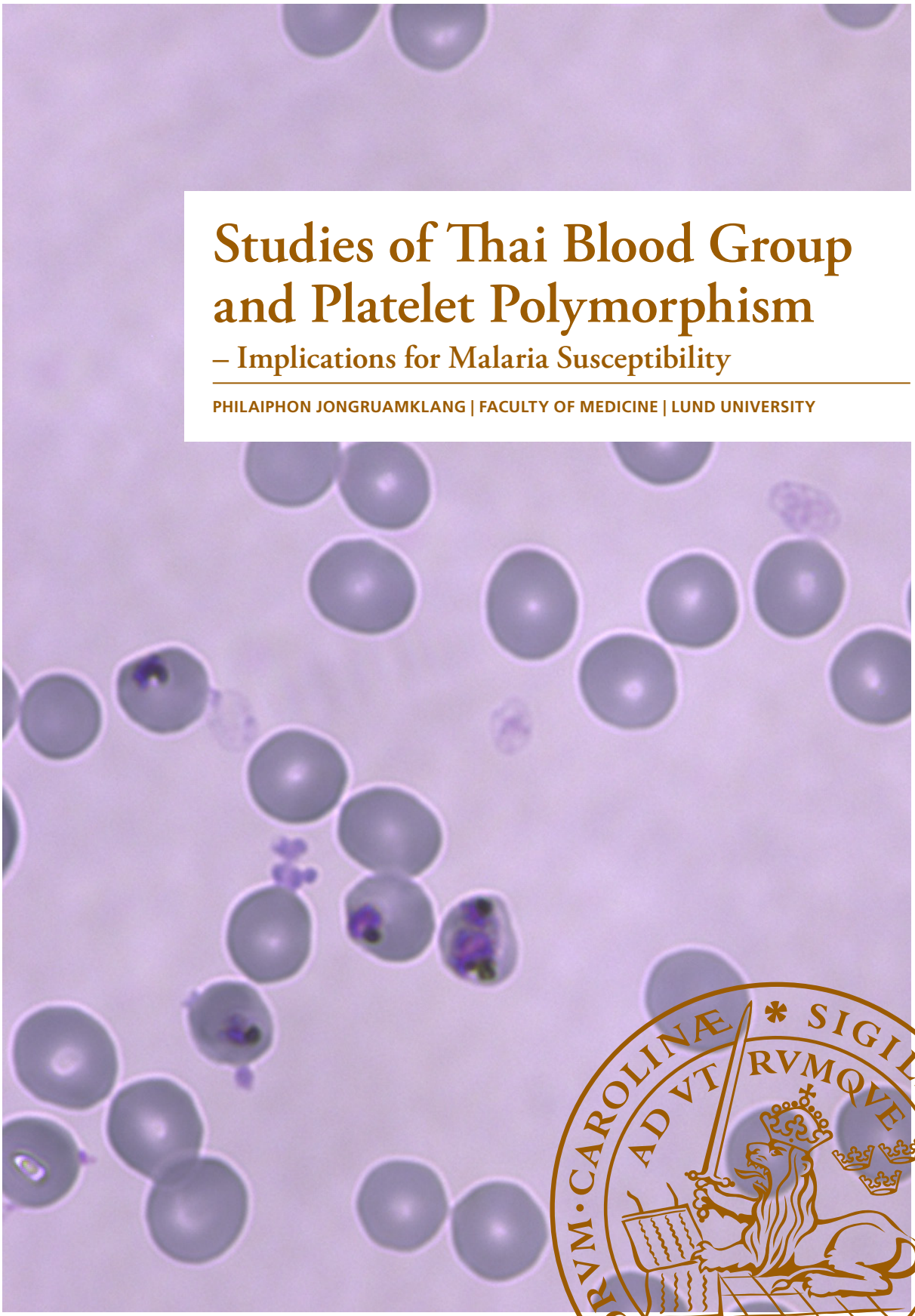

Studies of Thai Blood Group and Platelet Polymorphism

– Implications for Malaria Susceptibility

PHILAIPHON JONGRUAMKLANG | FACULTY OF MEDICINE | LUND UNIVERSITY

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Studies of Thai blood group and platelet polymorphism
- implications for malaria susceptibility

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Philaiphon Jongruamklang

DOCTORAL DISSERTATION

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This thesis explored initially blood group polymorphism among Thai blood donors from two different regions with the aim of looking for variation that might give potential protection against malarial infection. To do this, MALDI-TOF MS was initially employed in **Study I** as a powerful and efficient method for rapid routine genotyping to establish the common blood group and platelet antigen genotypes of 396 blood donors. Investigation of outliers identified variation among these samples, both expected and novel. The expected high prevalence of the Mi(a+) phenotype was observed, and identified that 2.3% of samples carried *FY* c.265T, a polymorphism carried on *FY*01* instead of *FY*02* as found in the Caucasian population. Of potential clinical relevance in a region where transfusion-dependent thalassemia is common, we identified two *RHCE*02* alleles known to encode an e-variant antigen. Allelic discrimination assays for extended testing of platelet polymorphism predicted the expected antigen distribution for Southeast Asia.

In **Study II**, homozygosity for a novel nonsense mutation (c.420T>G) in *B3GALNT1* (*GLOB*01N.13*) was identified in two Thai sisters suffering from recurrent spontaneous abortions due to the P^k phenotype, adding to the twelve null alleles already known in the GLOB system. Screening of the blood donor cohort revealed another individual carrying this allele, suggesting regional occurrence of this rare allele.

In **Study III**, DNA sequencing not only confirmed that *GYP*Mur* is the most frequent allele of the variant glycophorin genes in Thai blood donors, but also identified a *GYP*Bun*-like allele (designated *GYP*Thai*) that was shown to be relatively common (MAF = 0.01) in this group. These hybrid alleles have a previously uncharacterized effect on s antigen expression on RBCs, qualitatively and quantitatively, that has implications for reagent selection in transfusion medicine.

Study IV turned to platelets, which showed an inhibitory effect on RBC invasion by *P. falciparum* under physiological platelet:RBC ratios (approx. 1:10 - 1:40) in a dose-dependent manner and that was still effective at levels considered to be thrombocytopenic. At higher platelet concentrations, the trend was reversed so that platelets did not increase the inhibitory effect on RBC invasion further. However, platelet-dependent killing, as witnessed by increased extracellular parasites, remained effective suggesting platelets to be an important part of the host defence.

Much is known about the interaction of *Plasmodium* spp. and their interaction with erythrocytes but still new ligands on RBCs are still being discovered. This work also tried to examine the role of the Vel blood-group-carrying protein SMIM1 in invasion but so far, the results have been inconclusive. Despite all we know about blood group polymorphism, much remains to learn. This thesis has explored allelic variation in a region of the world where malaria has long been endemic and the observed blood cell traits highlight our intriguing ability to adapt.

Key words
Thai, Blood group polymorphism, Human platelet antigens, *Plasmodium falciparum*, Malaria susceptibility.

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Cover image: Giemsa-stained thin smear showing parasitized RBCs in a field of uninfected RBCs and platelets

Cover photo by Philaiphon Jongruamklang

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To My Family

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Abbreviations

AD	Allelic discrimination
AO	Acridine orange
bp	Base pair
CD	Cluster of differentiation
cDNA	Complementary DNA
DAF	Decay accelerating factor
DNA	Deoxyribonucleic acid
GP	Glycophorin
GPI	Glycosylphosphatidylinositol
GYP	Glycophorin genes
HDFN	Hemolytic disease of the fetus and newborn
HLA	Human leukocyte antigen
HPA	Human platelet antigen
HRM	High-resolution melting (analysis)
HTR	Hemolytic transfusion reaction
ISBT	International Society of Blood Transfusion
kb	kilobase pairs
MAF	Minor allele frequency
MALDI-TOF	Matrix-assisted laser desorption/ionization-time-of-flight
MCM	Malaria culture medium
MS	Mass spectrometry
NGS	Next-generation sequencing

P.	*Plasmodium*
PCR	Polymerase chain reaction
PCR-ASP	Polymerase chain reaction with allele-specific primers
PCR-RFLP	Polymerase chain reaction with Restriction fragment length polymorphism
PCR-SSP	Polymerase chain reaction with sequence-specific primers
qPCR	quantitative PCR
RBC	Red blood cell
SMIM1	Small integral membrane protein 1
rs	Reference SNP cluster ID
SNP	Single nucleotide polymorphism
TMP	Transmembrane protein

Studies included in this thesis

This thesis is based on the following studies:

I. **Jongruamklang P**, Gassner C, Meyer S, Kummasook A, Darlison M, Boonlum C, Chanta S, Frey BM, Olsson ML, Storry JR. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis of 36 blood group alleles among 396 Thai samples reveals region-specific variants. *Transfusion* 2018 Jul;58(7):1752-1762.

II. 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 at the *B3GALNT1* locus. *Transfusion Medicine* 2019 Jun;29(3):202-208.

III. **Jongruamklang P**, Grimsley S, Thornton N, Robb J, Olsson ML, Storry JR. Characterisation of *GYP*Mur* and novel *GYP*Bun*-like hybrids in Thai blood donors reveals a qualitatively altered s antigen. *Vox Sanguinis* 2020 (Published online 2020-03-23).

IV. **Jongruamklang P**, Rebetz J, Kapur R, Persson KEM, Olsson ML, Semple JW, Storry JR. Platelets inhibit erythrocyte invasion by *Plasmodium falciparum* at physiological platelet:erythrocyte ratios. (Manuscript submitted to *Transfusion Medicine* 2020-02-21)

Abstract

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Introduction

Blood groups

It was at the beginning of the twentieth century when Karl Landsteiner noticed that plasma from some individuals agglutinated the red blood cells (RBCs) from others and blood groups were discovered for the first time (Landsteiner, 1901). Around that time blood groups could only be explored through the visible "clumping" by antibodies that directly agglutinated RBCs. Not until nearly a half century later, in 1945 when Coombs and colleagues (Coombs et al., 1945) established the antiglobulin test, could we visualise non-agglutinating antibodies and discover more RBC antigens.

Currently, there are 367 authenticated blood group antigens, 330 of which fall into one of 39 blood group systems, genetically discrete groups of antigens controlled by a single gene or cluster of two or three closely linked homologous genes. Every blood group antigen is included in one of the systems, collections or low- or high-incidence series according to guidelines for blood group antigen and allele nomenclature defined by the International Society of Blood Transfusion (ISBT) Working Party for Red Cell Immunogenetics and Blood Group Terminology (Table 1). Most novel antigens are found to belong to known systems and of low or high incidence (Storry et al., 2019) but new blood group systems continue to be discovered, as exemplified by the very recent elucidation of PEL which is currently awaiting for ISBT approval (Azouzi et al., 2020). All antigens are given a name and are numbered sequentially, prefixed with the number of the system or series where it is placed.

Discovery of the ABO blood groups made blood transfusion achievable. The discovery of the Rh antigens led to the understanding, and consequent prevention, of hemolytic disease of the fetus and newborn (HDFN). While ABO and Rh are the most important systems in transfusion medicine, many other blood group antibodies can cause serious adverse effects such as hemolytic transfusion reaction (HTR) or HDFN. Screening for and identification of antibodies is an important part of transfusion medicine in order to find compatible, antigen-negative blood for transfusion. Further, antibodies to blood group antigens other than ABO maybe also be important in transplantation (Subramaniyan, 2017; Thornton and Grimsley, 2019).

Table 1 Summary of information on genes and gene products in the currently acknowledged blood group systems.

ISBT name	ISBT number	ISBT system symbol	Gene name(s)* (HUGO)	Chromosome location	Protein type†	Number of antigens	Key references
ABO	001	ABO	*ABO*	9q34.2	T2	4	(Yamamoto et al., 1990a, 1990b)
MNS	002	MNS	*GYPA, GYPB, (GYPE)*	4q31.21	T1	49	(Siebert and Fukuda, 1987, 1986)
P1PK	003	P1PK	*A4GALT*	22q13.2	T2	3	(Steffensen et al., 2000; Thuresson et al., 2011; Westman et al., 2018; Yeh et al., 2018)
Rh	004	RH	*RHD, RHCE*	1p36.11	M-12	55	(Arce et al., 1993; Avent et al., 1990; Cherif-Zahar et al., 1990; Kajii et al., 1993; Le Van Kim et al., 1992)
Lutheran	005	LU	*BCAM*	19q13.2	T1	27	(Parsons et al., 1995)
Kell	006	KEL	*KEL*	7q33	T2	36	(Lee et al., 1991)
Lewis	007	LE	*FUT3*	19p13.3	T2	6	(Kukowska-Latallo et al., 1990)
Duffy	008	FY	*ACKR1*	1q21-q22	M-7	5	(Chaudhuri et al., 1993)
Kidd	009	JK	*SLC14A1*	18q11-q12	M-10	3	(Olives et al., 1995)
Diego	010	DI	*SLC4A1*	17q21.31	M-14	22	(Bruce et al., 1994)
Yt	011	YT	*ACHE*	7q22	GPI	5	(Bartels et al., 1993; Rao et al., 1993)
Xg	012	XG	*XG, MIC2*	Xp22.32	T1	2	(Darling et al., 1986; Ellis et al., 1994; Möller et al., 2018; Yeh et al., 2018)
Scianna	013	SC	*ERMAP*	1p34.2	T1	7	(Wagner et al., 2003)
Dombrock	014	DO	*ART4*	12p13-p12	GPI	10	(Gubin et al., 2000)
Colton	015	CO	*AQP1*	7p14	M-6	4	(Smith et al., 1994)
Landsteiner-Wiener	016	LW	*ICAM4*	19p13.2	T1	3	(Bailly et al., 1994)
Chido/Rodgers	017	CH/RG	*C4A, C4B*	6p21.3	S	9	(Yu, 1991; Yu et al., 1986)
H	018	H	*FUT1*	19q13.33	T2	1	(Kelly et al., 1994)
Kx	019	XK	*XK*	Xp21.1	M-10	1	(Ho et al., 1994)
Gerbich	020	GE	*GYPC*	2q14-q21	T1	11	(Colin et al., 1986)

Table 1 (cont.) Summary of information on genes and gene products in the currently acknowledged blood group systems.

ISBT name	ISBT number	ISBT system symbol	Gene name(s)* (HUGO)	Chromosome location	Protein type†	Number of antigens	Key references
Cromer	021	CROM	*CD55*	1q32	GPI	20	(Telen et al., 1988)
Knops	022	KN	*CR1*	1q32.2	T1	10	(Moulds et al., 1991; Rao et al., 1991; Wong et al., 1989)
Indian	023	IN	*CD44*	11p13	T1	6	(Spring et al., 1988)
Ok	024	OK	*BSG*	19p13.3	T1	3	(Guo et al., 1998; Spring et al., 1997)
Raph	025	RAPH	*CD151*	11p15.5	M-4	1	(Karamatic Crew et al., 2004)
John Milton Hagen	026	JMH	*SEMA7A*	15q22.3-q23	GPI	7	(Mudad et al., 1995; Yamada et al., 1999)
I	027	I	*GCNT2*	6p24.2	T2	1	(Yu et al., 2001)
Globoside	028	GLOB	*B3GALNT1*	3q25	T2	2	(Hellberg et al., 2002)
Gill	029	GIL	*AQP3*	9p13	M-6	1	(Roudier et al., 2002)
Rh-associated glycoprotein	030	RHAG	*RHAG*	6p12.3	M-12	3	(Tilley et al., 2010)
FORS	031	FORS	*GBGT1*	9q34.13-q34.3	T2	1	(Svensson et al., 2013)
JR	032	JR	*ABCG2*	4q22.1	M-6	1	(Zelinski et al., 2012)
LAN	033	LAN	*ABCB6*	2q36	M-8	1	(Helias et al., 2012)
Vel	034	VEL	*SMIM1*	1p36.32	T2	1	(Ballif et al., 2013; Cvejic et al., 2013; Storry et al., 2013)
CD59	035	CD59	*CD59*	11p13	GPI	1	(Anliker et al., 2014)
Augustine	036	AUG	*SLC29A1*	6p21.1	M-11	4	(Daniels et al., 2015)
KANNO	037	KANNO	*PRNP*	20p13	GPI	1	(Omae et al., 2019)
Sid	038	SID	*B4GALNT2*	17q21.32	T2	1	(Lo Presti et al., 2003; Montiel et al., 2003; Stenfelt et al., 2019)
CTL2	039	CTL2	*SLC44A2*	19p13.2	M-10	2	(Vrignaud et al., 2019)

*As defined by the HUGO Gene Nomenclature Committee http://www.genenames.org/. () no gene product on normal RBCs. †T1 type 1, single membrane span, external N-terminus; T2 type 2, single membrane span, internal N-terminus; M-n is a multimembrane pass molecule that traverses the membrane n times; GPI is a molecule anchored to the RBC membrane via a glycosylphosphatidylinositol link; S is a molecule that is found in its soluble form in plasma but has been adsorbed to the RBC membrane and covalently bound to lysine residues. Blue represents provisional assignment of blood group status.

Blood group antigens are polymorphisms of erythrocyte surface molecules, so classified because they have stimulated the production of specific alloantibodies in transfusion recipients and/or pregnant women. Blood group carrying molecules include proteins, glycoproteins or glycolipids residing in the RBC membrane. While the blood group antigen differences rarely have an effect on function, many of the blood-group-carrying molecules have well-defined roles on the RBC. The functions of others are presumed based on homology with non-RBC proteins, and yet others remain unknown. The list of known or putative functions is growing and a blood group can be assigned by its biochemical properties (Daniels, 2013) into functional assemblies including channels/transporters, receptor/adhesion, glycocalyx, enzyme, complement regulator and membrane structure molecules (Figure 1).

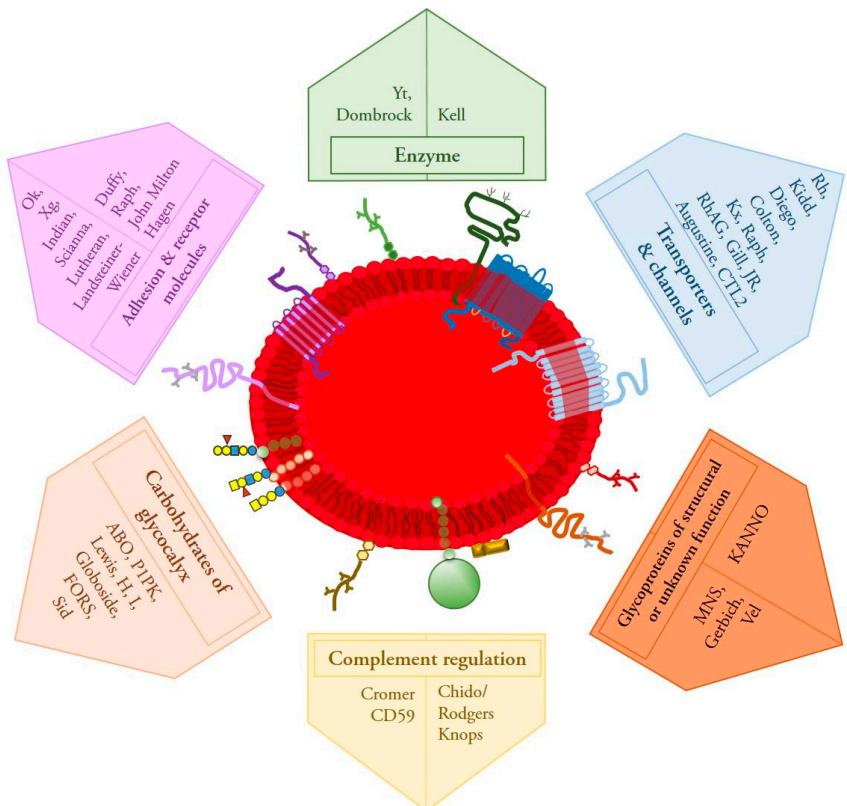


Figure 1 Schematic representation of blood group systems categorized by their known or putative functions on erythrocytes.

Blood group polymorphism

Polymorphism, originating from the Greek words *poly* (multiple) and *morph* (form), is a term used to describe multiple forms of an existing single gene in a population. In this regard, blood group polymorphisms are generated from alterations at the gene level through a number of various molecular mechanisms. Consequently, the altered genes cause the variants present on RBC membrane. Though the most common mechanism causing blood group diversity is the single nucleotide polymorphism (SNP), the changes of antigens caused by others can be found as listed in Table 2.

Single amino acid substitutions in a glycosyltransferase or a domain of a membrane protein due to missense mutations are common. Such SNPs can cause a difference in antigen expression, for instance generate a pair of antithetical antigens, create a novel antigen, or decrease the amount of expected antigen. Some examples of blood group polymorphisms created from SNPs can be found in Table 3. Another type of SNP is a nonsense mutation, which generates a premature stop codon and consequently no product encoded by the gene is expressed on the RBC membrane. Splice-site mutations, a molecular mechanism in which SNPs occur in important consensus sequences in introns, can result in skipping of exons from the mRNA, which in turn may cause a decrease in the copy number of the blood group molecule or abolish the expression of antigens from the RBC membrane.

Gene deletion is another mechanism causing polymorphism. In the Rh system, for example, the *RHD* gene is deleted resulting in the D-negative phenotype (Wagner and Flegel, 2000). Deletion of a single nucleotide can also have important consequences, for example, in the most well-known blood group system, ABO, the *O* and A^2 alleles both result from a nucleotide deletion (Yamamoto et al., 1992, 1990b). Conversely, novel antigens can be created as a result from duplication or insertion of genetic material, such as the Ls^a antigen in the Gerbich blood group system that is created from duplication of *GYPC* exon 3 (Reid et al., 1994).

Another more complex mechanism that contributes to diversity is gene rearrangement, which can occur from DNA crossover or gene conversion events. Recombination between the two homologous genes giving rise to hybrid proteins occur in the MNS and Rh blood group systems and creates many different kinds of phenotypes. Examples of the phenotypes created by these mechanisms include the common partial D variant, D^{VI}, which results from *RH(D-CE-D)* hybrid that belongs to the Rh system (Avent et al., 1997; Huang, 1997) and the Mi(a+) phenotypes carried on *GYP(B–A–B)* hybrid genes, which are created by DNA conversion of *GYPA* and *GYPB* in the MNS system (Blumenfeld and Huang, 1997).

Blood group genotyping

Since blood groups were first discovered in 1900, most blood group testing has been done by serology, *i.e.* immunohematological technique also known as hemagglutination. Not until the end of the twentieth century, with the application of gene cloning and sequencing of blood group genes, the prediction of blood group phenotypes from DNA sequence became possible.

For RBC typing, there are four main reasons for preferentially using molecular methods rather than serological methods:

i. There are no suitable RBCs easily available for serological typing (*e.g.*, this includes fetal typing and multitransfused or DAT positive patients)

ii. There is no serological reagent available

iii. Molecular testing will provide more or better information

iv. Molecular testing is more efficient or more cost effective than serological typing

Methods for blood group genotyping and phenotype prediction were developed soon after the cloning of blood group genes. When Yamamoto and colleagues first identified *A* and *B* alleles, they also found that the different polymorphisms could be digested with restriction endonucleases and utilized for diagnostic purposes (Yamamoto et al., 1990b). Though their technique, *i.e.* PCR-RFLP is still used nowadays, a great number of molecular based genotyping principles for blood groups have been implemented. For example, PCR with allele-specific primers (PCR-ASP), PCR with sequence-specific primers (PCR-SSP) or more advanced by real-time PCR, *e.g.* allelic discrimination (AD) or allele-specific real-time PCR, and many more. Sanger sequencing is another option to identify genetic variants among blood groups, long considered to be a gold standard method for genotyping.

In recent years, high-throughput genotyping methods based on MALDI-TOF MS (Study I; Gassner et al., 2013; Meyer et al., 2015), Luminex (Finning et al., 2016; Goldman et al., 2015) or qPCR (Denomme and Schanen, 2015) have been deployed for large-scale blood group genotyping or even for routine purposes. These methods are PCR-based and rely on SNP detection. Even if they are specific and can be high-throughput and automated, they are limited to detect only what they have been designed for (Westhoff, 2019).

More recently, next-generation sequencing (NGS) also known as massively parallel sequencing, a method that enables unbiased sequencing of all relevant genes in parallel for many samples at the same time, has been introduced for blood group genotyping

purposes and many studies have been published recently (Chou et al., 2017; Fichou et al., 2016, 2014; Jakobsen et al., 2019; Lane et al., 2016; Wu et al., 2018). Among others, Lane et al. have shown the comprehensive prediction of blood group and platelet phenotypes from a single person (Lane et al., 2016) and later reported the use of the whole genome sequencing (WGS) based RBC and PLT antigen typing in a bigger cohort (Lane et al., 2018). Since knowledge and the list of blood group antigens and their underlying genetic bases are growing, methods that are more accurate, cost effective and high-throughput with a capacity of adequate coverage of all blood group genes will become necessary to provide the required information to decipher the full blood group phenotype. In parallel, software and databases to interpret the large amount of data will be key. Such tools have already been developed and should be open and free to reference laboratories and scientists worldwide (Möller et al., 2016; Lane et al. 2019).

In the end, some kind of NGS-based approach is likely to become the most reliable blood typing test. To what degree this can be implemented in clinical routine practice in different parts of the world remains to be seen. The rapid decrease in price for targeted NGS or even whole exome or whole genome approaches provides some hope but we are not there yet.

Table 2 Molecular mechanisms that create blood group diversity.

Type of change	Molecular mechanism	Example of gene event	Phenotypic consequence
Antithetical antigen	Missense SNP	*KEL* c.578C>T	k → K
		GYPB c.143T>C	S → s
Novel antigen	Missense SNP	*GYPB* c.161G>A	Mit+
	Unequal crossover between homologous genes	*GYP(B-A)*	S–s+^{w}U–, Dantu+
	DNA conversion between homologous genes	*RH(D-CE-D)*	D^{VI}, BARC+
		GYP(B-A-B)	Mi(a+)
	Exon duplication	*GYPC* Exon 3	Ls(a+)
Decreased amount of expected antigen	Missense SNP	*ABO* c.646T>A	A$_{x}$
		FY c.265C>T	Fy^{x}
		CROM c.596C>T	Dr(a–)
	Splice site mutation	*GYPB* c.270+5g>t	S–s–U+^{w}
		XK IVS2+5g>a	McLeod phenotype
	Modifying gene	*KLF1* variants	Lu(a–b–)
No protein product	Premature stop codon	*GLOB* c.420T>G	P–
	Nucleotide deletion	*CO* c.232delG	Co(a–b–)
	Mutation in transcription factor binding motif	*FY* c.–67T>C	Fy(a–b–)
	Splice site mutation	*DO* c.145–2a>g	Gy(a–)
	Gene deletion	*ΔRHD*	D–

*SNP, single nucleotide polymorphism; *Δ*, deletion.

Table 3 Some examples of important blood group polymorphisms arising from SNPs.

ISBT system symbol	Polymorphism	Critical SNP*	Amino acid change†
ABO	A/B	c.796C>A, c.803G>C	p.Leu266Met, p.Gly268Ala
MNS	M/N	c.59C>T, c.71G>A, c.72T>G	p.Ser20Leu, p.Gly24Glu
	S/s	c.143T>C	p.Met48Thr
RH	C/c	c.307T>C	p.Ser103Pro
	E/e	c.676C>G	p.Pro226Ala
LU	Lu^a/Lu^b	c.230A>G	p.His77Arg
	Au^a/Au^b	c.1615A>G	p.Thr539Ala
KEL	K/k	c.578T>C	p.Met193Thr
	Kp^a/Kp^b	c.841T>C	p.Trp281Arg
	Js^a/Js^b	c.1790C>T	p.Pro597Leu
FY	Fy^a/ Fy^b	c.125G>A	p.Gly42Asp
	Fy^b/Fy	c.–67T>C	p.0 (Not coding)
JK	Jk^a/Jk^b	c.838G>A	p.Asp280Asn
DI	Di^a/Di^b	c.2561T>C	p.Leu854Pro
	Wr^a/Wr^b	c.1972G>A	p.Glu658Lys
YT	Yt^a/ Yt^b	c.1057C>A	p.His353Asn
SC	Sc1/Sc2	c.169G>A	p.Gly57Arg
DO	Do^a/Do^b	c.793A>G	p.Asn265Asp
CO	Co^a/Co^b	c.134C>T	p.Ala45Val
LW	LW^a/LW^b	c.299A>G	p.Gln100Arg
CROM	Tc^a/Tc^b	c.155G>T	p.Arg52Leu
KN	Kn^a/Kn^b	c.4681G>A	p.Val1561Met
IN	In^a/In^b	c.137C>G	p.Pro46Arg
OK	Ok(a+)/Ok(a–)	c.274G>A	p.Glu92Lys
RAPH	MER2+/MER2–	c.511C>T	p.Arg171Cys
JMH	JMH+/JMHK–	c.619C>T	p.Arg207Trp
I	I+/I–	c.1049G>A	p.Gly350Glu
GLOB	P+/P-	c.420T>G	p.Tyr140Ter
GIL	GIL+/GIL–	c.710+1g>a	Alternative splicing
RHAG	Duclos+/Duclos–	c.316C>G	p.Gln106Glu
FORS	FORS+/FORS–	c.887G>A	p.Arg296Gln
JR	Jr(a+)/Jr(a–)	c.376C>T	p.Gln126X
LAN	Lan+/ Lan–	c.717G>A	p.Gln239X
CD59	CD59:+1/ CD59:–1	c.266G>A	p.Cys89Tyr
AUG	At(a+)/At(a–)	c.1171G>A	p.Glu391Lys

*Counting from first nucleotide of translation-initiating methionine codon, †Counting from translation-initiating methionine.

Genetic diversity underlying blood group variation

The study of the genes underlying blood group polymorphisms has been very successful. We understand much about the structure of the gene products, and the antigens themselves, their importance biologically, and the likely reasons for why and how they have arisen. For the majority, there is a clear and reasonable explanation but still not completely for all. Many blood group systems appear to distribute their polymorphisms differently around the globe. ABO is a good example of a blood group system that has been well-studied and provides a great example of the genetic diversity and its benefits for humans. Since 1919, Hirszfeld and Hirszfeld (Hirszfeld and Hirszfeld, 1919) noticed a clear difference in distribution of blood group A and B among various populations. The genetic basis of the ABO system was elucidated after the gene encoding the ABO transferases was first cloned in 1990 (Yamamoto et al., 1990b). It is believed that the null phenotype of this system, *i.e.* blood group O, resulting from *ABO* c.261Gdel which abolishes the transferase activity to convert H antigen to A antigen, arose before humans emigrated from Africa (Cserti and Dzik, 2007). Group O RBCs show a greater survival advantage over group A RBCs in severe *Plasmodium falciparum* malaria (Fry et al., 2008; Rowe et al., 2007). The binding capacity of parasitized RBCs to uninfected RBCs, *i.e.* rosetting, a phenomenon observed in severe malaria is more conspicuous in blood group A patients (Rowe et al., 2007). The adhesive determinant, *i.e.* PfEMP1, have been demonstrated to bind primarily to A and B RBCs (Barragan et al., 2000). Further, parasite RIFIN molecules were recently found to bind to the A antigen (Goel et al., 2015).

Individuals with a non-O blood group have a higher risk of venous thromboembolism than those with the O blood group. The reason behind this is likely to be that the expression of A and B antigens on the N-glycans of von Willebrand factor plays a role in the half-life of this protein, subsequently A and B individuals have much higher plasma levels of von Willebrand factor than group O individuals (Cserti and Dzik, 2007). The increased tendency of blood clot formation has been presumed to be a survival advantage for the early human (Zivelin et al., 2006). Furthermore, blood group O is likely to have advantage against other diseases such as myocardial infarction and pancreatic cancer, but a greater likelihood to be a risk factor for infections such as *Vibrio cholera*, *Escherichia coli*, or *Helicobacter pylori* (Anstee, 2010). At the time of thesis writing, blood group O is believed to have a lower risk for the infectious disease of COVID-19, a severe respiratory disease caused by the virus SARS-CoV-2, compared with non-O blood groups, whereas blood group A individuals may be more vulnerable to the infection. (https://www.scmp.com/news/china/society/article/3075567/people-blood-type-may-be-more-vulnerable-coronavirus-china-study). However, further studies are required to confirm this association.

Polymorphisms in other blood group systems also demonstrate advantages over existing diseases. The Duffy blood group system is one of the best examples: the null Fy(a–b–) phenotype, resulting from the disruption in a transcription factor binding motif of *FY, i.e. ACKR1* gene, prevents RBCs from *P. vivax* and *P. knowlesi* infection since these parasites use ACKR1 protein exclusively as their invasion receptor (Mason et al., 1977; Miller et al., 1975). The phenotype has a very high prevalence in West Africa, where malaria is endemic.

The MNS system is another well-known example of a blood group system in which polymorphism is believed to have arisen from malaria-driven pressure. RBCs lacking GPA, En(a–), and GPB, (S–s–) are resistant to *P. falciparum* infection (Pasvol et al., 1982). Further, RBCs carrying the hybrid GPA-GPB protein, Dantu, have also been proven to resist invasion by *P. falciparum* (Field et al., 1994; Leffler et al., 2017). Those variants are reported mostly in Africa where malaria is endemic. In other parts of the world, the GP(B-A-B) variant, GP.Mur, has a relatively high prevalence in South East Asia, and has been speculated not only to provide an advantage related to malaria survival in itself but also through elevated expression of Band 3 in these RBCs (Hsu et al., 2009). In Papua New Guinea the common occurrence of the Gerbich-negative phenotype, Ge:−2,−3, is also thought to be a result of a selective advantage from the endemic malaria in the area since GPC is also used by *P. falciparum* for invasion (Maier et al., 2003).

While there is no absolute proof that the occurrence of blood group variants is directly due to selective pressure from different diseases, it is important to remember that, as has been stated by Daniels (Daniels, 2013), evolution happens over a very long time. Thus, the causative elements or pathogens may have perished long ago, so there is no evidence left behind allowing us to identify them. In addition, migratory effects can further contribute to the picture we see today.

Malaria

Malaria, a life-threatening disease caused by *Plasmodium* parasites, is one of the major human diseases spread across large parts of the globe. According to WHO, there were an estimated 228 million cases of malaria in 2018 and an estimated 405 000 deaths of which tragically, children aged under 5 years accounted for 67% (272 000) of all malaria deaths worldwide (WHO, 2019).

Plasmodium spp. are intracellular parasites belonging to the phylum Apicomplexa. Among six species that have been reported causing human malaria: *Plasmodium falciparum*, *P. vivax*, *P. knowlesi*, *P. ovale*, *P. malariae*, and *P. cynomolgi*, *P. falciparum* is considered the deadliest one. However, a recent study by Naing et al. (Naing et al., 2014) revealed that the mortality and morbidity caused by *P. vivax* are likely to have been significantly underestimated. The incidence of malaria due to *P. ovale* and *P. malariae* is low while *P. knowlesi*, a zoonotic parasite of Southeast Asian macaques, has become an important cause of human malaria in Southeast Asia, even with as yet no concrete evidence of primary human-to-human transmission (Ahmed and Cox-Singh, 2015).

The complex life cycle of *Plasmodium* spp. involves two hosts; the definitive host or vector which is the *Anopheles* mosquitoes, and a vertebrate intermediate host *e.g.* human (Cowman et al., 2017). The infected mosquito injects sporozoites into the host during its blood meal. The sporozoites then migrate to the liver and invade hepatocytes within which they develop and expand to release thousands of merozoites with an ability to invade RBCs. These liver merozoites are released into the bloodstream at which point they invade RBCs and thereafter, develop through ring, trophozoite, and schizont stages, replicating to produce up to 32 daughter merozoites (Cowman et al., 2017). This is followed by rupture of the RBCs and their release into the circulation. These free merozoites invade new RBCs to continue the asexual blood-stage *i.e.* intraerythrocytic stage life cycle. Some intraerythrocytic stages develop into gametocytes, the sexual forms of the parasite and can be taken up by the mosquito during feeding and continue to develop with infected mosquito, eventually, into sporozoites readily to be injected into a human host during the next blood meal.

The clinical features of malaria, of which anemia is one, are associated with lysis of infected RBCs during merozoite egress. The development of *P. falciparum* from the invasion to merozoite replication usually takes 48 hours, causing a sporadic fever every second day; a classic symptom of malaria caused by *P. falciparum* (CDC, 2019). (https://www.cdc.gov/malaria/about/disease.html).

In the laboratory where an asexual erythrocytic stage of *P. falciparum* can be cultured in the presence of RBCs and human serum (or albumin supplement), the life cycle will take around 48 hours and the development of parasite can be observed under the light microscope (Figure 2).

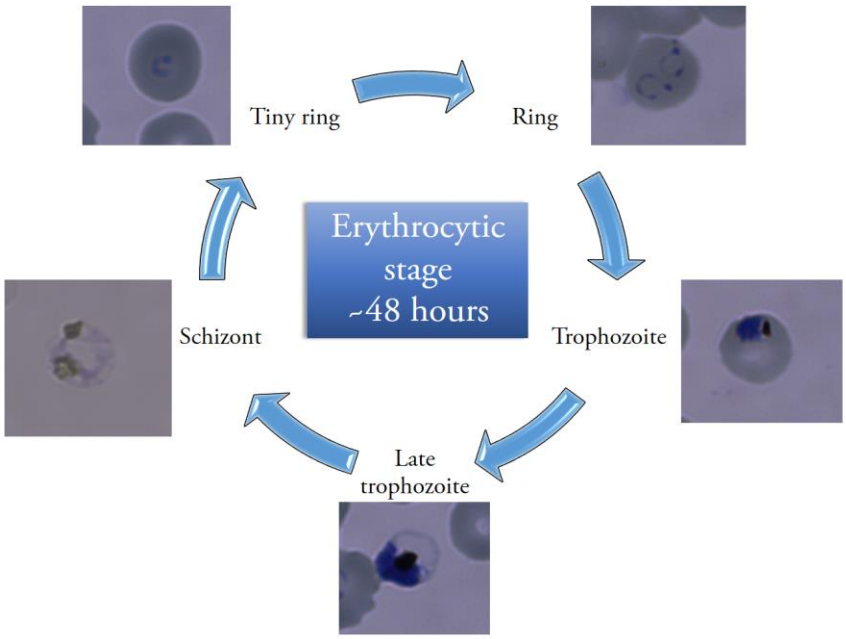


Figure 2 Schematic representation of *P. falciparum* erythrocytic stages during *in vitro* cultivation.

Malarial parasite invasion

Invasion of erythrocytes by the *P. falciparum* merozoite has many steps and involves many pairs of merozoite ligands and erythrocyte receptors. The stepwise event of merozoite invasion according to the review by Cowman et al. (Cowman et al., 2017) can be summarized here:

i. **Initial attachment:** merozoites attach to the erythrocyte membrane in a random orientation most commonly 'side on'.

ii. **Tight attachment:** the merozoite orientates to make a direct contact of its apical end with the erythrocyte membrane. Until now, the attachment is still reversible.

iii. **Tight junction formation:** now the attachment becomes irreversible. The interactions of merozoite EBL and PfRh proteins with their corresponding erythrocyte receptors promote the formation of tight junction at the interface of merozoite and erythrocyte membrane. The RON complex of parasite proteins is inserted into the erythrocyte membrane.

iv. **Penetration:** active invasion driven by the merozoite actin-myosin motor allows parasite to invade into a parasitophorous vacuole compartment created by secretion of the rhoptries into the host cell.

v. **Completion:** erythrocyte membrane is resealed and the invasion event is completed.

The entire process is completed within approximately 30 seconds. A schematic representation depicting the merozoite structure and invasion process are summarised in Figure 3.

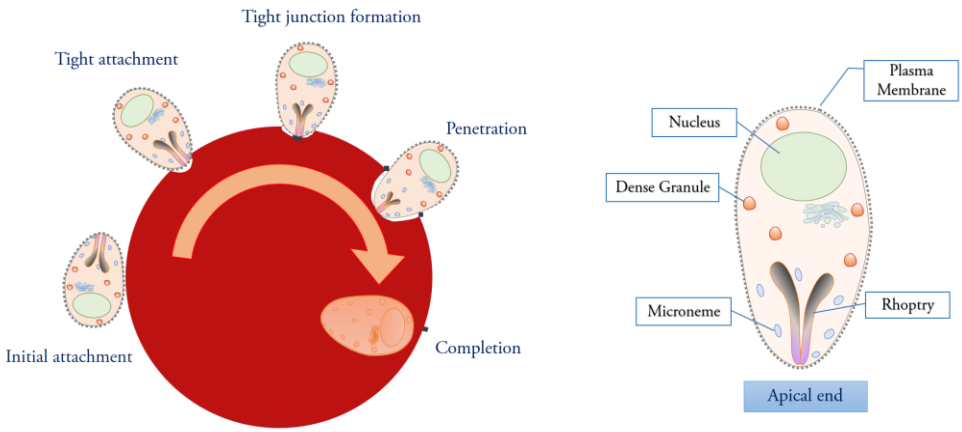


Figure 3 Schematic representation of the invasion process of *P. falciparum* and merozoite structure, adapted from Cowman et al (Cowman et al., 2017).

Erythrocyte receptors deployed by the parasite merozoite

Merozoites are uniquely adapted and designed for only one goal; to invade the next RBC. These relatively small cells carry organelles and structures, *e.g.* micronemes and rhoptries, at their apical ends which promote invasion when bound to the erythrocyte (Figure 3). Several pairs of ligands and erythrocyte receptors have been identified of which some have been shown to be widely used by most strains of *P. falciparum* while others are strain-specific. The well-studied ligands and their erythrocyte receptors such

as merozoite surface protein (MSP)-1/Band 3 are believed to be involved in initial attachment whereas others: erythrocyte-binding antigen (EBA)-175/GPA; EBL-1/GPB; EBA-140/GPC; *P. falciparum* reticulocyte-binding homologue (PfRh)4/CR1; and PfRh5/CD147 are involved during tight attachment and tight junction formation. Other proteins on the erythrocyte *e.g.* CD44, CD55, ICAM4, and recently identified ABCB6 have been shown to be involved in invasion with, as yet, unknown merozoite ligands. Conversely, erythrocyte receptors, *e.g.* the sialic acid (SA)-dependent receptors called W, Y, and Z, have been shown to bind to EBA-181, PfRh1, and PfRh2a/b, respectively although their identity remains unknown (Satchwell, 2016). The list of merozoite ligands are showed in Table 4. Many of the erythrocyte receptors are known to be classified into one of the 39 blood group systems, as represented in Figure 4.

Table 4 Selected parasite ligands and corresponding erythrocyte receptors.

Parasite ligands	Erythrocyte receptors
Duffy binding-like (DBL) family and erythrocyte binding-like (EBL) proteins	
PfEBA-140	Glycophorin C
PfEBA-175	Glycophorin A
PfEBA-181	Unknown (W)
PvDBP	DARC
PkαDBP	DARC
Reticulocyte (homolog) binding-like (RBL/Rh) family	
PfRh1	Unknown (Y)
PfRh2a	Unknown (Z)
PfRh2b	Unknown (Z)
PfRh4	Complement Receptor 1 (CD35)
PfRh5/PfRIPR	Basigin (CD147/EMMPRIN)
PvRBP1	Unknown
PvRBP2b	Transferrin receptor 1 (CD71)
Thrombospondin related anonymous protein (TRAP) family	
PfTRAP	Unknown
PvTRAP	Unknown
PfMTRAP	Semaphorin-7a (CD108)
PfTLP	Unknown
PfCTRP	Unknown
Apical membrane antigen (AMA)	
PfAMA1	PfRON2*
PvAMA1	PvRON2*

Pf, *P. falciparum*; Pv, *P. vivax*; Pk, *P. knowlesi*. * injected into erythrocyte membrane during invasion by merozoite.

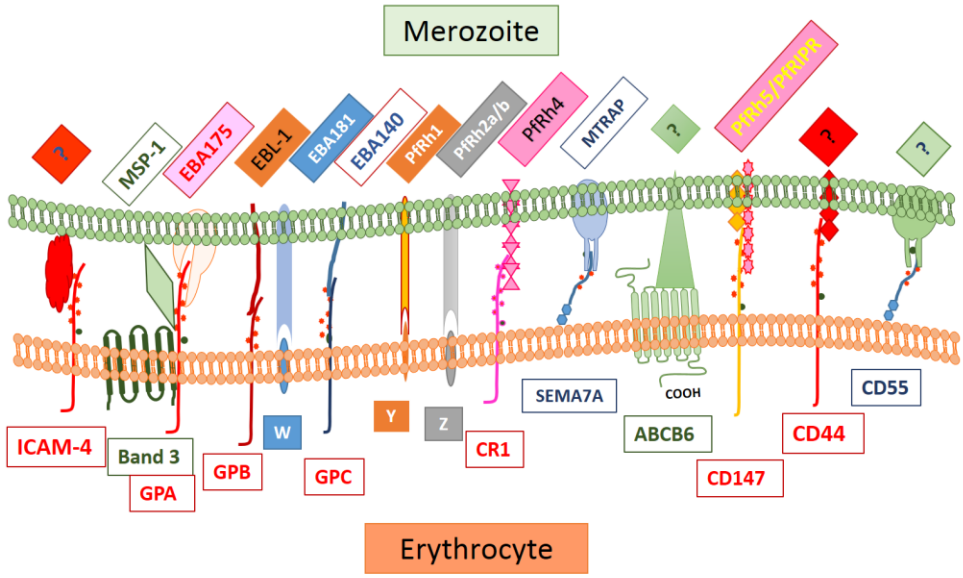


Figure 4 Schematic representation of human erythrocyte *P. falciparum* 'invasion receptors' presented with their corresponding merozoite-binding proteins. Receptor dimensions are not drawn to scale.

Evolutionary pressure of malaria on blood group diversity

As mentioned above, blood group antigens serve no function in transfusion or pregnancy, therefore, why and how they have arisen is of great evolutionary interest. Since RBCs are the primary target for malarial parasite *Plasmodium* species, one of the principal theories is that blood group polymorphism cannot only indirectly increase the herd immunity effect but also reduce infectability of the RBC.

As also discussed by Cserti and Dzik (Cserti and Dzik, 2007), Weatherall (Weatherall, 2008), and Anstee (Anstee, 2010), among other factors, malaria is likely to have been one of the strongest selective pressures influencing blood group polymorphism from an evolutionary perspective. Strong evidence for this is that certain blood group phenotypes are found at high frequency in malarial endemic areas but are rare elsewhere. The Fy(a–b–) phenotype described above for instance, has a very high prevalence in West Africa whereas it is found to be a rare phenotype in non-African descendants.

The correlation between other blood groups and *P. falciparum* has been observed, however, none of them are as outstanding as it is between the Duffy glycoprotein and

P. vivax. There are some blood group systems in which an involvement of *Plasmodium* spp. invasion and malaria survival has been reported, mostly identified using null phenotype RBCs as presented in Table 5.

Table 5 Blood group systems that have been reported involving malaria.

Blood group system	Interaction in brief	References
MNS	Both GPA and GPB are important receptors for *P. falciparum* though multiple ligands.	(Field et al., 1994; Leffler et al., 2017; Pasvol et al., 1982)
DI	Band 3/GPA complex is receptor for MSP1.	(Goel et al., 2003)
LW	*In vitro* invasion in the presence of synthetic protein binding to ICAM4 showed an 80% drop.	(Bhalla et al., 2015)
Kx	Kx protein is a receptor for AMA1. McLeod (Kx null) reduced *P. falciparum* invasion.	(Kato et al., 2005)
GE	GPC is a receptor for EBA140. Ge-negative RBCs are resistant to *P. falciparum*.	(Pasvol et al., 1982)
CROM	Knockdown of CD55 reduced up to 30% of *P. falciparum* invasion. CD55 null RBCs reduced invasion.	(Egan et al., 2015)
KN	CR1 is a receptor for PfRh4. RBCs carrying low copy number of CR1 form less rosettes. Sl(a–) RBCs form fewer rosettes.	(Krych-Goldberg et al., 2002)
IN	Knockdown of CD44 showed a 30% drop of parasitemia.	(Egan et al., 2015)
OK	Basigin (CD147) binds to PfRh5. Ok(a–) RBCs show reduced invasion of *P. falciparum*.	(Crosnier et al., 2011)
JMH	Semaphorin 7a is a receptor for MTRAP.	(Bartholdson et al., 2012)
LAN	Lan null RBCs are resistant to *P. falciparum*.	(Egan et al., 2018)
SID	Cad/Sd(a++) RBCs are strongly resistance to *P. falciparum*.	(Cartron et al., 1983)

Platelets and platelet polymorphism

Platelets, abundant small anucleate cell fragments in the periphery, are well-known for their physiological role in haemostasis, but more recently their roles in inflammation (Leslie, 2010), innate and adaptive immunity (Semple et al., 2011) and in other diseases have been reported. Platelets express many glycoproteins (GPs) on their surface membrane in order to support their functions through ligand-receptor interactions and these GPs are presented in polymorphic forms similar to the RBC blood group antigens, predominantly as a result of SNPs in their encoding genes. Platelet antigens, like erythrocyte antigens, can also stimulate antibodies in individuals lacking certain antigens through the exposure from transfusion or pregnancy. Antibodies against human platelet antigens (HPAs) are recognized as a major cause of several immune platelet disorders, such as foetal and neonatal alloimmune thrombocytopenia (FNAIT) platelet transfusion refractoriness (PTR), and post-transfusion purpura (PTP) (Kroll et al., 1998).

The clinical importance of HPA was first reported by van Loghem et al. in 1959 (Kroll et al., 1998) when they observed the post-transfusion purpura in a patient and the antigen was named "Zw^a"($P1^{A1}$, HPA-1a). Thereafter, 35 HPAs have been described. There are seven different platelet GPs, namely GPIa, GPIbα, GPIbβ, GPIIb, GPIIIa, CD109 and the latest identified GPIX, that express the polymorphic HPAs as presented in Table 4. Six biallelic systems (HPA-1, -2, -3, -4, -5 and -15) and other alloantigens are defined following the guidelines provided by the Platelet Nomenclature Committee (PNC), a collaboration between the ISBT platelet working party and the International Society on Thrombosis and Haemostasis (ISTH) scientific subcommittee on platelet immunology (Metcalfe et al., 2003). The frequencies of HPAs have shown to be varied among the different alleles as well as among ethnic groups (Table 6).

Several different HPAs can be carried on the same glycoprotein since this is due to polymorphisms residing on the same coding gene, however they are not organised into formal systems as are the RBC blood group antigens. The most polymorphic glycoprotein is GPIIIa which, to date, carries 19 antigens. Moreover, GPIIIa forms a complex on platelet surface with GPIIb resulting in the GPIIbIIIa complex, the most abundant molecule on platelet surface, which carries a total of 27 HPAs.

Table 6 Human platelet antigens.

Antigen systems	Glycoprotein	Encoding gene (HGNC)	Nucleotide change*	Amino acid change*
HPA-1	GPIIIa	*ITGB3*	c.176T>C	p.Leu33Pro
HPA-2	GPIbα	*GP1BA*	c.482C>T	p.Thr145Met
HPA-3	GPIIb	*ITGA2B*	c.2621T>G	p.Ile843Ser
HPA-4	GPIIIa	*ITGB3*	c.506G>A	p.Arg143Gln
HPA-5	GPIa	*ITGA2*	c.1600G>A	p.Glu505Lys
HPA-6	GPIIIa	*ITGB3*	c.1544G>A	p.Arg489Gln
HPA-7	GPIIIa	*ITGB3*	c.1297C>G	p.Pro407Ala
HPA-8	GPIIIa	*ITGB3*	c.1984C>T	p.Arg636Cys
HPA-9	GPIIb	*ITGA2B*	c.2602G>A	p.Val837Met
HPA-10	GPIIIa	*ITGB3*	c.263G>A	p.Arg62Gln
HPA-11	GPIIIa	*ITGB3*	c.1976G>A	p.Arg633His
HPA-12	GPIbβ	*GP1BB*	c.119G>A	p.Gly15Glu
HPA-13	GPIa	*ITGA2*	c.2483C>T	p.Thr799Met
HPA-14	GPIIIa	*ITGB3*	c.1909_1911delAAG	p.Lys611del
HPA-15	CD109	*CD109*	c.2108C>A	p.Ser682Tyr
HPA-16	GPIIIa	*ITGB3*	c.497C>T	p.Thr140Ile
HPA-17	GPIIIa	*ITGB3*	c.662C>T	p.Thr195Met
HPA-18	GPIa	*ITGA2*	c.2235G>T	p.Gln716His
HPA-19	GPIIIa	*ITGB3*	c.487A>C	p.Lys137Gln
HPA-20	GPIIb	*ITGA2B*	c.1949C>T	p.Thr619Met
HPA-21	GPIIIa	*ITGB3*	c.1960G>A	p.Glu628Lys
HPA-22	GPIIb	*ITGA2B*	c.584A>C	p.Lys164Thr
HPA-23	GPIIIa	*ITGB3*	c.1942C>T	p.Arg622Trp
HPA-24	GPIIb	*ITGA2B*	c.1508G>A	p.Ser472Asn
HPA-25	GPIa	*ITGA2*	c.3347C>T	p.Thr1087Met
HPA-26	GPIIIa	*ITGB3*	c.1818G>T	p.Lys580Asn
HPA-27	GPIIb	*ITGA2B*	c.2614C>A	p.Leu841Met
HPA-28	GPIIb	*ITGA2B*	c.2311G>T	p.Val740Leu
HPA-29	GPIIIa	*ITGB3*	c.98C>T	p.Thr7Met
HPA-30	GPIIb	*ITGA2B*	c.2511G>C	p.Gln806His
HPA-31	GPIX	*GP9*	c.368C>T	p.Pro123Leu
HPA-32	GPIIIa	*ITGB3*	c.521A>G	p.Asn174Ser
HPA-33	GPIIIa	*ITGB3*	c.1373A>G	p.Asp458Gly
HPA-34	GPIIIa	*ITGB3*	c.349C>T	p.Arg91Trp
HPA-35	GPIIIa	*ITGB3*	c.1514A>G	p.Arg479His

* Nucleotide and protein substitutions are shown as changes from the more common form (a) to the less common form (b). More information can be found at https://www.versiti.org/hpa.

HPA genotyping methods

Identification of the genetics underlying platelet polymorphisms allowed for the development of molecular methods for platelet genotyping. As a result, several molecular-based techniques have been implemented in order to screen for clinically significant HPAs. Although the most commonly used method is PCR with sequence-specific primers (PCR-SSP) as shown in Table 7, newer and more automated high-throughput methods gain more popularity, especially for a large pool of samples such as donor screening or frequency analysis.

In recent years, many high-throughput HPA genotyping methods have been developed for a large-scale screening or frequency analysis. For example, Bead arrays (Dunbar, 2006; Hashmi et al., 2007), Microarray (Avent et al., 2007; Beiboer et al., 2005), High-resolution melting (HRM) method (Hayashi et al., 2012; Kjeldsen-Kragh et al., 2007; Liew et al., 2006; Zhou et al., 2012), MALDI-TOF MS (Garritsen et al., 2009), PCR sequence-based typing (SBT) (Xu et al., 2009), Multiplex SNP genotyping with oligonucleotide extension (Shehata et al., 2011) and Amplicon-based Next-generation sequencing (NGS)(Vorholt et al., 2020). These high-throughput methods allow genotyping of a large number of samples in a short time, moreover, it can be automated which means the risk of contamination is lower however, since expensive instruments, reagents and computer software are required (Curtis, 2008), these platforms are now mainly available for research purposes and in large blood centres.

One crucial step of HPA typing by molecular methods (as with genotyping in general) is DNA isolation must be free of contamination and of a sufficient quantity and quality so as to avoid false positive and negative results. Similarly, the contamination with nucleases must be prevented since this can cause false negative results. Other important points that need to be taken into the consideration *e.g.* optimum PCR conditions, the correct quality control, and carefully interpretation (Hurd et al., 2002).

Table 7 Distribution of HPA-1 to -5 and -15 and methods used for genotyping.

Population	Method employed*	HPA-1		HPA-2		HPA-3		HPA-4		HPA-5		HPA-15		References
		a	b	a	b	a	b	a	b	a	b	a	b	
African-American	Reverse dot blot & PCR	0.920	0.080	0.82	0.18	0.63	0.37	1	0	0.79	0.21	NT	NT	(Kim et al., 1995)
Central African	PCR-SSP & PCR-RFLP	1	0	0.607	0.393	0.500	0.500	1	0	0.595	0.405	0.698	0.302	(Halle et al., 2005)
Argentinean	PCR-SSP	0.878	0.122	0.875	0.125	0.612	0.388	1	0	0.927	0.073	0.511	0.489	(Elena et al., 2008)
German	PCR-SSP	0.812	0.188	0.914	0.086	0.523	0.477	1	0	0.926	0.074	NT	NT	(Legler et al., 1996)
Danish	PCR-SSP	0.831	0.168	0.917	0.082	0.625	0.374	1	0	0.921	0.078	NT	NT	(Steffensen et al., 1996)
Finnish	PCR-ASRA	0.86	0.14	0.91	0.09	0.59	0.41	NT	NT	0.95	0.05	NT	NT	(Kekomäki et al., 1995)
Norwegian	MCA	0.867	0.133	0.943	0.057	0.471	0.529	1	0	0.929	0.071	0.505	0.495	(Randen et al., 2003)
Dutch	PCR-ASRA	0.846	0.154	0.934	0.066	0.555	0.445	1	0	0.902	0.098	NT	NT	(Simsek et al., 1993)
Slovenian	PCR-SSP	0.809	0.191	0.891	0.109	0.591	0.407	0.997	0.003	0.934	0.066	NT	NT	(Rozman et al., 1999)
Australian	PCR-SSP	0.858	0.142	0.927	0.073	0.619	0.381	1	0	0.905	0.095	NT	NT	(Bennett et al., 2002)
Pakistani	PCR-SSP	0.885	0.115	0.920	0.080	0.690	0.310	1	0	0.900	0.100	0.590	0.410	(Bhatti et al., 2010)
Japanese	PCR-PHFA, -SSP, -RFLP	0.998	0.002	0.898	0.102	0.594	0.406	0.990	0.010	0.960	0.040	NT	NT	(Fujiwara et al., 1996; Tanaka et al., 1996, 1995)
Korean	Reverse dot blot & PCR	0.995	0.005	0.870	0.130	0.670	0.330	1	0	0.970	0.030	NT	NT	(Kim et al., 1995)
Han Chinese	PCR-SSP	0.994	0.006	0.952	0.048	0.595	0.401	0.995	0.005	0.986	0.014	0.532	0.468	(Feng et al., 2006)
Taiwanese	PCR-SSP	0.996	0.004	0.965	0.035	0.558	0.442	0.998	0.002	0.985	0.015	0.531	0.463	(Pai et al., 2013)
Vietnamese	PCR-SSP & PCR-RFLP	0.986	0.014	0.953	0.047	0.486	0.514	1	0	0.972	0.028	0.477	0.533	(Halle et al., 2004)
Filipino	PCR-RFLP	0.980	0.002	0.975	0.025	0.530	0.470	0.995	0.005	0.965	0.035	0.480	0.520	(Shih et al., 2003)
Indonesian	PCR-SSP	0.977	0.023	0.940	0.060	0.507	0.493	0.948	0.052	0.968	0.032	0.551	0.449	(Asmarinah et al., 2013)
Burmese	PCR-SSP	0.946	0.054	0.970	0.030	0.603	0.397	0.995	0.005	0.984	0.016	0.498	0.502	(Phuangtham et al., 2017)
Karen	PCR-SSP	0.979	0.021	0.963	0.037	0.558	0.443	0.998	0.002	0.994	0.006	0.525	0.475	(Phuangtham et al., 2017)
Thai	PCR-SSP	0.985	0.015	0.952	0.048	0.560	0.440	1	0	0.968	0.032	0.491	0.509	(Kupatawintu et al., 2005)
Northeastern Thais	PCR-SSP	0.972	0.028	0.938	0.058	0.533	0.467	1	0	0.963	0.037	0.498	0.502	(Phuangtham et al., 2017; Romphruk et al., 2000)
Thais	MALDI-TOF & AD	0.974	0.026	0.940	0.060	0.539	0.461	1	0	0.968	0.032	0.552	0.448	**Study I and additional unpublished work**

*PCR-ASRA, Allele specific restriction assay polymerase chain reaction; PCR-PHFA, PCR-preferential homoduplex formation assay; MCA, Melting curve analysis. NT, not tested.

The present investigation

Aims

This thesis has explored primarily blood group polymorphism among the Thai people with the aim of looking for variation that might give potential protection against malarial infection. Furthermore, we sought a better understanding of the interaction between erythrocyte surface molecules and malaria parasites, and to provide some information about the function of blood group antigens in malarial invasion.

The specific aims of each study included here are as follows:

Study I Characterize the blood group and HPA allele profile of 396 Thai blood donors by the MALDI-TOF MS platform, and correlate the results with RhD, RhCE, MNS, K/k phenotype, as well as resolve apparent discrepancies. To specifically target known and potential malaria receptors, for example, ACKR1, MNS, Vel, and CD147, and look for further polymorphism in this cohort

Study II Determine the genetic background underlying the P^k phenotype in two Thai sisters suffering from multiple spontaneous abortions

Study III Identify the variant glycophorin genes in our sample set and to define the s antigen carried by these GP(B-A-B) hybrids using a panel of anti-s reagents

Study IV Evaluate the effect of platelet concentration and/or platelet:erythrocyte (P:E) ratios on *Plasmodium falciparum* erythrocyte invasion

Methods

The methods used in these studies are well-established and validated. A list of methods utilised in this thesis is described briefly in Table 8.

Table 8 Methods used in this thesis.

Methods	Study	Brief description and usage
Genomic DNA extraction	I, II, III	Silica-membrane-based nucleic acid purification was used to prepare DNA from whole blood samples (Qiagen AB).
MALDI-TOF MS	I	A high-throughput blood group genotyping platform used to characterize 36 blood group-related SNPs in Thai cohort (Gassner et al., 2013).
Allelic discrimination real-time PCR	I, II	TaqMan SNP genotyping assays were used in a real-time PCR system to discriminate SNPs, *SMIM1* rs1175550, *ACKR1* rs118062001 and SNPs in HPA-2, -3, -4, and -15. (ThermoFisher Scientific)
Allele-specific real-time PCR	I	Allele-specific primers with real-time PCR was used to screen for *RHCE* rs14735308. Primers were designed in-house.
PCR-ASP	II	PCR with allele(sequence)-specific primers was used for screening of *B3GALNT1* c.420T>G. Primers were designed in-house.
DNA cloning	I	Insertion of DNA fragments into a plasmid vector used for *GYPB* cDNA analysis.
Flow cytometry	I, II, III, IV	Laser-based detection of single cells using fluorochrome-conjugated antibodies or fluorescent dye. Flow cytometry was widely used in this thesis for analysis of antigen expression on RBCs and parasitized RBCs.
Sanger sequencing	I, II, III	Fluorescence-based DNA sequencing by capillary electrophoresis used to identify genetic variants in *GYPB* exon 3-4 and *B3GALNT1.*
Hemagglutination	I, II, III	Standard immunohematological techniques using commercially available reagents or human antibodies to identify existing blood group antigens on RBCs or use known-antigen RBCs in order to identify antibody.
Total RNA extraction	I	A phenol-chloroform based RNA isolation used to extract RNA from buffy coat samples.
Short tandem repeat analysis	II	A molecular biology method used to compare allele repeats at specific loci in DNA in Study II to estimate kinship.
Malaria culture / Parasite culture	IV	*In vitro* malarial parasite cultivation under low oxygen environment (Ménard, 2013; Moll et al., 2013).
Co-culture/ invasion assay	IV	Malaria parasite culture with the presence of platelets used to estimate the effect of platelets on erythrocyte invasion of *P. falciparum.*

Summary of Results

Study I: Thai blood groups by mass spectrometry

A new high-throughput technique for genotyping, MALDI-TOF MS platform, was chosen to characterize the blood group allele profile of 396 Thai blood donors, and correlate the results with RhD, RhCE, MNS, K/k phenotype, as well as resolve apparent discrepancies. We targeted known and potential malaria receptors, for example, ACKR1, MNS, Vel, and CD147, and looked for further polymorphism among our cohort. Since the MALDI-TOF MS platform is focused primarily to detect SNPs of importance in transfusion medicine, we extended our investigation to include other selected blood group–related polymorphisms with potential relevance in malaria. Thus, our data set provides new information regarding these receptors.

Genotyping results showed 100% concordance with the phenotyping results for RhD, K, and S/s. Interestingly, the ratio of group O to group B was opposite in the two donor groups: in Lampang, the ratio of O:B was 1.6:1, whereas in Saraburi the ratio was 1:1.24.

Investigation of 3 *RHCE* outliers with the R_1R_z phenotype revealed an e-variant antigen encoded by the *RHCE*02.22* allele, which had been described previously in nine samples in a study of RhCE variants, and was at that time identified in individuals of Caucasian descent (Pham et al., 2011). We speculated that this allele might be masked in R_1R_1 samples, which account for more than 60% of the samples in the cohort. Therefore, screening for rs147357308 (*RHCE* c.667T) was performed in real-time using allele-specific primers and confirmed by sequencing. We found that the frequency of this SNP was 3.3% of *RHCE*02* alleles, of which five samples carried *RHCE*02.22*, and seven samples carried the *RHCE*02.04* allele as defined by sequencing. This finding was surprising, because *RHCE*02.04* (*RHCE*CeVA*) had been previously described only in whites, and was associated with an altered C antigen (Noizat-Pirenne et al., 2002).

Our study shows the c.667T polymorphism to be relatively common in the Thai and amounted to 3.3% among the *RHCE*02* alleles. When investigating the prevalence of rs147357308 in tables of human variation, such as 1000 Genomes and ExAC (accessed through www.ensembl.org), the minor allele of this SNP was shown to be present only among the Chinese Dai in Xishuangbanna at approximately 1% in the East Asian superpopulation. The Thai are not represented in the East Asian superpopulation; however, this subpopulation bears the most similarity to the Thai people. The

prevalence is similar to that in the African superpopulation, where c.667C>T is found on the *RHCE*01.07* alleles (approx. 1.6%) and also encodes a variant e antigen.

In the MNS blood group system, MN typing discrepancies in 41 samples revealed glycophorin variants of which 40/41 were due to Mi^a-carrying hybrid proteins. Samples that were genotypically *GYPA*01* homozygotes but also carried glycophorin hybrids reacted with anti-N. This is due to the hybrid that carries ´N´ and which cross-reacts with routine anti-N reagents. Through PCR-ASP of our cohort (Hsu et al., 2013), we found that 66 of 396 samples (16.7%) carried an Mi^a-encoding glycophorin gene. Strikingly, a higher prevalence was observed in the northern region (Lampang; 21.3%) compared to the central region (Saraburi; 12.1%), which is significantly different (P = 0.015). Our data reinforce the gradient from north to south among the different ethnic groups that had been seen also by Kaset and colleagues (Kaset et al., 2015). Furthermore, the incidence of hybrid alleles in our cohort was higher than that seen previously, where the average has been between 9 and 10%. This may be due to a relative insensitivity of serological testing in the earlier studies, and may explain the higher than expected incidence of anti-Mi^a in transfused patients (Kupatawintu et al., 2010).

As expected, a high prevalence of Fy(a+) individuals (98.4%) was observed. However, we also observed an unexpected level of polymorphism among these *FY*01* alleles. Nine samples (2.3%) carried the Fy^x-characteristic weakening mutation, c.265C>T, on the *FY*01* allele, of which six were identified in blood donors from the north. As predicted Fy^a antigen expression was weaker in those samples carrying c.265T. This polymorphism has been shown to weaken the expression of Fy^b in Caucasian populations where it is carried on *FY*02*, most often in linkage disequilibrium with *FY* c.298A (Olsson et al., 1998). In our samples, no c.298A was detected and this is consistent with the report of Arndt et al. who described the *FY*01W.01* allele in a Vietnamese individual (Arndt et al., 2015). In contrast, we did not see an effect on Fy^a antigen expression by flow cytometry in RBCs from donors carrying *ACKR1* rs118062001 (c.199C>T; p.Leu67Phe), which was found in 100 samples. Even though there was no apparent effect on antigen expression, the Duffy protein is a known ligand for *P. vivax*, which is endemic in this region and malaria is more prevalent in the north and the northwest of Thailand. The prevalence of *FY*01* is 89.1% in our cohort and one may speculate that modification of this protein by c.265C>T or by c.199C>T may reduce susceptibility to invasion. The polymorphism c.265T on an *FY*B* background has been shown to reduce invasion by *P. vivax* (Albuquerque et al., 2010) but the effect of c.199T and c.265T on a *FY*A* allele remains to be investigated.

Six samples (1.5%) were heterozygous for the *JK*02N.01* allele, of which five genotyped as *JK*02*/*JK*02N.01* and one as *JK*01*/*JK*02N.01*. The latter typed

Jk(a+b–) as expected. The null alleles were more common in the central region (5/6 samples).

Screening for the SMIM1 c.64_80del polymorphism underlying the Vel-negative type revealed all samples to be wildtype. Flow cytometry using monoclonal anti-Vel was used to analyze Vel antigen expression on 223 samples of which 216 samples were homozygous for rs1175550A and the remaining seven samples genotyped as rs1175550A/G. Homozygosity for rs1175550A showed lower median fluorescence intensity (MFI) for Vel antigen expression than heterozygous samples ($P = 0.017$).

Vel antigen expression on RBCs is known to be influenced by the rs1175550 genotype and resulted in considerable variation from one person to another. Individuals homozygous for the common rs1175550A polymorphism show weaker expression (Christophersen et al., 2017). In this study, all but seven samples tested were rs1175550A homozygotes and showed weak but variable expression of Vel as determined by flow cytometry. This might explain the curious findings of Chandanayingyong and colleagues (Chandanayingyong et al., 1967), who identified 4 of 328 samples (1.2%) as Vel-negative by serology. Given that antigen expression is so variable, we suspect the Vel negative samples reported might, in fact, have been weakly Vel-positive.

Five samples genotyped *DI*01*/*DI*02*, and were confirmed serologically to be Di(a+b+). A single sample each of the following genotypes was detected: *CO*01*/*CO*02*; *YT*01*/*YT*02*, *IN*01*/*IN*02*. Since basigin (CD147), the carrier of the Ok blood group, is a malaria receptor (Crosnier et al., 2011) we analysed 165 RBC samples by flow cytometry using monoclonal anti-CD147. They were all positive and no apparent variation in protein expression was observed.

Study II: A novel nonsense *B3GALNT1* mutation in Thai sisters

In this study, we investigated the molecular background behind the rare P^k phenotype in two Thai sisters who had suffered multiple early spontaneous abortions due to the presence of naturally occurring anti-P in their plasma. The GLOB blood group system consists of two antigens namely P and PX2. P is widely distributed including on fetal heart and liver, as well as the placenta and makes these organs a ready target for antibody-mediated destruction. The gene responsible for P and PX2 antigen expression, *B3GALNT1*, is located on 3q26.1 and encodes 3-β-N-acetylgalactosaminyltransferase, which catalyses the addition of *N*-acetylgalactosamine (GalNAc) in β1–3 linkage to P^k, the precursor of P (Figure 5). The sera of P-negative individuals contain naturally occurring antibodies directed against the lacking antigens.

The most potent antibody is anti-P, which can cause serious intravascular hemolytic transfusion reactions because of its ability to bind and activate complement. Moreover, women with the rare P_1^k /P_2^k phenotypes have a higher incidence of early spontaneous abortions due to cytotoxic attack by anti-P of IgG_3 type on the globoside-rich placental tissue (Cantin and Lyonnais, 1983; Lindstrom et al., 1992).

The two sisters had suffered in total 19 miscarriages, most of which occurred in the first trimester. Their RBCs were tested for the presence of P/P1/PX2/P^k antigens as well as their corresponding antibodies using hemagglutination and flow cytometry. The proposita's RBCs typed as P–P1+PX2–P^k+, *i.e.* P_1^k phenotype, whereas her sister's typed as P–P1–PX2–P^k+, *i.e.* P_2^k phenotype. Anti-P and anti-PX2 were found in their plasmas. Sequencing results revealed that both sisters were homozygous for a novel nonsense mutation, c.420T>G, in *B3GALNT1*. This substitution introduces a premature stop codon, p.Tyr140Ter, which is predicted to severely curtail the 3-β-N-acetylgalactosaminyltransferase, *i.e.* the P synthase. A fully functional 3-β-N-acetylgalactosaminyltransferase contains 331 amino acids. The c.420T>G substitution found in the two sisters, lead to a premature stop after 139 residues, severely truncating the *B3GALNT1* coding region, losing the enzymatically active domain downstream of the nonsense mutation (Paulson and Colley, 1989). Thus, even if no attempts to measure the enzymatic activity of the *B3GALNT1* product were performed, it is reasonable to assume that the detected mutation is causative of the phenotype found, given that 58% of the coding region is lost. Furthermore, it is likely that the resulting transcripts may be subjected to nonsense-mediated mRNA decay, thereby eliminating the product altogether (Kervestin and Jacobson, 2012).

Since inactivating mutations in *B3GALNT1* and other blood group genes are often regional in their distribution, and no previous report about any *B3GALNT1* polymorphism among the Thai population has been described, a PCR allele-specific primer (ASP) assay was designed to detect c.420G and the Thai blood donor cohort was screened to determine the prevalence of this polymorphism. Heterozygosity in one individual in 384 donors was observed, suggesting a prevalence of 0.13%.

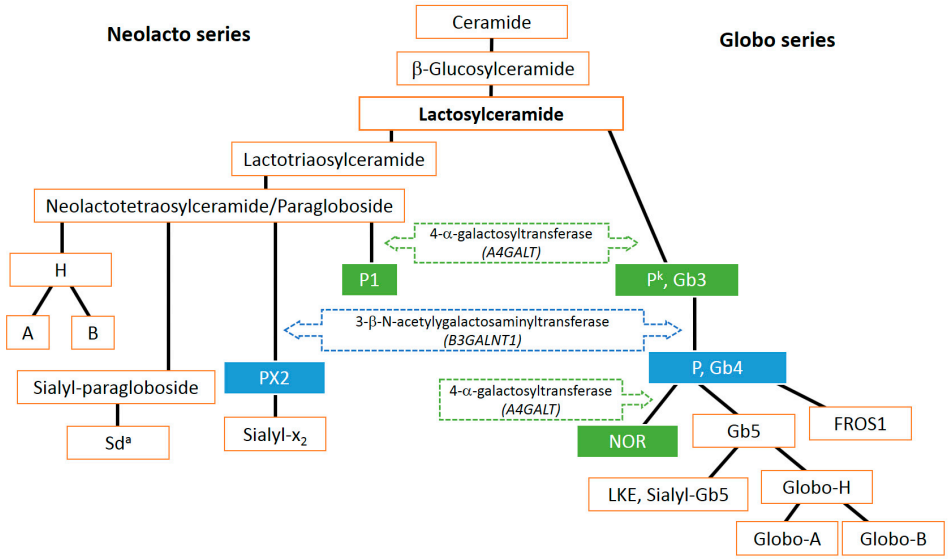


Figure 5 Schematic representation of the synthesis pathways of glycosphingolipids in the neolacto and globo series. Antigens involved in Study II are showed in green boxes for P1PK or in blue boxes for the GLOB blood group system. The glycosyltransferases responsible for glycan chain elongation and encoding genes are given in dashed arrow blocks. (Figure inspired by J Westman's PhD thesis)

It is also interesting to note that the donor's hometown, located in the Northeastern region of Thailand, is approximately 350 km away from the proposita's village, located in the Upper Central region of Thailand. Since the geography cannot rule out lineage, a microsatellite analysis between the donor and the sisters was performed to compare STRs at 13 loci. It revealed high homology between the samples, indicating possible kinship. It is possible that these markers are more frequent in the Thai population, making it difficult to assess if 0.13% is an over- or under-estimation of the frequency in the general Thai population.

In conclusion, a novel nonsense mutation (c.420T>G) in *B3GALNT1* (*GLOB*01N.13*) was described, thereby adding to the twelve alleles already known in the GLOB system, all published by the group in Lund.

Study III: Altered s antigen on GP(B-A-B) hybrid proteins

We focused on the molecular characterisation of hybrid glycoproteins that were identified in Study I. These hybrid molecules are well-established to be more common in SE Asia and this was borne out by the data in Study I that established the frequency of Mi(a+) glycoproteins as 16.7%.

DNA from 63 Thai blood donor samples in which a *GYP* hybrid gene had been amplified by PCR were investigated further by sequencing. Both polyclonal and monoclonal anti-s were used by flow cytometry for semiquantitative analysis of s expression and correlated with the glycophorin genotype.

The DNA sequencing results are summarised in Table 9. As expected the predominant hybrid allele present was *GYP*Mur* but in addition we identified two different *GYP.Bun*-like alleles, here designated *GYP*Thai* and *GYP*Thai II*. Seven samples (7/63) were *GYP*Thai* heterozygotes and thus, this allele is relatively common in our population (Minor Allele Frequency; MAF = 0.011).

Table 9 Summary of sequencing results of 63 samples previously identified to carry a *GYP* hybrid gene.

	Predicted S/s phenotype		
Genotype	**S–s+**	**S+s+**	**Total**
*GYP*Mur/GYPB*04*	51	0	51
*GYP*Mur/GYP*Mur*	3	0	3
*GYP*Thai/GYPB*04*	6	0	6
*GYP*Thai/GYPB*03*	0	1	1
*GYP*Thai/GYP*Mur*	1	0	1
*GYP*Thai II/GYP*Mur*	1	0	1
Total	62	1	63

Phenotype was predicted based on genotyping results by MALDI-TOF (Study I).

All samples carrying a hybrid allele were confirmed Mi(a+) by serological testing. *GYP*Mur* homozygotes had significantly lower reactivity when tested with IgM monoclonal anti-s P3BER when compared to *GYPB*04/04* controls: $P = 4x10^{-5}$. *GYP*Mur* heterozygotes were also lower: $P = 7.9x10^{-4}$. Reactivity with polyclonal anti-s was significantly increased with the homozygotes ($P = 1.6x10^{-5}$) and trending towards a slight increase for heterozygotes *(*$P = 0.054$). Reactivity of *GYP*Thai* heterozygous RBCs were shown to react similarly to those of *GYP*Mur* heterozygous RBCs. It is interesting to note that four samples that did not carry a *GYPB*04* allele

were nonreactive with clone P3BER whilst enhanced reactivity was observed with polyclonal anti-s. A third anti-s (monoclonal, P3YAN3) could not differentiate consistently between s antigen carried on normal GPB or hybrids. Surprisingly however, one sample identified to be a *GYP*Mur*/*GYP*Thai* compound heterozygote had significantly increased reactivity when tested with this anti-s. This phenomenon was also found in *GYP*Mur* homozygous RBCs.

We have demonstrated that the s antigen carried on these hybrids is qualitatively and quantitatively altered. IgM monoclonal anti-s, P3BER, did not recognize the s antigen on these GP hybrids at all. These results infer that this antibody requires a native GPB sequence.

This is quite remarkable but does agree with biochemical analysis performed earlier by Dahr who identified the amino acids $\mathbf{T}^{44}\mathbf{E}^{47}\mathbf{T}^{48}\mathbf{V}^{52}\mathbf{H}^{53}$ in the native GPB sequence, and their potential glycosylation, to be involved in s antigen recognition (Dahr, 1986). Examining the amino acid sequence on GP.Mur and GP.Bun, only one amino acid, p.Thr44, disappears (Figure 6). These results are in strong contrast to the work of Halverson (Halverson et al., 2009) who reported that the reactive epitope for serologically specific murine monoclonal anti-s appears to be downstream of p.Thr48 and defined by a core peptide sequence, QLVHRF, that could inhibit the antibodies independently of p.Thr48.

Even though studies regarding the GP(B-A-B) hybrids and their frequency in Thai populations have also been carried out by others (Chandanayingyong and Pejrachandra, 1975; Palacajornsuk et al., 2007), none of these studies report the *GYP*Bun*-like allele that we identified in our study. However, this allele was first identified in two Chinese from a large cohort of blood donors (Wei et al., 2016). Interestingly, we identified this allele in as much as 8 of 63 Mi(a+) samples (12.6%) which is relatively high in this group and suggests that this is a common variant in Thais. At the phenotypic level, this allele encodes a GP.Bun hybrid that carries the characteristic Hop antigen.

Another remarkable result is the novel *GYP*Bun*-like allele identified in this study (*GYP*Thai II*), which is predicted to express a GP.Bun hybrid protein but which has a much longer insertion of *GYPA* intron 3. This sample showed an intermediate expression of s antigen that was clearly different from that of GP.Bun or normal s antigen and surprisingly, typed Hop–. A possible explanation could be that the conformation required for Hop antigen presentation was altered since the glycophorins are known to assemble in the membrane as homo- and heterodimers, and this sample was a compound heterozygote together with *GYP*Mur*.

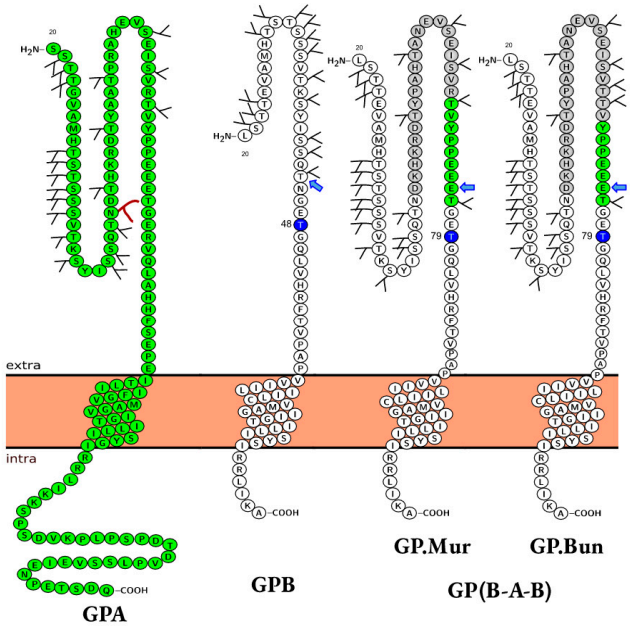


Figure 6 Schematic representation of GPA, GPB, GP.Mur and GP.Bun proteins in the RBC membrane. The amino acids in the hybrid proteins are derived from *GYPB* (open circles) and *GYPA* (filled green circles). The blue filled circle indicates the threonine that defines the s antigen. Gray circles show amino acids derived from *GYPB* pseudoexon 3 that are different to those encoded by *GYPA* exon 3. The Y shapes in black, attached to the polypeptides, represent potential O-glycosylation, the Y in red represents N-glycosylation. The blue arrow indicates p.Thr44 in GPB, which can be O-glycosylated, compared with the corresponding sequence in GP(B-A-B) hybrids. It should be noted that *GYP*Thai* encodes a hybrid protein that is identical to GP.Bun.

Study IV: Platelets inhibit *P. falciparum* erythrocyte invasion

The effect of platelets was explored on invasion of erythrocytes by *Plasmodium falciparum*. One of the signs of malaria in addition to anemia is thrombocytopenia, that is, low levels of platelets. Interestingly, other groups have shown that platelets are directly involved in killing of *P. falciparum* (Kho et al., 2018; McMorran et al., 2012, 2009).

In order to investigate the effect of different concentrations of platelets on erythrocyte invasion, we used the standard laboratory-adapted strains of *P. falciparum* parasites, W2mef and FCR3S1.2. Parasites were cultured in human group O RhD+ peripheral blood erythrocytes in 0.5% albumax-complete RPMI medium at 1% hematocrit at 37°C in low oxygen environment using the candle jar technique (Ménard, 2013; Moll et al., 2013) and supplement with platelets at predetermined ratios. We found that at platelet:erythrocyte (P:E) ratios ranging from 1:100 to 1:20, platelets inhibited the parasite's invasion in a dose-dependent manner. Invasion efficiency of FCR3S1.2 and W2mef of *P. falciparum* as measured by flow cytometry and compared to the control culture, decreased to the lowest levels of 56% and 64%, respectively when platelets were present at a ratio of 1:20. At ratios of 1:10, 1:5 and 1:2, the invasion efficiency was found to be widespread from very low at 36% and up to 112%. Overall, the presence of platelets in the cultures had an inhibitory effect on *P. falciparum* in both strains (FCR3S1.2 $P = 6.72 \times 10^{-9}$; and W2mef $P = 2.87 \times 10^{-7}$). All results are summarized in Table 10.

Table 10 Invasion efficiency (%) of *P. falciparum* in presence of P:E ratios ranging from 1:100 to 1:2 estimated by flow cytometry.

P:E ratio of	%Invasion efficiency			
	FCR3S1.2		W2mef	
	n	mean±SD	*n*	mean±SD
Invasion control	9	100±0.1	9	100±0.3
1:100	9	92.0±7.1	9	96.0±14.2
1:50	7	78.9±5.7	7	91.6±10.6
1:20	7	56.3±7.2	7	63.6±7.2
1:10	9	66.7±17.2	9	65.4±21.2
1:5	7	83.9±12.2	7	92.4±9.9
1:2	6	69.7±21.3	6	77.3±16.9

We suspect that the apparent invasion effect at higher platelet concentrations may result from aggregation and attachment of platelets to erythrocytes. To test this theory, the invasion efficiency at the ratios of 1:10 and 1:20 estimated by flow cytometry and manual counts under microscopy were compared. The result was not confirmed by inspection of the thin smears as shown in Table 11.

Moreover, although microscopic evaluation revealed aggregation of platelets and attachment of platelets to erythrocytes at high P:E ratios as expected, this was not found to specifically to parasitized erythrocytes (as shown in Fig. 2 in Study IV). Even if invasion does not show the same linear correlation at high platelet numbers, platelet-dependent killing, as witnessed by increased extracellular parasites, remains effective.

Table 11 Invasion efficiency (%) estimated by flow cytometry detecting the DNA staining using acridine orange and manually counting of Giemsa-stained thin blood smears.

	% Invasion efficiency (mean±SD, *n*= 4)			
	FCR3S1.2		**W2mef**	
P:E ratio	Flow cytometry	Thin blood smear	Flow cytometry	Thin blood smear
1:20	52.0±5.5	74.0±9.6	64.0±6.2	86.5±14.9
1:10	63.3±8.7	71.3±8.9	66.5±13.1	73.0±16.5

This study shows that platelets have an effect even at low concentrations, and appear to contradict early studies (Peyron et al., 1989) in which increased numbers of platelets beyond the physiological range showed greater inhibition.

We used two laboratory *P. falciparum* strains, W2mef and FCR3S1.2, which both require sialic-acid for successful invasion: both have been shown to have different invasion efficiency into trypsin-treated erythrocytes indicating different erythrocyte receptors are required for erythrocyte invasion (Triglia et al., 2005). The *P. falciparum* parasite W2mef strain was derived from the IndoChina III/CDC clone originally isolated from a Laotian man while the FCR3S1.2 was originally isolated in Gambia, West Africa. (https://www.m.ehime-u.ac.jp/school/parasitology/eng/Strain1.htm). Thus, the two strains can be said to represent two cases of clinical malarial infection in Africa and Asia.

Platelets can inhibit the invasion of *P. falciparum* strains W2mef and FCR3S1.2 at P:E ratios that mirror not only physiological platelet numbers but also at ratios that would ordinarily be considered as thrombocytopenic. As shown in a 1:100 P:E ratio, a reduced

parasitemia as measured by flow cytometry was observed and this effect increased until the ratio reached 1:20. While a ratio of 1:10, still within physiological range, did not show a clear cut effect with flow cytometry, in visual inspection of thin smears, fewer parasitized erythrocytes were observed suggesting that platelets were still inhibitory. Our results do not support the data of Peyron et al. (Peyron et al., 1989) since no increased effect on inhibition was observed in our study at P:E ratios beyond physiological numbers.

Microscopic examination showed the presence of platelet aggregates and of platelet-erythrocyte adherence. While we might have expected adherence to infected erythrocytes, this was not the case and no specific pattern was observed. This correlates to some degree with a previous report by Crosnier et al. (Crosnier et al., 2011) who showed that increased parasite staining by flow cytometry was due to extracellular parasites and debris. We observed extracellular parasites in the thin smears at all ratios however, adherence of aggregated platelets to the parasites was observed more often when platelets were present at higher ratios. Thus, it is difficult to determine cause or effect. Activated platelets may directly inhibit parasite growth and/or erythrocyte invasion, or the complexes of extracellular parasite-aggregated platelets may be incorrectly counted as parasitized erythrocytes by flow cytometry. This could cause either over- or under-estimation of parasitemia. In this study, no attempt to determine the activation state of the platelets was undertaken so this question remains to be answered.

Additional unpublished work

The original aims of the thesis included to seek a better understanding of the interaction between erythrocyte surface molecules and malaria parasites, and to provide information about the function of blood group antigens in malarial invasion. Therefore, some questions of interest were asked:

- Does the difference of causative agents (between Southeast Asia and Africa) involve polymorphism of host receptors?
- Do the rare variants of RBC surface proteins among healthy Thai blood donors have any effects to *Plasmodium* spp. invasion susceptibility?

According to WHO, *Plasmodium* spp. primarily responsible for malaria in WHO South-East Asia Region, including Thailand, are *P. falciparum* and mixed (52%), and *P. vivax* (48%) whereas malaria in Africa mostly due to infection by *P. falciparum* (more than 76%)(WHO, 2019). As has been discussed above, much is known about invasion receptors for different malaria species and strains but there are also many unanswered questions. One such question concerns an α-chymotrypsin-sensitive RBC receptor, sometimes designated W (Gilberger et al., 2003), for *P. falciparum* (see Table 4 and Fig. 4), which has not yet been identified. Given that my supervisor, Jill Storry, discovered a new erythroid protein that appeared to be sensitive for this protease (Storry et al., 2013), we decided to focus on this question together with collaborators Kristina Persson and Martin L Olsson.

Does SMIM1 play a role in RBC invasion by *P. falciparum*?

While the Vel blood group antigen was first reported in 1952 (Sussman and Miller, 1952) and the genetic background was disclosed 60 years later (Ballif et al., 2013; Cvejic et al., 2013; Storry et al., 2013), the function on RBC membrane of SMIM1 protein remains to be determined. Originally, we hypothesised that SMIM1 may be a receptor for *P. falciparum* and our theory was supported by the following:

- We had experimental evidence to support that the SMIM1 protein was a single-pass type 1 TMP with a GXXXG motif (Storry et al., 2013) similar to GPA, which has been shown to be a receptor for *P. falciparum*.
- SMIM1 does not appear to be glycosylated, yet resistant to trypsin and sensitive to α-chymotrypsin treatment. This matched well with the characteristics of an unknown erythrocyte receptor (receptor Z (Duraisingh et al., 2003) for the *P. falciparum* ligand *P. falciparum* reticulocyte binding homologue 2b (PfRh2b), which had been described as a trypsin resistant and chymotrypsin sensitive receptor (Sahar et al., 2011), but for which no specific receptor on erythrocyte membrane had yet been identified. It also matches the characteristics of the binding partner for EBA-181, which is also supposedly trypsin-resistant but α-chymotrypsin-sensitive. Interestingly, PfRh2b and EBA-181 appear to depend on each other and possibly work together (Lopaticki et al., 2011).
- SMIM1 was found to be phosphorylated on p.Ser22 and p.Ser28 in a study of the global phosphoproteome in erythrocytes infected with *P. falciparum*, along with 25 other human proteins, several of which are well-known erythroid proteins, such as Band 3, Glycophorin C, Protein 4.1, Kell, CD44, and Ankyrin-1(Solyakov et al., 2011).

It therefore seemed possible that SMIM1 protein could be part of malaria pathogenesis, and needed to be investigated.

Having established and optimised *P. falciparum* cultivation in our laboratory, we investigated the role of SMIM1 protein in erythrocyte invasion by *P. falciparum*. Vel-negative and Vel-positive erythrocytes from voluntary anonymised blood donors were collected and included in an invasion assay to compare the parasite invasion efficiency. In order to keep these rare erythrocytes for a longer period of time, RBCs were droplet-frozen using liquid nitrogen as described in *Judd's methods in immunohematology* (Judd et al., 2008).

The strains 3D7, W2mef, and FCR3S1.2, standard laboratory-adapted strains of *P. falciparum* parasites, were cultured and synchronized as described in Study IV. It can be noted that 3D7 but not W2mef were originally found to depend on the α-chymotrypsin-sensitive receptor for invasion (Gilberger et al. 2003). Interestingly, the same study also noticed that 3D7 had much stronger expression of EBA-181, a parasite protein acting as a ligand for an unknown RBC receptor.

For invasion assays, magnet-purified schizonts were added to acceptor erythrocytes at 1% hematocrit in complete RPMI (RPMI 1640 containing 25 mM HEPES and 24

mM $NaHCO_3$, supplemented with 20 mg/L hypoxantine, 25 µg/L gentamicin and 0.5% Albumax™ II) at an expected initial parasitemia of 0.5-1%. Vel-negative and a control Vel-positive RBCs of the same ABO blood group and collected at the same time, were tested in parallel. Assays were plated in 6 replicates of 100 µL using 96-well plates. Two replicates (0 hour) were stained with 1:1000 Acridine Orange (AO) for 60 minutes at 37°C and then fixed with 0.1%formaldehyde/glutaraldehyde-PBS solution. Four replicates were incubated at 37°C under low-oxygen environment. After 40 hours of incubation, three replicates (40 hours) were stained as above. All samples were acquired on a FACSCalibur flow cytometer using CellQuest v3.3 (Becton Dickinson) and analysed using FCS Express 6 Flow cytometry software (De Novo Software) to estimate the AO positive, *i.e.* parasitized, RBCs. Giemsa-stained thin smears were prepared from the last remaining replicate and examined under light microscope at 1000x magnification if required. The percentage of parasitized cells; %Parasitemia, was determined and parasite multiplication rate (PMR) was calculated by the formula:

$$PMR = \frac{\%Parasitemia\ at\ 40\ hours}{AVG\ of\ \%Parasitemia\ at\ 0\ hour}$$

From a very first experiment, we used the invasion assay to determine whether Vel status of RBCs affect the invasion of *P. falciparum* or not. By using different strains of *P. falciparum*, we found that they respond differently. The *P. falciparum* strain 3D7; a sialic acid (SA)-independent, surprisingly showed the better growth when culturing with Vel-negative than with Vel-positive RBCs. A similar result was observed for FCR3S1.2; a SA-dependent strain, whereas the opposite trend was observed for W2mef; another SA-dependent strain (Figure 7).

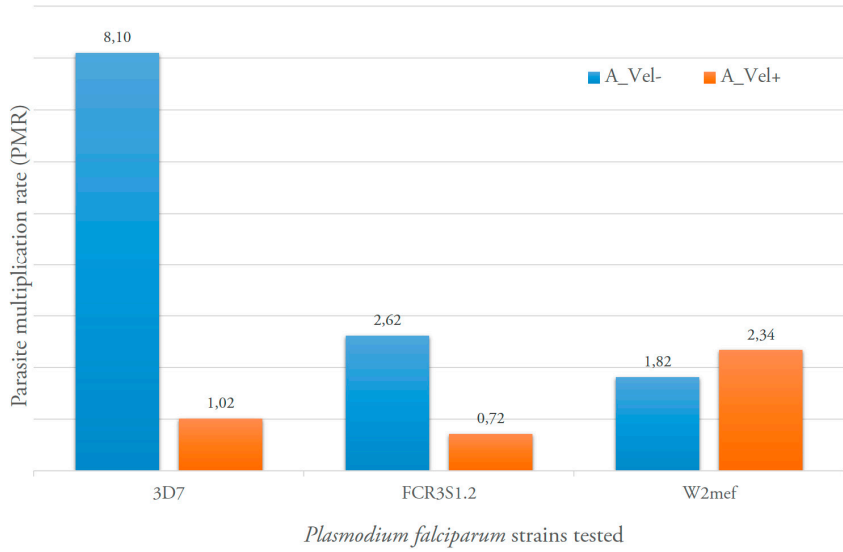


Figure 7 Parasite multiplication rate (PMR) after 40 hours-incubation. Three strains of *P. falciparum* were cultivated with group A Vel-negative (blue, A_Vel–) or Vel-positive (orange, A_Vel+) RBCs.

The subsequent experiments, which were performed using stably well-grown parasites, showed inconclusive results as listed in Table 12 and Figure 8. Though there are no clear cut results, *P. falciparum*, especially W2mef, seemed to preferentially parasitise Vel-negative RBCs or at least, grow better than when cultured with Vel-positive RBCs, particularly when tested with fresh RBCs as in experiments C and E (Figure 8).

Table 12 Parasite multiplication rate (PMR) when cultured with Vel-negative or Vel-positive RBCs.

P. falciparum strain used	*n**	PMR (Mean±SD)	
		Vel-negative RBCs	Vel-positive RBCs
FCR3S1.2	7	3.04±1.41	2.98±1.17
W2mef	4	2.76±1.294	1.91±0.706

* Number of experiments.

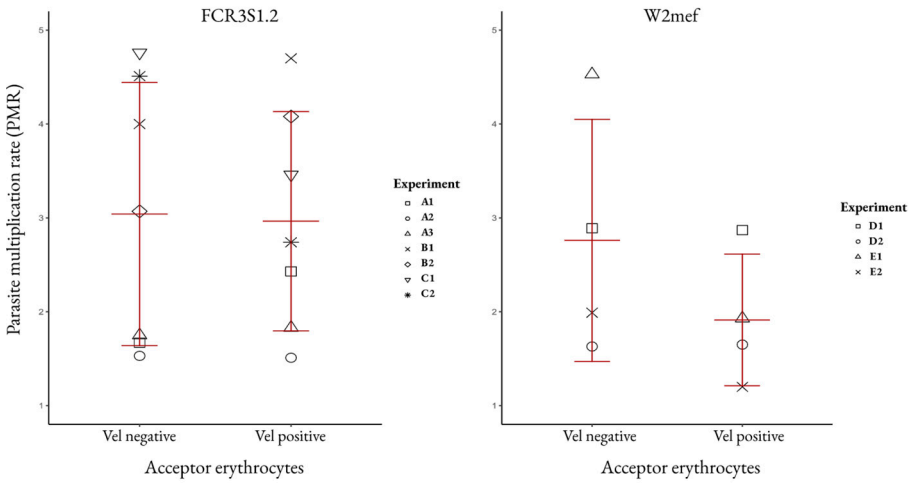
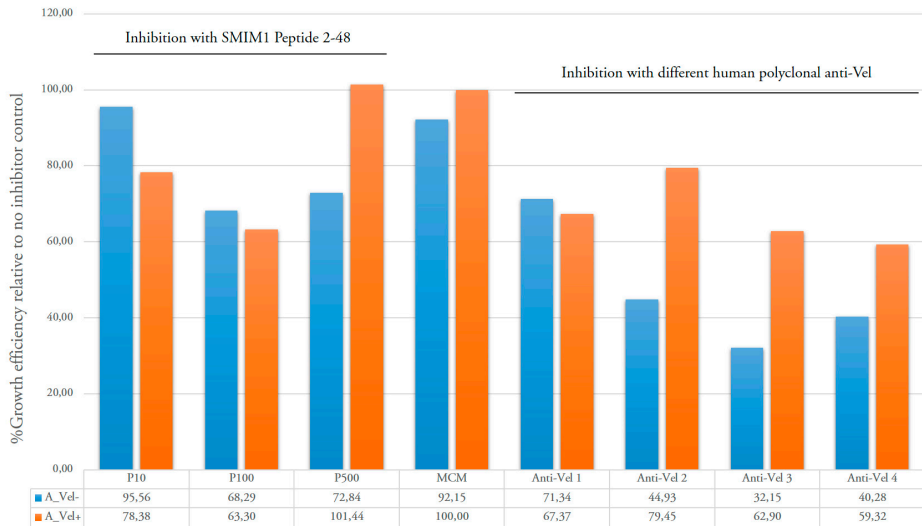


Figure 8 PMR from each experiment were plotted accordingly the acceptor RBCs used in the cultivation.

Inhibition of invasion assays with human polyclonal anti-Vel antibody or with a recombinant SMIM1 peptide (amino acids 2-48) were carried out as above with some modifications, specifically, anti-Vel sera at a final concentration of 10% or SMIM1 peptide at a desired concentration were added before the incubation. Both peptide and anti-Vel showed inhibitory effect to *P. falciparum* invasion as represented in Figure 9. The inhibitory effect of the peptide, however, seems to be a result from non-specific blocking by peptide or protein increasing in the cultivation environment and/or the dilution effect. The major pitfall in the experiment was that no scrambled peptide was tested in parallel and therefore, interpretation of the experiment is difficult. Further experiments, if needed, should be done by setting up a scrambled peptide control along with the tests. Also, the biochemical basis for our hypothesis changed. Recently, it had been proven that SMIM1 protein is a type II TMP with the short C-terminus on the outside of the erythrocyte (Arnaud et al., 2015; Kelley et al., 2019). This is in contrast with the original hypothesis, which postulated that SMIM1 has its N-terminus extracellularly. Thus, the peptide inhibition-invasion assay was performed with the N-terminal portion of SMIM1 and even if it showed signs of minor inhibition of the growth of *P. falciparum*, this effect is probably non-specific.

The anti-Vel inhibition-invasion assay showed the interesting result that an inhibitory effect of 10% plasma was shown in both cultures; either Vel-negative or Vel-positive, growth efficiency appeared to be increased when culture was performed in Vel-positive RBCs. Under the presence of anti-Vel, growth efficiency when cultured with Vel-

negative RBCs ranged from 32.15-71.41% (mean±SD = 47.2±17) whereas a higher growth percentage from 59.32-79.45 (mean±SD = 67.3±8.8) was observed in the presence of Vel-positive RBCs. A major omission from these experiments was a control with 10% inert plasma yet these initial results are striking. Binding of anti-Vel is abolished when the C-terminal cysteine is mutated or the three C-terminal amino acids are deleted. Thus, anti-Vel targets the C-terminus. However, anti-Vel has no target on Vel-negative RBCs so the result is difficult to explain. The results suggest that plasma in itself inhibits invasion *in vitro*, however the effect was less obvious in cultures with Vel-positive RBCs. Since anti-Vel reacts with Vel-positive RBCs no matter how the protein is orientated (the Vel epitope is by definition extracellular), this might lead to blocking of SMIM1 protein on the RBC membrane and this might mimick the tantalizing data that Vel-negative RBCs appear to be more readily invaded.



	P10	P100	P500	MCM	Anti-Vel 1	Anti-Vel 2	Anti-Vel 3	Anti-Vel 4
A_Vel-	95,56	68,29	72,84	92,15	71,34	44,93	32,15	40,28
A_Vel+	78,38	63,30	101,44	100,00	67,37	79,45	62,90	59,32

Figure 9 Growth efficiency (%) of *P. falciparum* strain W2mef cultured with Vel-negative (blue) or Vel-positive (orange) RBCs with presence of SMIM1 peptide 2-48 at final concentration of 10 (P10), 100 (P100), 500 (P500) µg/mL or different human polyclonal anti-Vel at final concentration of 10%. Percentage presented is relative to the parasite cultured in malaria culture medium (MCM) with same Vel phenotype as 100% control. Percent growth showed for Vel-negative in MCM is related to Vel-positive in MCM.

A study by Solyakov (Solyakov et al., 2011) showed that SMIM1 was phosphorylated after malarial infection along with several other erythroid proteins, and our preliminary results showed a lower parasite growth rate in Vel-positive RBC culture. We therefore speculated that phosphorylated SMIM1 may take part in parasite killing inside RBCs. This seems to support the fact that frequency of Vel-negative is higher in non-malaria areas such as in Sweden whereas it shown to be an even higher-frequency antigen in malaria endemic area, *e.g.* Thailand and Africa. However, much is still unclear regarding the role of SMIM1 in *P. falciparum* pathogenicity.

The experiments related to malaria parasite culture required a very long time for optimizing cultivation, validating the synchronization protocol, and verifying the flow cytometry acquisition. Until now, we only have some preliminary results that are insufficient to draw any conclusions but can serve as a good basis for follow-up experiments. Further investigation needs to be done and some of the below suggestions should be taken in to consideration when work continues:

- Cultures with a SA-independent strain of *P. falciparum*; 3D7 for instance, should be used to determine the function of candidate receptors involved in SA-independent invasion pathway. This seems to be particularly important given the data mentioned above (Gilberger et al., 2003).
- Since ABO and MNS blood group systems are known to be related with *P. falciparum* pathogenesis, these blood group polymorphisms should be selected carefully when testing blood samples are collected.
- If SMIM1 protein is to be proven to take part in parasite killing, the *SMIM1* rs175550A/G SNP related to Vel expression on RBCs could be tested further for its relationship with malarial susceptibility.
- If peptide blocking experiments should be carried out, not only should the N-terminal peptide used be changed for a C-terminal sequence but a scrambled peptide should be included as a negative control.
- Apart from Vel status, many other host factors differ between the Vel-positive and Vel-negative RBCs used so far. It would be optimal to be able to use a knock-out system to make sure only SMIM1 differs between the test and control target cells.

Platelet polymorphism in Thais

Based on the discrimination of SNPs performed using MALDI-TOF MS, the frequencies of HPA-1 and HPA-5 variants in the cohort were reported (Study I). We later used also allele-discrimination real-time PCR techniques described in Study I, to test for HPA-2, -3, -4, and -15 and the frequency of the most clinically significant HPA polymorphisms among Thais were established as presented in Table 13.

Table 13 Frequencies of HPA-1 to HPA-5 and HPA-15 in the Thai population.

		Genotype frequencies (%)			Allele frequencies	
HPA	***n***	**aa**	**ab**	**bb**	**a**	**b**
HPA-1	385	94.8	6.2	0.0	0.974	0.026
HPA-2	396	88.6	10.9	0.5	0.940	0.060
HPA-3	396	29.5	48.7	21.7	0.539	0.461
HPA-4	396	100	0	0	1	0
HPA-5	385	93.8	6.0	0.3	0.968	0.032
HPA-15	396	30.3	49.7	19.9	0.552	0.448

Result for HPA-1 and HPA-5 were obtained from MALDI-TOF MS while those for HPA-2, -3, -4 and -15 were obtained from allele-discrimination real-time PCR.

Genotype and allele frequency in our cohort showed the same trend compared to studies by others (Kupatawintu et al., 2005; Phuangtham et al., 2017; Romphruk et al., 2000), however, the frequency screening is only a beginning of our study. It is still on-going, with the aim to develop a multiplex assay using PCR-SSP to screen for common HPA variants. We do not only aim for setting up the valid single-tube PCR screening method but also for use of whole blood for direct screening, with no DNA isolation step needed. This has previously been developed as part of a B.Sc. project at the Nordic reference laboratory for screening of blood donors negative for high-frequency blood group antigens.

The most clinically significant HPAs belong to six biallelic systems (HPA-1, -2, -3, -4, -5 and -15) and will be identified by our multiplex PCR system. The set of primers targeting the HPA-1a, -2a, -3a, -4a, 5a, -15a and -15b alleles will be used for DNA amplification. The test will be developed based on a commercially available PCR system (Extract-N-Amp™ Blood PCR Kit, Sigma-Aldrich) which allows screening of HPA alleles directly from anticoagulated whole blood (Wagner et al., 2008).

This study is a continuing project following the work of another B.Sc. student, in which a multiplex assay for detection was attempted for four HPAs including 1a, 2a, 5a and 15a in a one-tube direct PCR. Problems were encountered with non-specific bands

when the HPA-3a primers were added into the mixture, and difficulties with HPA-15b amplification also arose. In addition, HPA-5a amplification needed to be improved since the band was faint. As we already established the distribution of the most clinically significant HPA polymorphisms among Thai cohort and these DNA are readily available, we will use the DNA from this cohort as a sample source for validating purposes.

A gel photography of a gradient annealing temperature PCR result for HPA-3a optimization is shown in Figure 10. Unfortunately, when we tried to combine all primers in a single tube, non-specific bands appeared. Primers have been redesigned more carefully and now attempts are on-going to set up in two multiplexes and perhaps extend the assay to identify more alleles such as HPA-1b, HPA-3b, and HPA-5b.

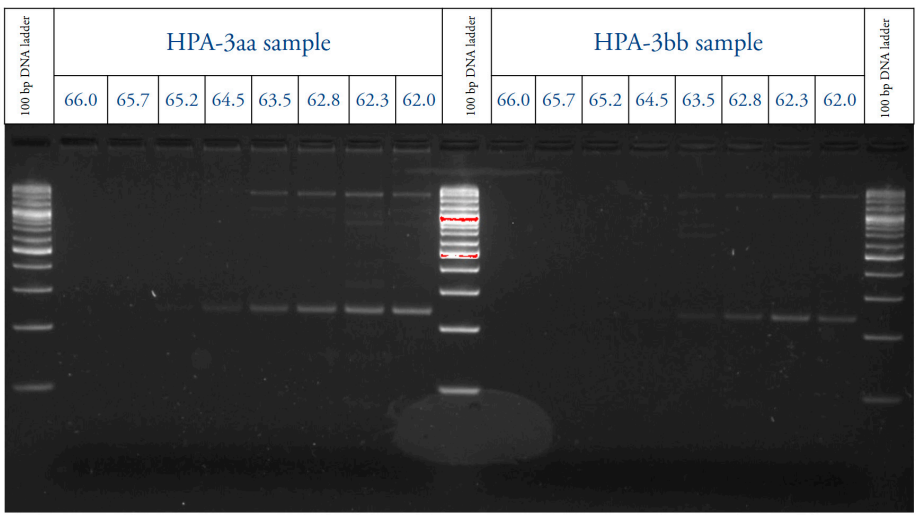


Figure 10 Primers for HPA-3a amplification were tested using gradient PCR. The specific product size is 251 base pairs. The result showed that an appropriate annealing temperature is 64.5°C at which the product from the positive sample (HPA-3aa) was observed whilst no product from the negative sample (HPA-3bb) was observed. However, amplification is rather weak in the HPA-3aa sample and may not be entirely negative with HPA-3bb either so further optimization may be needed.

General discussion

Old variants, new alleles

In Study I, we identified several interesting alleles which have not been previously reported in the Thai population. For example, *RHCE*02* c.667T which, with a prevalence of 3.3%, was shown to be relatively common. This c.667T polymorphism can be found in the African superpopulation but, interestingly, on the *RHCE*01* allele instead. Another allele identified here is *FY* c.265T, a polymorphism carried on *FY*02* in the Caucasian and other populations. In our population we found this polymorphism carried on *FY*01* and accounted for 2.3% which again, is relatively common. It is interesting that we found the same variants known to affect blood group expression but on different allelic backgrounds in the populations that live around equatorial belt where malaria is endemic. One may speculate that modification of these proteins may play a role in minimizing infection by *Plasmodium* spp.

The Vel phenotype in the Thai

Another blood group antigen that we investigated in depth is the Vel blood group antigen. The Vel-negative phenotype is rare, found in 1 in ~4 000 people worldwide and more frequent, 1 in ~1 700 in Scandinavians (Reid et al., 2012) and more specifically in 1 in ~1 200 in southern Sweden (Storry et al., 2013). In an early study, Chandanayingyong and colleagues reported the apparent finding of 4 Vel-negative individuals in 328 (1.2%) Thai (Chandanayingyong et al., 1967). The Vel antigen is carried on SMIM1. While the function of SMIM1 protein on the erythrocyte membrane is unknown, it was originally suggested to be a possible receptor molecule for *P. falciparum* (Storry et al., 2013). We have shown that all samples in our cohort were wildtype SMIM1 homozygotes, *i.e.* Vel-positive with 97% homozygosity for rs1175550A, a weaker expression polymorphism. We found that only 7 samples carried rs1175550G, the stronger expression polymorphism, and all 7 samples were heterozygous. Thus, the allele frequency of rs1175550G in Thai population is 0.018 which is as low as can be found in Asian population, whilst frequency among other populations are higher especially in African (https://www.ncbi.nlm.nih.gov/snp/rs1175550#frequency_tab). The rs1175550A is located in a regulatory motif involved in SMIM1 transcription (Christophersen et al.,

2017) and people homozygous for rs1175550A show variable but low Vel antigen strength and that it is quite possible that the 4/328 individuals in the early study were most likely very weakly Vel-positive.

Mi(a+) variants in malaria

Glycophorin-deficient RBCs demonstrated some degree of resistance to *P. falciparum* invasion (Daniels, 2013). We did not identify any GP-deficient phenotype in our cohort, but on the other hand, Mi(a+) GP(B-A-B) hybrid proteins were common with a prevalence of 16.7%, which we investigated further in Study III. As expected, *GYP*Mur* is the most common variant in our population and accounted for 88.9% of *GYP(B-A-B)* hybrids. This raises a question, does this variant of RBC surface proteins have any effects to *Plasmodium* spp. invasion susceptibility? One study performed in Thailand (Pruksa et al., 2013) has shown that, in fact, this GP variant seems to enhance *P. falciparum* erythrocyte invasion. The parasite ligand EBL-1 is known to bind with a sialylated glycan epitope residing on GPB during the invasion (Li et al., 2012). Even though no specific sequence has been identified yet, the invasion enhancement might be related to more sialylated glycans on the GP hybrid protein since the hybrid protein appears to be longer than a normal GPB and likely to have more O-glycosylation on its molecules (see Figure 6). As the frequency of the *GYP*Mur* allele and GP(B-A-B) hybrid variants is relatively high in our population, we had hoped to test these cells to determine their susceptibility to *P. falciparum* but the experiments have been put on hold because cultivation with frozen/thawed RBCs gave inconsistent results despite previous data showing that this should be fully possible (Gaur et al., 2003).

Variation in GLOB

The GLOB blood group system consisting of P and PX2 antigens is closely related to P1PK blood group system since P antigen is an extension of P^k antigen whereas NOR, the third antigen in P1PK blood group system, is an extension of P (see Figure 5). The null phenotype of this blood group system is a rare phenotype with a naturally occurring anti-P in the globoside-deficient individual's sera. Pregnant women who have anti-P in their plasma have a higher than normal rate of spontaneous abortion due to cytotoxic IgM and IgG3 antibodies directed against the P antigen (Reid et al., 2012). The prevalence of P antigen in all populations is more than 99.9% and P-negative individuals have mainly been found in Scandinavian, Arabian and Finnish people. In study II, we established the allele frequency for the novel *B3GALNT1* c.420T>G mutation as 0.13% in our cohort of Thai blood donors, however we cannot be sure whether it is an over- or under-estimation. It was shown in Study II that the single blood donor found positive for c.420G shared STR markers with the P^k sisters, which could signal kinship. If so, the frequency may be an overestimation of the general

population rate. Since we identified at least two homozygotes and possibility for three more heterozygotes; their parents and a younger sister, it could be assumed that the frequency of the mutation allele is higher than expected but based on a single family. We cannot exclude that this SNP is distributed among the Thai, particularly in the region where it was found. So far, one could say that this allele is a Thai-specific variant. Furthermore, anti-PP1P^{k} in Thais have been reported in previous studies in a total of 4 cases (Kupatawintu et al., 2010; Urwijitaroon et al., 1999). Even though the genetic background of these cases was not reported it may hint towards a higher frequency of this null phenotype related to the GLOB blood group system than currently known.

The carbohydrate blood group antigens are well-known to be widely used as pathogen receptors. Although, many of them are shown to be associated with the diseases caused by bacteria or viruses, the ABO and SID blood group systems have both been reported to be involved in pathogenesis of *P. falciparum.* Absence of A antigen on RBCs prevents severe malaria in *P. falciparum* infection (Fry et al., 2008; Rowe et al., 2007) while strong expression of Sd^{a} antigen (the Cad++ phenotype) has been reported to inhibit *P. falciparum* invasion (Cartron et al., 1983). In addition, sialic acid on the RBC surface is crucially important for many malarial strains to invade and glycophorins A, B and C are all very rich in this negatively charged sugar residue. In addition, the LKE antigen (SSEA-4) is a sialylated blood group antigen, but is not known to be involved in malaria so far. Until now, the function of GLOB blood group antigens is still a mystery and no evidences of involving in *P. falciparum* infection revealed so far whilst globoside (P antigen) serves as a receptor for several pathogens including P-fimbriated *E.coli* and Parvovirus B19 (Hellberg et al., 2013).

Platelets and P. falciparum

One of the signs of malaria in addition to anemia is thrombocytopenia, which is when lower than normal number of platelets are present in the peripheral blood. Though studies by others have shown that platelets are directly involved in killing of *P. falciparum*, these focus on normal and higher levels of platelets (Kho et al., 2018; McMorran et al., 2012; Peyron et al., 1989). Therefore, we wanted to investigate the effect of different concentrations of platelets on erythrocyte invasion, and in particular, the ratio that represented thrombocytopenia. We have showed in Study IV that platelets can inhibit the invasion of *P. falciparum* strains W2mef and FCR3S1.2 at P:E ratios that mirror the physiological platelet numbers and at ratios that would ordinarily be considered as thrombocytopenic. This study can be considered as a pilot study since we did not investigate the activation status of the platelets and more interestingly, the critical receptors involved in inhibiting invasion.

Conclusions

The main conclusions from this thesis are listed below.

Study I MALDI-TOF MS is a powerful and efficient method for rapid routine genotyping and investigation of outliers identified novel variation among our samples. The expected high prevalence of the Mi(a+) phenotype was observed from both regions tested. Of interest, we identified 9 sample (2.3%) that carried *FY* c.265T, a polymorphism carried on *FY*01* instead of *FY*02* as found in Caucasian population. Of potential clinical relevance in a region where transfusion-dependent thalassemia is common, we identified two *RHCE*02* alleles known to encode an e-variant antigen.

Study II A novel nonsense mutation (c.420T>G) in *B3GALNT1* (*GLOB*01N.13*) that disrupts the synthesis of P antigen and results in the clinically relevant P^k phenotype was described, adding to the twelve alleles already known in the GLOB system.

Study III DNA sequencing not only confirmed that *GYP*Mur* is the most frequent allele of the variant glycophorin genes in Thai blood donors, but also identified a *GYP*Bun*-like allele that we have shown to be relatively common (MAF = 0.01) in this group. Both alleles have a previously uncharacterized effect on s expression on RBCs, qualitatively and quantitatively.

Study IV Platelets showed an inhibitory effect on erythrocyte invasion by *P. falciparum* under physiological ratios (approx. 1:10 - 1:40) in a dose-dependent manner and still effective under thrombocytopenia-like conditions. At higher platelet numbers, the trend was reversed and platelets did not further increase the inhibitory effect on erythrocyte invasion. However, platelet-dependent killing, as witnessed by increased extracellular parasites, remained effective.

Popular scientific summary

An amazing 84% of all our cells are red blood cells (RBCs) and together with platelets (5%) and leucocytes (1.5%) blood is the single largest organ in our body. These cells will circulate around the body, and while RBCs deliver oxygen to various tissues in exchange for CO_2, platelets act as sentinels for vessel and tissue damage. The surface of both RBCs and platelets consists of many molecules that are important for cell function: so-called (glyco)proteins and (glyco)lipids, (where "glyco" means that these molecules also carry sugar chains). In the same way as we are all different from each other, so can these surface molecules on blood cells differ slightly from one person to another. These small differences are called antigens because they can stimulate production of antibodies if blood carrying an antigen is transfused into a patient whose RBCs lack that antigen. Similarly, pregnant women can make antibodies to antigens on the RBCs of their fetus that are inherited from the father. Antibodies to blood group antigens can cause RBC destruction of transfused RBCs or fetal RBCs, sometimes with severe clinical outcomes.

The difference in antigens of blood groups between individuals is called blood group polymorphism. The word is derived from the word *poly* (multiple) and *morph* (form). It is thought to have many origins but scientists agree that one of the most important causes is selective pressure from various pathogens, which results in the selection of genes that help increase the chances of survival. Blood group polymorphism can be seen in individuals from the same geographic region but also between different populations.

One of the greatest drivers of polymorphism in RBCs is malaria, caused by different species of the *Plasmodium* parasite. These organisms require RBCs for replication and have developed a sophisticated arsenal of different proteins in order to invade the cells, including those molecules that we recognise as blood group antigens. Malaria is endemic to all countries around the world's equatorial belt and in these different populations there is clear evidence that certain rare blood groups have arisen due to their protective effect. The best example is in West Africa, the RBCs of almost all individuals have a blood group called Duffy-negative, Fy(a–b–), in which a whole protein is absent. This protein, called ACKR1, has been shown to be the primary binding partner for *Plasmodium vivax*, and thus evolution has over the years, given an

advantage to people whose RBCs lack the protein. However, there are many other polymorphisms on RBCs that have been shown to be important in parasite invasion. Malaria is also endemic in Thailand and neighbouring countries and both *P. vivax* and *P. falciparum* are equally prevalent.

Thus the aims of this thesis were to investigate blood group and platelet polymorphism in Thailand as well as to conduct studies involving molecules on red blood cells and platelets related to the *P. falciparum* invasion of RBCs. Samples were collected from blood donors from two regional blood centres, Lampang and Saraburi. The rapid and efficient MALDI-TOF MS technology was used to analyse the blood group genes of these donors and the results were compared with their group antigen profile. Thus the blood group distribution at both the genetic and antigen level of these 396 donors could be mapped.

Some results were expected, for example, 16.7 % of the blood donors were Mi(a+), which correlated with previously reported frequencies of this blood group antigen in Southeast Asia. We continued our studies to expand the results of this large number of Mi(a+) blood donors. By using DNA sequencing, *GYP*Mur* was confirmed as the most common allele in the glycophorin hybrid group found in Thai donors. We also detected two new *GYP.Bun*-like alleles: *GYP*Thai*, which was the second most common allele in our study with a frequency of 1.1%; and *GYP*Thai II*, which was found in one sample only. *GYP*Mur* and *GYP*Thai* alleles were shown to have an effect on the detection of MNS antigens, both qualitative and quantitative. This is important in considering blood for transfusion in Mi(a+) patients.

Interesting variants were also identified in other blood group systems that are potential candidates for further studies on malaria invasion. These include a weakened form of the Fy^a antigen that, given this protein's role in *P. vivax* invasion, may be an advantage in these individuals. Similarly, a rare *RHCE* allele in the Rh blood group system that altered expression of the RhC and Rhe antigens, showed similarities with an *RHCE* allele in Africa, and which can cause problems with giving and receiving blood, especially in thalassemia patients who are regularly transfused.

In another study, the Thai donor samples were also tested for a rare mutation in the *B3GALNT1* gene (c.420T> G) after it was identified in two Thai sisters. Both had the very rare blood group phenotype called P^k. Women who have this blood type have a very high chance of miscarriage in the first trimester of pregnancy because the body produces antibodies that can destroy the cells of the fetus. This study allowed us to find and explain the cause of this special blood type at the genetic level. The new mutation was detected in one blood donor only, confirming that it is rare but also suggesting the presence of a Thai-specific allele.

Like RBCs, platelets also carry antigens (HPA) which can vary in frequency in different parts of the world. The Thai donor cohort was analysed genetically although the expected antigen frequencies were observed, a multiplex assay to screen for these HPA alleles directly from blood was investigated. While platelet polymorphism may not play an important role in *P. falciparum* invasion of RBCs, a role for platelets in general was investigated and therefore, the relationship between platelets and malaria invasion was studied. The results of the experiments show that platelets can inhibit the red blood cell invasion by *P. falciparum*, both at normal levels and at low levels.

This thesis examines the unexpected but subtle blood group diversity in Thailand, a region that has long been a malaria-endemic area and provides new avenues of potential investigation.

Popular scientific summary in Thai

เม็ดเลือดแดง (erythrocyte หรือ red blood cell; RBC) เป็นเซลล์ที่พบได้มากที่สุดในร่างกาย และหากรวมเข้ากับเกล็ดเลือด จะทำให้มีปริมาณมากถึงร้อยละ 90 ของเซลล์ทั้งหมดที่ประกอบขึ้นเป็นร่างกายของเรา เซลล์เหล่านี้จะเดินทางไปรอบ ๆ ร่างกาย โดยเม็ดเลือดแดงจะทำหน้าที่ขนส่งออกซิเจนให้กับเนื้อเยื่อต่าง ๆ ในขณะที่หน้าที่หลักของเกล็ดเลือด คือการซ่อมแซมความเสียหายที่เกิดขึ้นในหลอดเลือด บนผิวของเซลล์เหล่านี้มีโมเลกุลจำนวนมากที่มีความสำคัญต่อการทำงานของเซลล์นั้น ๆ ทั้งยังมีความแตกต่างกันไปในแต่ละคน โดยที่ความแตกต่างนี้ แม้เพียงเล็กน้อยก็สามารถส่งผลกระทบกับผู้ป่วยที่ได้รับเลือดหรือหญิงตั้งครรภ์ โดยการกระตุ้นให้มีการสร้างแอนติบอดีต่อโมเลกุลที่แตกต่างกันนี้ โมเลกุลที่อยู่บนผิวเซลล์เหล่านี้ เรียกว่า แอนติเจนของหมู่เลือด (blood group antigens) นอกจากจะมีความแตกต่างกันระหว่างแต่ละบุคคลแล้ว ยังมีความแตกต่างกันไปตามแต่ละพื้นที่หรือประเทศต่าง ๆ ทั่วโลกอีกด้วย

ความแตกต่างของแอนติเจนของหมู่เลือดระหว่างแต่ละบุคคล เรียกว่า โพลีมอร์ฟิซึมของหมู่เลือด (blood group polymorphism) ซึ่งมีรากศัพท์มาจาก คำว่า poly (multiple; มาก หลากหลาย) และ morph (form; รูปแบบ ลักษณะ) ความแตกต่างของแอนติเจนเชื่อว่ามาจากหลายสาเหตุ แต่นักวิทยาศาสตร์มีความเห็นสอดคล้องกันว่า สาเหตุสำคัญที่สุดคือ แรงกดดัน (selective pressure) จากเชื้อโรคต่าง ๆ ที่ทำให้เกิดการคัดเลือกจีนส์ (genes) ที่ช่วยเพิ่มโอกาสการรอดชีวิต และเชื้อโรคที่มีบทบาทมากที่สุดคือ ปรสิตที่ก่อโรคมาลาเรีย หรือเชื้อในสปีชี่ส์พลาสโมเดียมนั่นเอง

การกระจายของมาลาเรียในพื้นที่ต่าง ๆ ของโลก มีความสัมพันธ์กับการพบหมู่เลือดหายากอย่างชัดเจน เช่น ในอัฟริกาตะวันตก ซึ่งมีการระบาดของมาลาเรียจากเชื้อพลาสโมเดียมฟัลซิพารัม แต่ไม่พบว่ามาจากเชื้อพลาสโมเดียมไวแว็กซ์เลย ชาวอัฟริกันเหล่านี้เกือบทั้งหมดมีหมู่เลือดที่เรียกว่า ดัฟฟี่ลบ (Duffy-negative; Fy(a−b−)) ซึ่งส่งผลให้เชื้อพลาสโมเดียมไวแว็กซ์ ไม่สามารถรุกรานเม็ดเลือดแดงได้ ประเทศไทยรวมทั้งประเทศในแถบเอเชียตะวันออกเฉียงใต้นับเป็นพื้นที่ที่มีการระบาดของมาลาเรียเช่นกัน แต่พบว่านอกจากเชื้อพลาสโมเดียมฟัลซิพารัมแล้ว เชื้อพลาสโมเดียมไวแว็กซ์ยังเป็นสาเหตุของโรคมาลาเรียในผู้ป่วยเกือบครึ่งหนึ่งอีกด้วย นอกจากนี้หมู่เลือดหายากที่มีการศึกษามาแล้วว่ามีความสัมพันธ์กับการก่อโรคมาลาเรีย ยังไม่ค่อยมีรายงานหรือการศึกษาในชาวไทยมากนัก

วิทยานิพนธ์นี้ จึงได้ทำการศึกษาและรวบรวมข้อมูลของแอนติเจนหมู่เลือดและเกล็ดเลือดในกลุ่มตัวอย่างคนไทย รวมทั้งทำการศึกษาเบื้องต้นที่เกี่ยวข้องกับโมเลกุลบนเม็ดเลือดแดงและเกล็ดเลือดที่เกี่ยวข้องกับการบุกรุกเม็ดเลือดแดงของเชื้อพลาสโมเดียมฟัลซิพารัม โดยคาดหวังว่าจะสามารถเข้าใจและอธิบายความสัมพันธ์ของโมเลกุลที่แตกต่างกันบนผิวเม็ดเลือดแดงนี้ กับการรุกรานเม็ดเลือดแดงของเชื้อพลาสโมเดียมฟัลซิพารัมได้

ในขั้นต้น เราได้สำรวจความหลากหลายหรืออีกนัยหนึ่ง ความแตกต่างของหมู่เลือดของคนไทย โดยเก็บตัวอย่างเลือดจากผู้บริจาคโลหิตจากศูนย์บริการโลหิตสองแห่ง คือที่ลำปางและสระบุรี เพื่อนำมาตรวจวิเคราะห์หาแอนติเจนของหมู่เลือดที่อาจช่วยป้องกันการติดเชื้อมาลาเรียได้ ในการศึกษานี้เราใช้เทคโนโลยีที่เรียกว่า MALDI-TOF MS ซึ่งเป็นเทคโนโลยีที่ทันสมัยและมีประสิทธิภาพสำหรับการตรวจเพื่อจำแนกความแตกต่างระดับจีนส์ได้อย่างรวดเร็ว ด้วยเทคโนโลยีนี้ เราจึงตรวจความแตกต่างระดับจีนส์และแปลผล ซึ่งนำไปสู่การสร้างแผนภาพการกระจายของหมู่เลือดทั้งในระดับแอนติเจนและระดับจีนส์ของผู้บริจาคโลหิต 396 คน พร้อมกันนี้ เรายังทำการทดสอบเพิ่มเติมสำหรับกรณีที่ผลขัดแย้งกัน เพื่อหาสาเหตุและอธิบายความเป็นไปได้ให้มากที่สุด

เราพบความชุกของหมู่เลือดชนิด Mi(a+) สูงถึง 16.7% ซึ่งเป็นไปตามที่คาดไว้ สัมพันธ์กับรายงานการศึกษาที่เคยมีมาก่อน รวมทั้งหมู่เลือดนี้เป็นที่ทราบกันว่า มีความชุกสูงในพื้นที่เอเชียตะวันออกเฉียงใต้ เราได้ทำการศึกษาต่อเพื่อขยายผลจากการพบหมู่เลือดชนิด Mi(a+) เป็นจำนวนมากนี้ โดยใช้การตรวจลำดับดีเอ็นเอ (DNA sequencing) ซึ่งทำให้เรายืนยันได้ว่า *GYP*Mur* เป็นอัลลีล (รูปแบบของดีเอ็นเอที่เฉพาะและแตกต่างจากชิ้นส่วนดีเอ็นเออื่น) ที่พบบ่อยที่สุดในกลุ่มของไฮบริดไกลโคฟอริน (glycophorin hybrid) ที่พบในผู้บริจาคโลหิตคนไทย นอกจากนี้เรายังตรวจพบอัลลีลใหม่ ซึ่งเราให้ชื่อว่า *GYP*Thai* อัลลีลใหม่นี้ เป็นอัลลีลรองที่พบได้บ่อยในคนไทย โดยมีความชุกอยู่ที่ร้อยละ 1.1 เรายังพบอีกด้วยว่า อัลลีล *GYP*Mur* และ *GYP*Thai* มีผลกระทบต่อการตรวจหาแอนติเจน s ซึ่งเป็นแอนติเจนของหมู่เลือดระบบ MNS ทั้งในแบบที่ทำให้ตรวจไม่พบหรือให้ผลบวกอ่อน ซึ่งข้อมูลนี้ มีความสำคัญต่อการตัดสินใจเลือกน้ำยาที่ใช้สำหรับทดสอบหาแอนติเจน s ซึ่งมีความสำคัญในการให้และรับโลหิต เราพบการกลายพันธุ์ที่เรียกว่า c.265T บนจีนส์ของหมู่เลือดดัฟฟี่ในหลายตัวอย่าง สิ่งที่น่าสนใจคือ c.265T นี้พบบนอัลลีล *FY*01* แทนที่จะพบบน *FY*02* เหมือนที่พบในคนผิวขาวหรือคอเคเซียน และเรายังตรวจพบอัลลีล *RHCE*02* ซึ่งเป็นอัลลีลหายากของหมู่เลือดระบบอาร์เอช (Rh system) และมีความสัมพันธ์กับการเกิด e-variant ซึ่งสามารถสร้างปัญหาต่อการให้และรับเลือด โดยเฉพาะในผู้ป่วยธาลัสซีเมียที่เป็นกลุ่มที่มีโอกาสที่จะต้องรับเลือดเป็นประจำ

ในอีกการศึกษาหนึ่ง เราพบการกลายพันธุ์ของจีนส์ *B3GALNT1* ชนิด c.420T>G ซึ่งส่งผลให้หญิงไทยสองคนที่เป็นพี่น้องกัน มีหมู่เลือดพิเศษที่เรียกว่า P^k คนที่มีหมู่เลือดนี้ มีโอกาสสูงมากที่จะแท้งบุตรในช่วงไตรมาสแรก เนื่องจากร่างกายมีการสร้างแอนติบอดีที่สามารถทำลายเซลล์ของ

ทารกในครรภ์ได้ การศึกษานี้ทำให้เราพบและอธิบายต้นเหตุของหมู่เลือดพิเศษนี้ในระดับจีนส์ นอกจากนี้ เรายังตรวจพบอัลลีลนี้ในผู้บริจาคโลหิตอีกหนึ่งราย ซึ่งชี้ให้เห็นว่า อัลลีลหมู่เลือดหายากนี้มีความจำเพาะในคนไทย

เราให้ความสนใจกับเกล็ดเลือดด้วยเช่นกัน ดังนั้นเราจึงได้ทำการศึกษาถึงความสัมพันธ์ระหว่างเกล็ดเลือดและเชื้อก่อโรคมาลาเรีย ผลจากการทดลองแสดงให้เห็นว่า เกล็ดเลือดสามารถยับยั้งการบุกรุกเม็ดเลือดแดงของเชื้อพลาสโมเดียมฟัลซิพารัมได้ ทั้งในระดับที่เกล็ดเลือดอยู่ในระดับปรกติและอยู่ในระดับต่ำ นอกจากนี้ เรายังแสดงให้เห็นว่า เมื่อระดับของเกล็ดเลือดสูงขึ้น การยับยั้งยังคงมีอยู่ แต่มิได้เพิ่มประสิทธิภาพขึ้น

เป็นที่ทราบกันว่า เชื้อพลาสโมเดียมที่เป็นสาเหตุของโรคมาลาเรียนั้น อาศัยโมเลกุลบนเม็ดเลือดแดงเองในการเข้าสู่เม็ดเลือดแดง แม้ความรู้ในส่วนนี้จะมีอยู่มากแล้ว แต่ยังคงมีการค้นพบและรายงานถึงโมเลกุลใหม่ ๆ บนเม็ดเลือดแดงที่ถูกเชื้อพลาสโมเดียมใช้ในการรุกรานเม็ดเลือดแดงอยู่เสมอ ซึ่งเราเองก็ให้ความสนใจในประเด็นนี้ โดยเราได้พยายามตรวจสอบบทบาทของโปรตีน SMIM1 (เป็นโปรตีนที่ถูกจัดเป็นหมู่เลือด Vel) ในการเข้าสู่เม็ดเลือดแดงของเชื้อ แต่จนถึงขณะนี้ผลการทดสอบยังไม่สามารถสรุปได้ชัดเจน เป็นสิ่งที่สะท้อนภาพประการหนึ่งว่า แม้เรามีความรู้ ความเข้าใจเกี่ยวกับความหลากหลายและความแตกต่างของหมู่เลือดเป็นอันมาก สิ่งที่เรายังไม่ทราบหรืออธิบายได้ ยังคงมีมากกว่า

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