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Red Blood Cell Polymorphism and Susceptibility to *Plasmodium vivax*

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Abstract

Resistance to *Plasmodium vivax* blood-stage infection has been widely recognised to result from absence of the Duffy (Fy) blood group from the surface of red blood cells (RBCs) in individuals of African descent. Interestingly, recent studies from different malaria-endemic regions have begun to reveal new perspectives on the association between Duffy gene polymorphism and *P. vivax* malaria. In Papua New Guinea and the Americas, heterozygous carriers of a Duffy-negative allele are less susceptible to *P. vivax* infection than Duffy-positive homozygotes. In Brazil, studies show that the Fy^a antigen, compared to Fy^b, is associated with lower binding to the *P. vivax* Duffy-binding protein and reduced susceptibility to vivax malaria. Additionally, it is interesting that numerous studies have now shown that *P. vivax* can infect RBCs and cause clinical disease in Duffy-negative people. This suggests that the relationship between *P. vivax* and the Duffy antigen is more complex than customarily described. Evidence of *P. vivax* Duffy-independent red cell invasion indicates that the parasite must be evolving alternative red cell invasion pathways. In this chapter, we review the evidence for *P. vivax* Duffy-dependent and Duffy-independent red cell invasion. We also consider the influence of further host gene polymorphism associated with malaria endemicity on susceptibility to vivax malaria. The interaction between the parasite and the RBC has significant potential to influence the effectiveness of *P. vivax*-specific vaccines and drug treatments. Ultimately, the relationships between red cell polymorphisms and *P. vivax* blood-stage infection will influence our estimates on the population at risk and efforts to eliminate vivax malaria.

1. INTRODUCTION

The image of *Plasmodium knowlesi* 'pulling' its way into erythrocytes of *Macaca mulatta* is iconic in malaria research (Fig. 2.1) and sets the stage for reviewing the mechanisms of human resistance to *Plasmodium vivax*. Aikawa and colleagues described the events underlying erythrocyte invasion to involve an initial attachment to the red cell membrane by the parasite's apical end, invagination of the red cell membrane around the merozoite and sealing of the erythrocyte on completion of invasion (Aikawa et al., 1978). As brilliantly shown through their electron micrographs, evidence of a 'tight junction' formed through

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molecular interactions between the parasite and host continues to inspire malaria research. Identifying specific molecules involved in the formation and gliding motility of this junction is central to unravelling the mechanism of *Plasmodium* species' invasion of the red cell. Understanding how to inhibit, disrupt or block this intimate parasite–host interaction potentially leads to strategies for a vaccine against blood-stage infection, malaria morbidity and mortality.

In this chapter, we rely on a wide range of clinical, field and laboratory findings to illustrate our evolving understanding of the factors that influence resistance to *P. vivax* malaria and the selective barrier that has confronted this parasite. Reviewing this work according to a general chronological time frame will remind readers how our understanding of *P. vivax* infection and malaria has developed over the past 95 years. This approach also seeks to emphasise how medical and basic research scientists have applied available experimental strategies in collaborations across multiple generations to solve the important puzzle as to how malaria parasites infect red blood cells (RBCs) and cause a disease that has had significant impact on human health and the evolution of our genome.

2. THE ERA OF GREAT BIOLOGICAL DISCOVERY

2.1. Cell Biology and the Germ Theory

The late 1800s to the early 1900s was a revolutionary time period that began the integration of medicine and the sciences. Of paramount importance to this chapter is the germ theory that proposed that microorganisms were the cause of many diseases. Pasteur's experimental evidence showing that micro-organisms in nutrient broth did not arise through spontaneous generation (1860s) significantly demystified the relationship between disease and the microbial world, and Koch's series of objective criteria provided a formal test to link specific microbes to specific diseases (1890). Following this lead, in 1880, Laveran first linked human malaria to infection of RBCs by plasmodia (Laveran, 1880) (*P. falciparum* (Welch 1897), *P. vivax* and *P. malariae* (Grassi and Feletti, 1890) as well as *P. ovale* (James, 1929)). During this same time, the medical discipline of psychiatry was coming to understand that infection with the spirochaete bacterium *Treponema pallidum*, caused syphilis (Schaudinn, 1905; Schaudinn and Hoffman, 1905) and that the resulting disease could advance to cause numerous visceral forms and overlapping clinical outcomes (Merrit et al., 1946). Primary infections (marked by the appearance of a chancre at the site of the infection) would heal in 2 to 6 weeks without leaving a scar. Secondary stages would lead to clinical symptoms in approximately half of all cases, producing skin lesions, rash or other generalised inflammatory symptoms. Following a period of latency that could last for years, approximately one-quarter of patients would go on to develop tertiary stages (paretic and tabetic neurosyphilis; general paralysis of the insane (Brandt, 1985)). Despite an improved understanding of the relationship between the microbe and the natural history of the disease, treatment of syphilis remained based on administration of mercury by mouth, injection, dermatologic application or exposure to its vapours to purge the humour through salivation or sweating. Understandably, optimism greeted Paul Erlich's development of the arsenicals salvarsan (1910) and neosalvarsan as potential 'magic bullets' against syphilis; however, these treatments still exposed patients to significant risk and still failed to ward off or cure neurosyphilis (Stokes and Shaffer, 1924; Arnold, 1984; Jolliffe, 1993). Patients with neurosyphilis were difficult to manage, 'became completely demented and unable to care for themselves, dying most often in insane asylums' (Brown, 2000). Accurate syphilis prevalence data is difficult to come by as the disease carried significant social stigma; however, population estimates in Europe and the United States suggested that 15% of the general population had the disease (Stokes, 1918). With at least 10–30% of syphilis patients requiring long-term palliative care, mental institutions were being pushed beyond their effective operating capacities (Stokes, 1918; Chernin, 1984; Hook and Marra, 1992). As a

result, there was considerable interest in developing more effective treatments for neurosyphilis.

Sporadic reports had been published that mentally ill syphilitic patients who experienced bouts of fever showed signs of recovery or remission (Brown, 2000), and in 1876, Rosenblum reported that approximately 50% of psychiatric patients were cured after an attack of 'recurrent fever' (Chernin, 1984). It was also noted that observation of neurosyphilis was uncommon in malaria-endemic regions of Africa (Merrit et al., 1946). In an 1887 review, the Viennese psychiatrist Julius Wagner-Jauregg noted 163 incidents of psychoses remitting following typhoid, intermittent fevers or erysipelas. While findings of this nature seemed to encourage treatment of paretic patients by artificially inducing fever, the dangers of experimental treatment of human beings through exposure to agents such as tuberculin or injection of malarial parasites made physicians reluctant to perform these procedures for fear of legal repercussions (Brown, 2000).

Wagner-Jauregg's first treatments of neurosyphilis patients with malariotherapy occurred in 1917, when a shell-shocked soldier from the Mace-donian Front was admitted to the hospital in Vienna. Coincidently, this patient was experiencing malaria fevers and chills. With blood from this patient, Wagner-Jauregg was able to induce fevers in a small number of paretic patients (Withrow, 1990). While improvements were observed in this first series of malaria-treated patients, complications associated with malaria tropica (falciparum malaria) soon became apparent[1]. After establishing a steady supply of benign tertian malaria (vivax malaria), Wagner-Jauregg reported in 1921 that 25% of his first 200 patients were able to return to work (Brown, 2000). While malariotherapy was not without risk, the success reported by these early trials quickly led to widespread practice throughout Europe and treatment of paretic patients with malariotherapy was first attempted in the United States in 1922. In the United States, equally positive results as experienced in Europe were observed following treatment with tertian malaria as Paul O'Leary and colleagues described in a first report on malariotherapy at the Mayo Clinic (Minnesota) (O'Leary et al., 1926). Because of the impact of malaria treatment on neurosyphilis, Wagner-Jauregg was awarded the Nobel Prize in Medicine in 1927 (Withrow, 1990).

2.2. Malariotherapy and African-Based Resistance to *P. vivax*

Treatment of neurosyphilis in the United States significantly expanded the practice of malariotherapy. In the application of malariotherapy to African-Americans in particular, publications documented that certain individuals were observed to exhibit notable immunity to infection by *P. vivax*. Early evidence of this clinical observation was noted in published discussions of papers presented to the Dermatology and Syphilology section of the American Medical Association.

May 19, 1927; Washington, D.C.

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[1]Human malaria is caused by five parasite species (*Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and recently *P. knowlesi* in Malaysia (Cox-Singh and Singh, 2008)). Tertian malaria (recurring fevers at approximately 48-hour intervals) caused by *P. vivax* has been classified as 'benign', while disease associated with *P. falciparum* has been classified as 'malignant'. While *P. vivax* was preferred over *P. falciparum* by practitioners of malariotherapy (Becker, 1949, Chernin, 1984, Withrow, 1990), the literature notes that induced malaria was not without risks for patients. Furthermore, in a note on 'The Nomenclature of Malaria' by Bruce (1903), it is noted 'I have left out the commonly used terms, simple, benign, 'malignant', pernicious, as they are misleading. The so-called simple tertian may often be more severe than the so-called 'malignant' tertian' (Bruce, 1903). General differences between vivax and falciparum malaria have been described frequently (Zimmerman et al., 2004, Price et al., 2007). *Plasmodium vivax* shows a selective preference for infecting young red blood cells (reticulocytes) (Kitchen, 1938, Garnham, 1966), where *P. falciparum* infects a wider range of red cells (Kitchen, 1939); as reticulocytes comprise less than 1% of the circulating red blood cells, this preference may constrain *P. vivax* parasitaemia. *P. vivax* and *P. ovale* are noted to produce dormant liver stages, termed hypnozoites (Krotoski et al., 1982, Krotoski, 1989). *Plasmodium falciparum* and *P. malariae* do not produce hypnozoites. Hypnozoites can be reactivated after clearance of primary infection to cause relapses weeks to years later.

> Dr. Watson W. Eldridge, Jr., St. Elizabeth's Hospital, Washington, D.C. – I inoculated the first patient in December, 1922, and we have treated approximately 275 cases since … I should like to know whether, in the group in which the malaria failed to take, reinoculations were successful. I have had several inoculation failures, principally among coloured males.
>
> (O'Leary, 1927)
>
> Dr. Paul A. O'Leary, Mayo Clinic, Rochester, Minn. - … We have reinoculated as many as six times those patients who have not developed chills and fever and have not been successful in obtaining a 'take'.
>
> (O'Leary, 1927)

Resistance to vivax malaria was seen to be the primary disadvantage that would prevent malariotherapy from being used as a routine treatment of neurosyphilis. When carefully controlled studies were performed in the context of malariotherapy, details showed that African-Americans and Africans consistently displayed significantly higher levels of resistance to *P. vivax* strains from numerous geographic origins and inoculation doses compared to Caucasians (Young et al., 1955). Additionally, because African-Americans from nonmalarious regions of the United States were as refractory to *P. vivax* infection as those from malarious regions, this resistance was suggested to be natural rather than acquired (Boyd and Stratman-Thomas, 1933; Becker et al., 1946; Young et al., 1946; Young et al., 1955; Bray, 1958). Of further interest, to determine if a *P. vivax* infection once established in an African-American patient would acquire characteristics enabling more successful infection of resistant individuals, Young et al. used blood from a *P. vivax*-infected African-American to inoculate two resistant individuals of the same race (Young et al., 1955). Despite receiving inocula three to seven times higher than that routinely causing blood-stage infection of Caucasian patients, neither of the resistant individuals developed blood-stage parasitaemia. From these results it was concluded that the *P. vivax* strain would not be transformed to acquire characteristics that would enable subsequent infection of resistant individuals (Young et al., 1955). Interestingly, it was also reported that African-Americans displayed resistance to *P. knowlesi* and *P. cynomolgi* in addition to *P. vivax*. From their studies with patients at the Manhattan State Hospital, Milam and Coggeshall reported that African-American patients experienced significantly milder *P. knowlesi* infections compared to Caucasian patients as measured by a delayed time to first blood-stage parasitaemia and shorter duration of blood-stage infection (Milam and Coggeshall, 1938). Following their report of accidental human infections with *P. cynomolgi* (Eyles et al., 1960; Eyles, 1963), Beye et al. wanted to further test whether non-human primates could act as reservoirs of malaria. Their follow-up study included 7 African-American and 13 Caucasian volunteers from the US Penitentiary in Atlanta. An overall summary of their results showed that blood-stage parasites were not observed in any of the African-American study participants, but 12 of the 13 Caucasian patients did exhibit blood-stage parasitaemia (Beye et al., 1961). As *P. knowlesi* and *P. cynomolgi* were observed to have difficulties similar to *P. vivax* for causing malaria in African-American patients and study participants, results have suggested that these parasite species may infect the human RBC by similar invasion pathways.

In 1947, Butler and Sapero published an interesting exceptional report on resistance to *P. vivax* among African-Americans following natural exposure to *P. vivax* in the South Pacific. The authors wrote that, 'With the onset of the present war in the Pacific and the arrival of negro [sic] troops on highly malarious bases in the South Pacific (Melanesia), it was hoped that the negro [sic] might be spared the ravages of Pacific vivax malaria because of his racial tolerance to the United States strains' (Butler and Sapero, 1947).

The authors' study design indicated that the surveyed population included several thousand troops and that 28% were African-American (20–35 years of age; primarily from the Carolinas and Georgia), and virtually no malaria was noted among the study group during training procedures in the United States. Significant exposure to *Anopheles farauti* was noted once the troops were deployed in the South Pacific and while suppressive atabrine was provided, a sizable incidence of initial and recurrent malaria attacks indicated that this prophylactic treatment was not taken regularly. *Plasmodium* species infections were calculated monthly for primary and recurrent attacks. While these infections were not differentiated according to racial groups, the study observations suggested that this was not a serious omission. The results from the study showed that 90.5% of all re-admissions were due to *P. vivax* and that 41.5% of the re-admissions occurred in the African-American group. The authors conceded that if the entire 9.5% of the non-vivax re-admissions occurred in the African-American group, 32% of African-American re-admissions would have correlated with *P. vivax* infection (Butler and Sapero, 1947). In contrast to reports from malariotherapy trials, the conservative evaluation of this study population suggested that African-American troops were highly susceptible to blood-stage infection by Pacific strains of *P. vivax* when naturally exposed to the parasite.

2.3. Human Variation and Blood Groups

A theoretical synthesis similar to the one occurring in the cell biology and infectious disease world also gained momentum in genetics and evolution at the dawn of the twentieth century. At this time, Darwin's *On the Origin of Species* had stimulated wide-ranging controversy throughout the scientific community. Although Mendel's re-discovered work with garden peas provided a foundation for understanding the dynamics of heredity, opposing factions debating the role of natural selection in evolution took his observations to argue that evolution resulted from successive leaps *or* gradual change; the role of mutation in natural selection of new species was at first questioned (Mayr and Provine, 1981). The population biologists Fisher, Wright and Haldane promoted ideas that genes worked together to bring about genetic variation in populations. From this population-based perspective, genetic variations that optimised fitness would be transmitted from one generation to the next (Mayr and Provine, 1981). The Malaria Hypothesis, first proposed by Haldane, is a well-known but counterintuitive twist of population biology and selection theory. This hypothesis proposes that otherwise harmful mutations (e.g. the thalassaemias) are transmitted at higher frequencies in some populations because they confer selective advantages against plasmodia and balance susceptibility to malaria (Haldane, 1949).

Practical implications regarding the influence of mutation on phenotype, heritability and human population biology came to light in studies on blood transfusion, where many of the first human genetic polymorphisms were identified through serological cross-reactivity, recognising variations in blood group antigens (Race and Sanger, 1950; Mourant et al., 1976). Karl Landsteiner made the first observations that serum from healthy humans had an agglutinating effect on the blood corpuscles of other humans, leading to the identification of the ABO blood group system in 1901 (Landsteiner, 1901). Blood group systems enabled early human geneticists to test heritability of these polymorphic traits, provided consistent opportunity to test population genetic hypotheses and allowed them to make some of their first observations regarding genetic similarities and differences between races and ethnicities. At present, there are 32 blood group systems inclusive of over 600 specific antigens, encoded by 42 genes; overall polymorphism is captured among 1312 alleles (Table 2.1). Cross-reacting antibodies recognise extracellular epitopes of proteins imbedded in the RBC membrane. Malarial parasites would naturally encounter these epitopes when contacting the red cell prior to blood-stage infection. Therefore, it is not surprising that a

number of these blood group proteins have been implicated in influencing susceptibility to malaria (Table 2.1).

3. RESISTANCE TO *P. VIVAX* AND INSIGHTS ON MALARIA RED CELL INVASION

Investigator Profile: An Interview with Louis Miller, M.D., with Vicki Glaser.

(Glaser, 2004)

[In the early 1970s] we knew that invasion was very specific – each type of Plasmodium goes to a specific host – but we did not know why. At that time I was working on a monkey parasite, P. knowlesi … The parasite would only invade certain types of RBCs [in tissue culture], such as human or monkey cells, and it would not invade others such as mouse … As host red cell specificity was likely based on surface molecules that act as receptors, I began to study red cells … null for various blood groups in the hope that one would be the receptor for P. knowlesi invasion of human red cells. I found that P. knowlesi was not able to invade Duffy blood group negative red cells. I went to the library that night, and I knew right away that I had discovered the missing factor for the resistance of West Africans to P. vivax.

(Louis Miller)

3.1. Serological Recognition of Duffy (Fy) Blood Group Polymorphism

The Duffy blood group antigen (Fy ^{a}) was first observed in 1950 on erythrocytes using alloantisera found in a multiply transfused haemophiliac (named by permission of the patient) at the time a haemolytic transfusion reaction was observed (Cutbush et al., 1950). The expected Fy^{b} antisera was discovered in Berlin shortly thereafter (Ikin et al., 1951); surveys of European populations suggested frequencies for the co-dominantly expressed Fy^{a} and Fy^{b} antigens of 35% and 65%, respectively (Cutbush et al., 1950; Ikin et al., 1951). Upon screening, a series of blood samples from African-American donors to the Knickerbocker Blood Bank of New York City, Sanger et al. observed that 68% of the samples did not react with either the Fy ^{a} or Fy ^{b} antisera (Sanger et al., 1955). Additional analysis of Nigerian families showed that the null phenotype was inherited in Mendelian manner and provided an opportunity to investigate Fy ^{a} copy number. In an earlier study, Race et al. found that the antiserum Pri reliably distinguished between single and double donors for Fy ^{a} (Race et al., 1953). Results obtained from tests on three African-Americans who were phenotypically Fy(a+b+) and two who were Fy(a+b−) all suggested that Fy ^{a} was observed to be present in single-dose quantity, as compared to double-dose quantities observed in Fy(a+b−) Europeans (Sanger et al., 1955) (Duffy blood group nomenclature is summarised at the conclusion of Section 3.3; Table 2.2). These observations suggested that those of African ancestry possessed either a different antigen, Fy^{c}, or they did not express the Duffy antigen and carried a Duffy-null allele, Fy^{0}. Blood group researchers hypothesised that identification of an Fy^{c} antigen would be forthcoming as a result of large numbers of blood transfusions involving African donor and Caucasian recipient pairs, or through their attempts to stimulate anti-Fy^{c} reactivity through injection of Fy(a−b−) red cells into European volunteers (Sanger et al., 1955). The failure to discover the Fy^{c} antigen and therefore the possibility of a Duffy-null phenotype was not unanticipated. A comparable observation had been made previously in the MNSs system (Table 2.1), where an occasional African blood sample reacted with neither the anti-S nor the anti-s antisera (Sanger et al., 1955).

3.2. The Genetic Resistance Factor to *P. vivax* – Duffy Negativity

The impressive distribution of the Fy(a−b−) phenotype in diverse African populations has been a fascination to population geneticists, evolutionary biologists and infectious disease physicians and biologists. Based on the overlapping distribution of the Fy(a−b−) phenotype, the very low prevalence of *P. vivax* in African populations (Bray, 1957) and the desire to identify 'receptors' used by malarial parasites to invade human erythrocytes (Butcher et al., 1973; Miller and Dvorak, 1973), Louis Miller and colleagues at the US National Institutes of Health performed a series of studies in the mid-1970s to determine if red cells deficient for any of the human blood group systems would resist infection using newly developed abilities to culture malarial parasites in the laboratory (Butcher and Cohen, 1971). In their studies, Miller and colleagues first observed that the non-human primate malarial parasite *P. knowlesi* was not able to infect erythrocytes from Fy(a−b−) African-Americans *in vitro* (Miller et al., 1975), while the parasite easily infected erythrocytes from Fy(a+b−), Fy(a+b+) and Fy(a−b+) donors. From these results, Miller et al. hypothesised that Fy(a−b−) would explain the absence of *P. vivax* from West Africa where blood group system surveys were reporting that Fy(a−b−) frequency was nearly 100% (Mourant et al., 1976). Miller's *in vitro* findings led directly to a study testing the *in vivo* susceptibility of Duffy-negative and Duffy-positive individuals to *P. vivax* blood-stage infection (Miller et al., 1976). In this study, 17 consenting prisoner volunteers were first characterized for their Duffy blood group phenotype serologically. *P. vivax*-infected mosquitoes were then allowed to take blood meals, first from Fy(a−b−) African-Americans, and then following interruption, were allowed to continue feeding on Fy(a+b−), Fy(a+b+) and Fy(a−b+) Caucasian and African-Americans. Results showed that none of the five Fy(a−b−) study subjects developed blood-stage parasitaemia despite evaluation of daily blood smears for 90–180 days, while all 12 of the Duffy-positive individuals developed blood-stage infection within 15 days (Miller et al., 1976).

While this study showed strong evidence that *P. vivax* required the Duffy blood group antigen to be present on the erythrocyte surface to invade the cell successfully and continue its life cycle, no information beyond basic susceptibility to blood-stage infection was produced. Studies at this point of the investigation neither tested for differences in susceptibility based on Fy^a vs. Fy^b, nor were they on individuals who were heterozygous for the Duffy-negative allele, expressing a single gene dose of the Duffy blood group protein. Further comparisons regarding *P. vivax* susceptibility among these Duffy phenotypes could have provided important insight towards understanding the relative selective differences among the Fy^a, Fy^b and Duffy-negative alleles.

In an attempt to explain how the frequency of Duffy blood group negativity had risen to 100% corresponding with the absence of *P. vivax* from vast regions of malaria-endemic West Africa, Miller and colleagues offered the following hypotheses. 'Although *P. vivax* infection rarely causes death, it may decrease survival in African children with malnutrition and other endemic diseases … as the frequency of the Duffy-negative gene increased in the population, the number of susceptible persons decreased below a critical level, and *P. vivax* disappeared from the region' (Miller et al., 1976). This hypothesis suggests that the Duffy-negative phenotype increased the fitness of human populations against vivax malaria and would have led to the evolution of a human host population in which *P. vivax* was not able to reproduce with enough success to maintain its life cycle. This, and queries about other pathogens that may interact with the Duffy antigen have fuelled the debate surrounding the relationship between *P. vivax* and evolution of the Duffy-negative phenotype for decades. (Livingstone, 1984; Carter, 2003; Kwiatkowski, 2005; Rosenberg, 2007).

3.3. The Molecular and Cellular Basis of Duffy Blood Group Polymorphism

As identification of Fy^c was not forthcoming, understanding the molecular differences responsible for this and the other Duffy blood group polymorphisms would rely on the advance of molecular biology. Methodical progress towards cloning the Duffy gene can be marked through attempts to purify the Duffy protein (Moore et al., 1982; Hadley et al., 1984; Chaudhuri et al., 1989) and identification of a series of Duffy epitopes: Fy3 (Albrey et al., 1971), Fy4 (Behzad et al., 1973), Fy5 (Colledge et al., 1973) and Fy6 (Nichols et al., 1987) and their respective antisera. These antisera have been used to illustrate that the Duffy protein was characterized by a number of different epitopes. It is therefore important to note that all these epitopes were absent from Fy(a−b−) African individuals. Beginning in 1988, Chaudhuri and colleagues at the New York Blood Centre described a series of experiments using the murine anti-Fy6 monoclonal antibody to affinity purify Duffy antigens from solubilised erythrocytes (Chaudhuri et al., 1989). Chaudhuri et al. further studied these Duffy peptides by amino acid sequencing and synthesis of a DNA probe to identify a gene-specific cDNA molecule (Chaudhuri et al., 1993). Through this process, they identified a 338 codon open reading frame (ORF) sequence exhibiting significant homology to the human interleukin 8 receptor, predicting seven transmembrane segments, an extracellular amino terminus, three extracellular loop domains, three intracellular loop domains and a carboxy-terminal cytoplasmic tail (Fig. 2.2) (Chaudhuri et al., 1993). Further studies on the genomic organisation of the Duffy gene sequence confirmed early predictions that the gene locus was present in a peri-centromeric region of human chromosome 1 (1q22-23) (Donahue et al., 1968; Dracopoli et al., 1991; Mathew et al., 1994). While the role of the Duffy blood group antigen is potentially of great interest in allergy (Vergara et al., 2008), cardiovascular disease (Reich et al., 2009), cancer biology (Shen et al., 2006) and HIV-AIDS (He et al., 2008), we will not cover these topics here. Additional details related to Duffy antigen chemokine receptor biology (Pruenster et al., 2009) are provided in the legend to Fig. 2.2.

Specific analysis of Duffy cDNA molecules (5-RACE) produced evidence that the gene was composed of two exons (Iwamoto et al., 1996). Exon 1 was observed to encode seven amino acids, MGNCLHR; exon 2 was found to encode 338 amino acids. It was subsequently shown that the primary transcript of the Duffy gene was composed of codons 1–7 from exon 1 joined to codons 10–338 from exon 2 encoding a protein of 336 amino acids; this splice variant is expressed in erythroid lineage cells. Additional studies characterizing the Duffy gene were also successful in identifying a single nucleotide polymorphism (SNP) in codon 42 associated with the Fy^a (GGT; encodes glycine) and Fy^b (GAT; encodes aspartic acid) antigens (Chaudhuri et al., 1995; Iwamoto et al., 1995; Tournamille et al., 1995b). Then, after acknowledging no additional polymorphism compared to the $FY{\star}B$ allele, suggesting that Africans carried no important disruption in the Duffy ORF (Chaudhuri et al., 1995; Tournamille et al., 1995a), Tournamille et al. discovered a T to C SNP 33 nucleotides upstream from the primary transcription starting position (−33) in the Duffy gene promoter (originally positioned at nucleotide −46) (Tournamille et al., 1995a), resulting in an $FY{\star}B^{ES}$ allele (ES = erythroid silent). Duffy-negative Africans were homozygous for this polymorphism that was shown to occur in a tissue-specific GATA1 transcription-factor-binding motif. *In vitro* assays showed that this polymorphism blocked gene expression in erythroid lineage cells but did not block expression in non-erythroid cells. Results of this study provided the molecular genetic explanation for erythrocyte Duffy negativity.

Since the identification of the Fy^a, Fy^b and Duffy-negative SNPs, additional less-common variants have been identified to provide a more complete description of Duffy genotype and serological phenotype polymorphisms. Following identification of the $FY{\star}B^{ES}$ allele, Zimmerman et al. sought to determine if the Duffy gene in Papua New Guineans living in *P. vivax*-endemic regions would be characterized by accumulation of any functional polymorphism. A survey of the Duffy gene promoter and ORF polymorphisms identified

above revealed that the same promoter SNP found on the African *FY★B*^{ES} allele was observed on the resident Papua New Guinea (PNG) *FY★A* allele (suggests *FY★A*^{ES}) (Zimmerman et al., 1999). Flow cytometry comparing anti-Fy6 (Nichols et al., 1987) antibody binding to erythrocytes from six PNG homozygous wild-type individuals and six PNG heterozygous individuals showed that individuals with two erythroid-functional alleles expressed approximately twice the amount of the Fy^{a} antigen compared to individuals with one erythroid-functional allele. Since the time that *FY★A*^{ES} was reported in PNG, this allele has also been found in Tunisia (Sellami et al., 2008). Before describing additional polymorphism, the Duffy-negative phenotype has been observed in association with a 14-nucleotide deletion in the Duffy coding sequence (Mallinson et al., 1995).

Further variation in Duffy serology, originally described by Chown et al., was observed as a result of low Fy^{b} expression levels (termed Fy^{x}) (Chown et al., 1965), observed primarily in Caucasian families. Again, application of molecular genetic strategies enabled identification of nucleotide sequence changes associated with this serological phenotype. First, descriptions of the mutation underlying the Fy^{x}, or Fy^{bweak}, variant identified polymorphism in codon 89 of the *FY★B* allele, changing the amino acid sequence from arginine (codon CGC) to cysteine (codon TGC) in Fy^{b} and Fy^{bweak} antigens (Olsson et al., 1998; Parasol et al., 1998; Tournamille et al., 1998). This polymorphism has been observed in association with an alanine to threonine amino acid substitution at codon 100 (GCA to TCA), and an additional alanine to serine substitution at codon 49 (GCA to TCA) (Castilho 2004). The substitution these *FY★X* alleles all share is arginine to cysteine at codon 89; to date, this polymorphism has not been observed on the *FY★A* allele. The Fy^{x} polymorphism occurs within the first intracellular loop of the Duffy protein and is associated with reduced cell surface expression of Duffy (Olsson et al., 1998; Tournamille et al., 1998). The frequency of the *FY★B*^{weak} allele is approximately 2% in Caucasians (Chown et al., 1965; Olsson et al., 1998). Finally, weak expression of Fy^{b} has been reported in association with deletion of a ‘C’ nucleotide residue, between −76 and −74, of the Duffy gene promoter in an Sp1 regulatory site (Moulds et al., 1998).

Overall, flow cytometry studies testing the association between Duffy promoter and Fy^{bweak} polymorphisms have demonstrated consistent relationships. Relative levels of erythroid expression have shown that heterozygous carriers of a Duffy-negative allele express approximately 50% the level of the Duffy antigen on their red cells compared to the red cells from individuals homozygous for Duffy-positive alleles. The *FY★X* allele is associated with approximately 10% of the expression compared to the *FY★A* and *FY★B* alleles (Tournamille et al., 1998). The overall Duffy phenotype is dependent on both promoter and coding region SNPs. Expression phenotypes relative to the 15 different genotypes possible from the five known Duffy alleles (*FY★A*, *FY★B*, *FY★X*, *FY★A*^{ES}, *FY★B*^{ES}) are summarised in Table 2.2. Additional observations that Duffy expression is highest on reticulocytes (Woolley et al., 2000; Woolley et al., 2005) may contribute to the observation of preferential invasion of these immature red cells by *P. vivax* (Kitchen, 1938).

Comparative sequence analyses of Duffy gene orthologues in non-human primates have shown that the *FY★B* allele is the ancestral state (Palatnik and Rowe, 1984; Chaudhuri et al., 1995; Li et al., 1997; Tournamille et al., 2004; Demogines et al., 2012; Oliveira et al., 2012). The Duffy-negative allele *FY★B*^{ES} bears clear signatures of strong and recent positive selection in African populations (Hamblin and Di Rienzo, 2000; Hamblin et al., 2002). Interestingly, the *FY★A* allele predicted to have arisen after *FY★B*^{ES} (Li et al., 1997) has also been characterized by levels of polymorphism lower than would be expected by a neutral model of evolution in a sample of Chinese individuals (Hamblin et al., 2002). Phylogenetic comparisons of this nature for the *FY★X* and *FY★A*^{ES} alleles have not yet been performed. That *P. vivax* would be considered to be the agent behind the selection

observed in human-specific alleles is curious given the long-held considerations that *P. vivax* infection is rarely lethal in humans. As a growing number of clinical research studies are establishing connections between *P. vivax* and severe malaria and malaria mortality (e.g. (Genton et al., 2008; Tjitra et al., 2008; Anstey et al., 2009; Baird, 2009; Kochar et al., 2009)), further consideration is necessary to determine how vivax malaria exerts its pressure as an agent of natural selection (Anstey et al. discuss details of clinical vivax malaria in Chapter 3 in Volume 80 of this special issue).

3.4. The Duffy Binding Protein

Several studies have now described the parasite ligand interacting with the Duffy blood group antigen. *P. knowlesi* Duffy binding proteins (Haynes et al., 1988; Adams et al., 1990) and *P. vivax* DBP (PvDBP) (Wertheimer and Barnwell, 1989; Fang et al., 1991) have molecular weights of approximately 140 kD; *P. knowlesi* α (Pkα) binds to the Duffy antigen (Chitnis and Miller, 1994). A 330-amino acid cysteine-rich region of the DBP is predicted to be the protein domain responsible for binding to Duffy-positive human RBCs (Ranjan and Chitnis, 1999). The DBP is expressed in the micronemes and on the surface of *P. knowlesi*, and by homology, *P. vivax* merozoites. A number of structural features of the DBPs are shared with erythrocyte binding proteins of other malarial parasites. As such, these proteins have come to be known as Duffy-binding-like erythrocyte binding proteins (DBL-EBP) (Adams et al., 1992) Shared features among EBPs include two cysteine-rich regions (Region II – containing 12 conserved cysteine residues; Region VI containing 8 conserved cysteine residues), a highly polymorphic region (Regions III to V) and numerous aromatic amino acids (tryptophan, phenylalanine and tyrosine) (Adams et al., 1992). Because of the overall significance of EBPs in blood-stage infection, it is important to consider further the interaction between the PvDBP and the Duffy antigen.

Despite the difficulties in growing *P. vivax* in culture, a number of *in vitro* studies have built a body of information on the importance of DBP–Duffy antigen interaction to invasion of the RBC. As noted above, while *P. knowlesi* merozoites would successfully contact and reorient their apical surfaces in apposition to the membrane of both Duffy-positive and Duffy-negative erythrocytes, the tight junction between merozoite and erythrocyte membranes did not form with Duffy-negative cells (Miller et al., 1979). These observations have suggested that some aspect of the parasite's invasion mechanism failed to engage in the absence of the Duffy antigen. Experiments more specifically focused on the *P. vivax* DBP have demonstrated competitive interference between recombinant PvDBP expressed on COS cells and the Duffy receptor on donor erythrocytes using a 35-amino-acid peptide (amino acid 8–42) from the receptor's NH_2-terminal domain, the monoclonal antibody anti-Fy6 and the chemokine MGSA (CXCL1) (Chitnis and Miller, 1994; Chitnis et al., 1996). These same studies have also demonstrated that sulphation of tyrosine 41 is critical for optimal DBP binding to the Duffy receptor *in vitro* (Choe et al., 2005). Further studies employing this COS-cell-binding affinity assay showed that Duffy-negative heterozygous, compared to homozygous positive, erythrocytes exhibit consistently lower affinity for PvDBP transfected cells (Michon et al., 2001). Additionally, elevated levels of amino acid sequence polymorphism in the DBP binding region, as well as antibody responses from people living in *P. vivax*-endemic regions recognising DBP, suggest that this molecule may be under selective pressure (Tsuboi et al., 1994; Ampudia et al., 1996; Fraser et al., 1997; Michon et al., 1998; Cole-Tobian et al., 2002) by the human immune system. A number of years later, Singh et al. have now shown that *P. knowlesi* merozoites of Pkα knockout strain are not able to form a junction with or invade Duffy-positive human RBCs (Singh et al., 2005). That the Pkα knockout continues to successfully invade rhesus erythrocytes suggests that the *P. knowlesi* β and γ proteins bind other receptors and enable Duffy-independent red cell invasion (Chitnis and Miller, 1994; Ranjan and Chitnis, 1999).

Interrogation of the parasite ligand–host receptor relationship has since been examined through studies to characterize more specifically the interaction of PvDBP with the Duffy antigen. Recombinant chimeric proteins have been used to localise PvDBP-Duffy binding to a 170-amino-acid segment of the parasite ligand between cysteines 4 and 7 (Ranjan and Chitnis, 1999). Further studies have gone on to suggest that there are discontinuous epitopes within this segment that are predicted to be important for Duffy antigen interaction (VanBuskirk et al., 2004; Hans et al., 2005) and that receptor binding residues and polymorphic residues under immune pressure map to opposing surfaces of PvDBP (Singh et al., 2006). More recent studies have shown that both patient-derived and polyclonal rabbit antibodies specific for PvDBP are able to inhibit *P. vivax* invasion of human RBCs *in vitro* (Grimberg et al., 2007; Russell et al., 2011).

4. EVOLVING PERSPECTIVES ON RESISTANCE TO *P. VIVAX*

Molecular and cell biology technologies have provided powerful strategies for cloning and expressing proteins from malarial parasites to study their interactions with human red cells *in vitro* (Adams et al., 1992). Molecular diagnostic methods for interrogating genetic polymorphisms and diagnosing infection have transformed strategies for studying the epidemiology of malaria (Greenwood, 2002). Over the past 20 years, application of these methods has significantly influenced our perspectives on the frequency and distribution of *P. vivax* as well as the role played by genetic polymorphism and the interaction between *P. vivax* and the human RBC.

4.1. Further Influence of Duffy Polymorphism on Resistance to *P. vivax* Malaria

Discovery of the $FY{\star}A^{ES}$ allele in PNG (Zimmerman et al., 1999) provided a new opportunity to evaluate the association between a Duffy-negative allele and susceptibility to *P. vivax* infection and disease. Although no individual has been identified to be homozygous for the $FY{\star}A^{ES}$ allele in PNG, the opportunity was provided to determine if there was any selective advantage associated with being a heterozygous carrier of a Duffy-negative allele. In these studies, Kasehagen and colleagues performed cross-sectional malaria prevalence surveys in the same PNG communities where the $FY{\star}A^{ES}$ allele had been identified. These Wosera villages, north of the PNG Central Ranges, are highly endemic for all four human malarial parasite species (Genton et al., 1995a; Kasehagen et al., 2006; Lin et al., 2010). *Plasmodium* species infection status was evaluated by conventional blood smear light microscopy and semi-quantitative polymerase chain reaction (PCR)-based strategies. In both unmatched and matched (adjusted for age, sex and village of residence) longitudinal cohort analyses, results showed that Duffy-negative heterozygotes ($FY{\star}A/{\star}A^{ES}$) were partially protected from *P. vivax* blood-stage infection compared to those homozygous for wild-type alleles ($FY{\star}A/{\star}A$) (Kasehagen et al., 2007). In these same study cohorts, there were no differences in susceptibility to *P. falciparum* infection between $FY{\star}A/{\star}A^{ES}$ and $FY{\star}A/{\star}A$ study participants (Kasehagen et al., 2007).

Additional analyses evaluated parasitaemia by a semi-quantitative PCR assay between $FY{\star}A/{\star}A^{ES}$ and $FY{\star}A/{\star}A$ study participants, in age group categories less than and greater than 15 years. Among $FY{\star}A/{\star}A^{ES}$ children under 15 years of age, the mean *P. vivax* fluorescent signal intensity (corresponds with parasitaemia (McNamara et al., 2006)) was significantly lower among $FY{\star}A/{\star}A^{ES}$ (mean = 2.37, $\log_{10}$ transformed) compared to $FY{\star}A/{\star}A$ children (mean = 2.96) (Mann–Whitney U:$P = 0.023$). Interestingly, this was similar to the difference in parasitaemia observed between $FY{\star}A/{\star}A$ individuals older vs. younger than 15 years of age (mean fluorescent signal intensity 2.23 vs. 2.81, respectively; Mann–Whitney U:$P < 0.0001$). This suggested that the difference in parasitaemia attributed to the difference in genotype ($FY{\star}A/{\star}A^{ES}$ and $FY{\star}A/{\star}A$) was similar to the difference that would otherwise be attributed to acquired immunity of older individuals. Similar to

results from the longitudinal cohort studies, no association was observed between susceptibility to *P. falciparum* parasitaemia and the Duffy genotype. The findings from this study provided the first evidence that Duffy-negative heterozygosity reduced erythrocyte susceptibility to *P. vivax* infection (Kasehagen et al., 2007). Reduced susceptibility to *P. vivax* was not associated with an increased susceptibility to *P. falciparum* malaria as may have been predicted by studies that previously reported reduced severity of falciparum malaria conferred by exposure to *P. vivax*. Similarly, cross-sectional studies in the Amazon Basin of Brazil (Cavasini et al., 2007; Sousa et al., 2007) and Rio Grande do Sul (Albuquerque et al., 2010) have shown significantly reduced prevalence of *P. vivax* infection among Duffy-negative heterozygotes (either $FY{\star}A/{\star}B^{ES}$ or $FY{\star}B/{\star}B^{ES}$) when compared with subjects from the same endemic areas with the $FY{\star}A/{\star}A$, $FY{\star}B/{\star}B$ or $FY{\star}A/{\star}B$ genotypes. These results suggest that Duffy-negative heterozygosity confers significant protection from vivax malaria and may provide some insight regarding a selective advantage that led to the $FY{\star}B^{ES}$ allele reaching genetic fixation in Africa.

The frequency of the $FY{\star}X$ allele at 2% in Caucasian populations may limit opportunities to perform epidemiological studies to determine if the Fy^{bweak} antigen is associated with reduced risk of *P. vivax* malaria. In contrast, the $FY{\star}A$ allele is widely distributed in Southeast Asia (Howes et al., 2011), where *P. vivax* is proposed to have evolved from origins as a parasite of Old World monkeys (Carter, 2003; Escalante et al., 2005; Culleton et al., 2011), and in South America, where *P. vivax* is the predominant malaria parasite in human infections (Oliveira-Ferreira et al., 2010; Arevalo-Herrera et al., 2012).

In vitro studies have shown that the *P. knowlesi* DBP interacts with stronger affinity to the Fy^b compared to the Fy^a antigen. After observing similar results with the PvDBP (40–50% decreased binding to Fya+b− vs. Fya−b+ erythrocytes (King et al., 2011); $P < 0.0001$), King et al. performed a cohort study in the Brazilian Amazon to determine if the *in vitro* results translated to *in vivo* protection from clinical vivax malaria in association with the $FY{\star}A$ compared to the $FY{\star}B$ allele (King et al., 2011). Study participants ($n = 400$; 5–74 years of age) lived along the Iquiri River where the annual incidence rates of *P. vivax* and *P. falciparum* malaria during the 14-month study period were 0.31 and 0.17, respectively (124 cases of *P. vivax*, 66 cases of *P. falciparum*, 31 cases of *P. vivax* + *P. falciparum* malaria). Overall, when compared to the $FY{\star}A/{\star}B$ genotype ($n = 140$), individuals with the $FY{\star}A/{\star}B^{ES}$ and $FY{\star}A/{\star}A$ genotypes experienced 80% ($n = 35$; risk ratio, 0.204 (95% confidence interval (CI), 0.09–0.87)) and 29% ($n = 52$; risk ratio, 0.715 (95% CI, 0.31–1.21)) reduced risk of clinical vivax malaria, respectively. Consistent with stronger affinity between PvDBP and the Fy^b antigen, individuals with the $FY{\star}B/{\star}B^{ES}$ and $FY{\star}B/{\star}B$ genotypes experienced 220–270% increased risk of clinical vivax malaria, respectively, when compared to $FY{\star}A/{\star}B$ ($FY{\star}B/{\star}B^{ES}$: $n = 76$; risk ratio, 2.17 (95% CI, 0.91–4.77); $FY{\star}B/{\star}B$: $n = 87$; risk ratio, 2.70 (95% CI, 1.36–5.49). As in the studies performed by Kasehagen et al., there was no association between the *FY* genotype and risk for *P. falciparum* in the multivariate analysis (overall risk ratio 1.08, 95% CI 0.87–2.38, $P = 0.42$). While it will be helpful to see if additional epidemiological studies corroborate these findings, results from Cavasini et al. are of interest (Cavasini et al., 2007). In their studies, $FY{\star}A/{\star}B^{ES}$ was observed less frequently among 312 *P. vivax* patients (10.9%) than in 330 healthy blood donors (Brazilian blood bank; 18.8%). Results from King et al. would suggest that like the $FY{\star}B^{ES}$ allele, the frequency of $FY{\star}A$ has increased in frequency (reaching genetic fixation in many populations) to improve human fitness against *P. vivax* malaria (King et al., 2011).

4.2. Duffy-Independent Red Cell Invasion by *P. vivax*

With consistent observation of resistance to *P. vivax* malaria associated with Duffy negativity, reduced susceptibility to *P. vivax* associated with lower Duffy expression and/or

reduced affinity between the Duffy antigen and the parasite invasion ligand, it is understandable that the Duffy antigen has come to be regarded as an *essential* receptor for *P. vivax* red cell invasion. It has therefore been of keen interest that an increasing number of studies have reported *P. vivax* PCR positivity in Duffy-negative people. These studies have included *P. vivax* in Duffy-negative people in the Nyanza Province of Western Kenya (Ryan et al., 2006) and the Amazon Basin (Brazil) (Cavasini et al., 2007). However, another large-scale PCR-based survey covering nine different African countries detected only one *P. vivax*-positive person in over 2500 samples, and this individual was Duffy positive (Culleton et al., 2008). With a history of clinical reports of *P. vivax* malaria occurring in Europeans returning from holiday or business travel to Africa (Phillips-Howard et al., 1990; Gautret et al., 2001; Mendis et al., 2001; Muhlberger et al., 2004; Guerra et al., 2010), new questions have arisen with regard to the Duffy-negative *P. vivax* resistance factor (Rosenberg, 2007).

In an effort to understand the epidemiology of malaria throughout Madagascar, Ménard and colleagues initiated a series of blood sample collections in 2006 from the country's four major malaria-transmission regions (Ménard et al., 2010). These surveys provided new insight into the basic prevalence of *Plasmodium* species infections and drug resistance. They also opened the opportunity to investigate the intersection of malaria infection in a human population characterized by unique origins and admixture.

The peopling of Madagascar is recent in human history and is suggested to have been initiated by sea-faring people of Indonesia or Malaysia (Nias Island of western Sumatra or Borneo, respectively) with evidence that founding individuals arrived 2300 years before present (Burney et al., 2004). Upon Bantu migration from Africa (Tanzania and Mozambique) during the second and third centuries and new waves of Malayo-Indonesian immigration from the eighth century onwards, significant cultural assimilation and genetic admixture has occurred. Malaria is likely to have been transported to Madagascar through the earliest human settlers more than 2000 years ago. It is more difficult to predict when during the first millennium of human settlement the human population numbers and density became favourable to support endemic transmission of the four common species of human malaria parasites that are observed in Madagascar today.

In this setting, surveys of school-aged children revealed *P. vivax* PCR positivity in 8.8% of asymptomatic Duffy-negative children ($n = 476$) (Ménard et al., 2010). During surveys to assess *in vivo* efficacy of drugs recommended by the Madagascar Ministry of Health to treat malarial illness, nine Duffy-negative people were identified who had PCR-confirmed, mono-infection *P. vivax* malaria (4.9% of 183 participants). Given the unusual prevalence of vivax malaria in people considered to be resistant to this disease, Ménard et al. took additional steps to validate this finding by microscopy to provide the first evidence to confirm Duffy-independent blood-stage infection and development by *P. vivax* (Fig. 2.3) (Ménard et al., 2010). Microscopy results included the observation of sexual-stage gametocytes necessary to continue the parasite life cycle through mosquito transmission. Consistent with observations reported by many blood group laboratories, flow cytometry analysis of erythrocytes from Malagasy study participants who had experienced clinical *P. vivax* malaria showed that Duffy-negative genotype and phenotype were 100% concordant. Additionally, DNA sequence analysis of the Duffy gene confirmed that the Duffy-negative allele identified in Madagascar was identical to the $FY{\star}B^{ES}$ allele observed in West Africa (included > 2550 bp of the gene's proximal promoter and full coding sequence).

Population-level observations from Ménard et al. provide further insight regarding the unique parasite–host relationships discovered in Madagascar (Ménard et al., 2010). In the communities with the highest frequencies of Duffy negativity, there was little to no

prevalence of *P. vivax* infection detected in either Duffy-positive or Duffy-negative people. In contrast, with an increase in the frequency of Duffy positivity, *P. vivax* prevalence increased and was not significantly different between Duffy-positive and Duffy-negative people (Fig. 2.4; χ^2 results: Miandrivazo $P = 0.733$; Maevatanana $P = 0.278$; Tsiroanomandidy $P = 0.09$). These results suggest that high population levels of Duffy negativity may act similarly to herd immunity to reduce transmission and consequently protect Duffy-positives from vivax malaria. In populations with higher frequencies of Duffy positivity, opportunities for the parasite to attempt invasion of the Duffy-negative RBCs are more frequently available as antibody reactivity against *P. vivax* merozoites, PvDBP and PvMSP-1 indicate that liver-stage infection (and therefore hypnozoite formation) commonly occurs in Duffy-negative people (Spencer et al., 1978; Michon et al., 1998; Herrera et al., 2005; Culleton et al., 2009). It is important to note that a survey of clinical malaria from this Madagascar study did suggest that Duffy negativity was associated with protection from malarial illness. Finally, it is of interest that genotyping results from six unlinked *P. vivax*-specific microsatellites suggested that multiple *P. vivax* strains were present in the blood samples from Duffy-negative infections.

With additional reports of *P. vivax* PCR-positive Duffy-negative people from Equatorial Guinea (Rubio et al., 1999; Mendes et al., 2011), Gabon (Mendes et al., 2011) and Mauritania (Wurtz et al., 2011), evidence of Duffy-independent *P. vivax* blood-stage infection has been observed across geographically distant communities of the Duffy-negative population of sub-Saharan Africa. It will be important to continue surveillance of *P. vivax* strains capable of Duffy-independent red cell invasion because of implications regarding the potential that *P. vivax* may compound the burden of clinical malaria in Africa and complicate *P. vivax*-specific vaccine development and malaria elimination.

4.3. Global Distribution of Duffy Polymorphism and the Population at Risk of *P. vivax* Malaria

Much like the maps used to illustrate an overlap between malaria endemicity and the distribution of the sickle cell allele (Piel et al., 2010), α- and β-thalassaemias (Weatherall and Clegg, 2001) and glucose-6-phosphate dehydrogenase (G6PD) deficiency (Howes et al., 2012), population surveys of Duffy blood group variants have been used to map the polymorphisms' spatial distribution. These maps provide an important overview of the ongoing relationship between *P. vivax* and human malaria. Recently, Howes et al. have collated a comprehensive geographically referenced database of available Duffy phenotype and genotype survey data to refine the global cartography of the common Duffy variants ($FY{\star}A$, $FY{\star}B$ and $FY{\star}B^{ES}$) (Howes et al., 2011). Results of this effort are summarised in Fig. 2.5. (For information on source data for this study, see Howes et al. Supplemental Information for 320 references). Recalling that non-human primate studies provide evidence that $FY{\star}B$ (green) is the ancestral allele in the hominid lineage, this map suggests that the $FY{\star}B^{ES}$ (red to orange) originated in Africa and has spread across a vast geographical and ethnic landscape. The map also indicates that $FY{\star}A$ (blue) has reached genetic fixation in regions of the world where the non-human malaria parasite ancestors originated and dispersed (noted above). From these potential Asian origins, $FY{\star}A$ has admixed with $FY{\star}B$ and also spread into the Americas. Areas of the map appearing in different shades of grey identify regions where populations are characterized by heterogeneous frequencies of $FY{\star}A$, $FY{\star}B$ and $FY{\star}B^{ES}$ ranging from 20–50%. In these latter regions, *P. vivax* is likely to have been exposed to a range of RBC phenotypes with varying contact affinities between PvDBP and the Duffy receptor, including heterozygous and homozygous Duffy negativity. In the struggle to survive, it seems reasonable to hypothesise that *P. vivax* strains have optimised effective contact of the apical invasion mechanism across a gradient of Duffy

polymorphism, which now includes absence of this receptor, to engage the moving junction needed for successful red cell invasion.

With increasing evidence that *P. vivax* is not restricted to a Duffy-dependent invasion pathway, it is important to consider how this might affect the estimation of the population at risk of *P. vivax* malaria (PvPAR). To date, Malaria Atlas Project estimates of the PvPAR have applied a biological exclusion criterion based on 100% protection from *P. vivax* infection by Duffy negativity (Guerra et al., 2010). With potential that a model based on this conservative perspective may significantly underestimate the global PvPAR, we were interested in considering how reduced Duffy- negative exclusion would alter this metric in global and regional estimates. Figure 2.6 shows that even if Duffy-negative protection were reduced to 50%, the overall global burden of *P. vivax* infection would remain heavily focused in Asia. Not surprisingly, the most significant changes in PvPAR would be observed in the Africa, Saudi Arabia and Yemen region (Africa+) with a projected fivefold increase. Interestingly, this increased PvPAR in Africa would surpass the estimated risk in the Americas by threefold.

4.4. Association of Non-Duffy Gene Polymorphisms with *P. vivax* Resistance

Recently, a number of groups have begun to consider the possibility that non-Duffy human gene polymorphisms associated with protection against falciparum malaria may also affect susceptibility to infection and disease attributable to *P. vivax*.

4.4.1. G6PD Deficiency—Among the RBC variants considered to date, G6PD deficiency is important to examine for a combination of reasons. The enzyme catalyses the first reaction in the pentose phosphate pathway leading to the formation of NADPH needed by cells to counter oxidative stress (Cappellini and Fiorelli, 2008). The enzyme deficiency was discovered because of its association with haemolytic anaemia following administration of primaquine (Beutler, 1994), the only clinically validated medication against *P. vivax* hypnozoites (Wells et al., 2010). Because of the widespread distribution of G6PD deficiency in malaria-endemic regions, administration of primaquine and therefore, elimination of *P. vivax* is problematic. The gene (13 exons distributed over 18.5 kb; ≥140 mutations (Cappellini and Fiorelli, 2008; Minucci et al., 2012)) is located in the telomeric region of the human X chromosome and is therefore characterized by classical X-linked inheritance patterns, hemizygosity in males and X-inactivation in females (Beutler, 1996). The most common West African G6PD variant, *G6PD A-* (Val → Met, codon 68; moderate (10–60%) activity variant), has been associated with significant reduction in the risk of severe falciparum malaria in male hemizygotes (Ruwende et al., 1995; Guindo et al., 2007) and in heterozygous females (Ruwende et al., 1995). More recently, Louicharoen et al. found that the *G6PD-Mahidol*^{487A} mutation (Gly → Ser, codon 163; moderate (10–60%) activity variant) was associated with reduced *P. vivax*, but not *P. falciparum*, parasite density (Louicharoen et al., 2009). Whether the *G6PD-Mahidol*^{487A} mutation reduces the severity of clinical vivax malaria was not reported. Leslie et al. have more recently reported that the G6PD-Mediterranean type (Med; Ser → Phe, codon 188; severely deficient (1–10% activity)) is associated with protection from clinical vivax in a case-control study of Afghan refugees living in Pakistan (Leslie et al., 2010). Howes et al. further discuss details regarding the complexities of G6PD deficiency and vivax malaria in Chapter 4 of this Volume.

4.4.2. Haemoglobinopathies—To date, very few studies have been performed to investigate associations between the major haemoglobinopathies and *P. vivax* (Taylor et al., 2012). Increased susceptibility to *P. vivax* infection has been observed in association with α-thalassaemia (−α/−α) in Vanuatu (Williams et al., 1996) and PNG (Allen et al., 1997) and

with HbE β-thalassaemia in Sri Lanka (O'Donnell et al., 2009). It is suggested that α-thalassaemia may increase susceptibility to *P. vivax* infection because of higher overall red cell turnover increasing reticulocytaemia (Weatherall and Clegg, 2001). As *P. vivax* shows a strong preference for infecting reticulocytes (Kitchen, 1938), increased reticulocyte counts associated with the thalassaemias would produce more target cells for the merozoites to infect. In one additional study in India, reduced susceptibility to *P. vivax* infection was reported in association with HbE (Kar et al., 1992). Although red cell remodelling and pathogenesis is markedly different among human malaria species parasites, the association of reduced susceptibility to *P. vivax* with HbE may be more consistent with observations from falciparum malaria studies where α-thalassaemia confers protection against malaria. This protection is proposed to occur because erythrocytes bind higher levels of antibody from sera of malaria-exposed individuals (Luzzi et al., 1991) and were observed to be more readily phagocytised by blood monocytes (Yuthavong et al., 1988) compared with normal red cells.

Interestingly, the concept that α-thalassaemia can increase susceptibility to *P. vivax* infection in young children while being associated with decreased susceptibility to *P. falciparum* has led to a hypothesis that the predilection to *P. vivax* may lead to cross-immunity between parasite species, which protects against falciparum malaria later in life. This hypothesis has stirred a lively debate regarding benefits and risks of cross-species infections (Bruce and Day, 2003; Snounou, 2004; Zimmerman et al., 2004). While this debate will continue, it must be acknowledged that *P. vivax* is responsible for causing severe illness and death (Baird, 2009) and that mixed infections with *P. vivax* and *P. falciparum* can be more severe than mono-infections by these same two species (Tjitra et al., 2008).

4.4.3. Southeast Asian Ovalocytosis—More consistent with *P. vivax* and the Duffy receptor interactions, there is significant interest in the influence of mutations contributing to Southeast Asian ovalocytosis (SAO) and protection against vivax malaria. Two proteins associated with red cell ovalocytosis in Melanesians include the solute carrier family 4, anion exchanger, member 1 (SLC4A1, also known as erythrocyte membrane protein band 3) and glycophorin C (GYPC). SLC4A1 is expressed in the red cell membrane and functions as a chloride/bicarbonate exchanger involved in CO_2 transport from tissues to lungs. Three domains of the protein are structurally and functionally distinct. First, the 40 kDa N-terminal cytoplasmic domain (400 amino acids) contains an 11-aminoacid segment (residues 175–185) that acts as an attachment site for the red cell skeleton by binding ankyrin (Chang and Low, 2003; Stefanovic et al., 2007). Second, a hydrophobic, polytopic transmembrane domain (481 amino acids; 14 membrane-spanning segments) carries out anion exchange (Abdalla et al., 1980). Third, the cytoplasmic tail at the extreme C-terminus of the membrane domain (41 amino acids) binds carbonic anhydrase II. Association with glycophorin A (GYPA) promotes the correct folding and translocation of the SLC4A1 protein. This protein is predominantly dimeric but forms tetramers in the presence of ankyrin (Alper, 2009). A number of single amino acid substitutions in SLC4A1 give rise to the Diego blood group system (Poole, 2000) and a 27 base pair deletion removing 9 amino acids (*SLC4A1*Δ*27*; codons 400–408) near the first transmembrane region of the protein leads to SAO (Jarolim et al., 1991; Mgone et al., 1996; Mgone et al., 1998). GYPC is a physiologically important monomer (128 amino acids, apparent 35 kDa, integral membrane sialoglycoprotein) (Cartron et al., 1993) that interacts with the peripheral membrane protein 4.1 to mediate attachment of the submembranous cytoskeleton to the erythrocyte membrane. Deletion of *GYPC* exon 3 (*GYPCDex3*) results in the Gerbich-negative blood group phenotype (Colin et al., 1989; High et al., 1989) and has also been associated with ovalocytosis in PNG in the absence of *SLC4A1*Δ*27* (Patel et al., 2001).

In vitro studies have shown that SAO compared to normal RBCs show resistance to both *P. falciparum* and *P. knowlesi* (Kidson et al., 1981; Hadley et al., 1983). Although trypsin treatment had previously been shown to render resistant Duffy-negative red cells susceptible to *P. knowlesi* infection (Mason et al., 1977; Miller et al., 1979), this same experimental strategy was not successful with SAO cells (Hadley et al., 1983). The mechanism of resistance was suggested to be increased rigidity of the RBC membrane (Mohandas et al., 1984). Both SAO and Gerbich negativity have been shown to reduce the severity of falciparum malaria (Cattani et al., 1987; Serjeantson, 1989; Genton et al., 1995b; Allen et al., 1999). These overall *in vitro* and *in vivo* observations prompted the investigation of the impact of SAO on susceptibility to *P. vivax* malaria in PNG.

It is important to note that *P. vivax* malaria in PNG is observed to peak by approximately 3 years of age (Kasehagen et al., 2006; Mueller et al., 2009) and evidence of significant protection from clinical symptoms associated with *P. vivax* is observed in young school-aged children (Michon et al., 2007). The impact of SAO was studied through multiple childhood cohorts. Results from this study showed that in a cohort of infants 3–21 months of age SAO was associated with a 55% reduction in the risk of clinical *P. vivax* episodes with parasitaemia greater than 500 infected cells/μl (adjusted IRR (incidence rate ratio) = 0.54; CI_{95} (0.34, 0.59), $P < 0.0001$). Additionally, in a treatment-time to re-infection cohort of 5–14 year olds, SAO children experienced a 52% reduction in *P. vivax* re-infection diagnosed by light microscopy (CI_{95} (23, 87), $P = 0.014$) (Rosanas-Urgell et al., 2012). Further studies showed that while Duffy antigen expression was not significantly different on SAO compared to normal erythrocytes, high-level PvDBP-specific binding inhibitory antibodies (>90% binding inhibition) were observed significantly more often in sera from SAO than non-SAO children (SAO, 22.2%; non-SAO, 6.7%; $P = 0.008$)(Rosanas-Urgell et al., 2012). Consistent with *in vitro* observations, interactions leading to reorientation and apical contact of the *P. vivax* merozoite are likely to occur *in vivo*. Results indicating that PvDBP-specific binding inhibitory antibodies were more common in SAO children suggest that PvDBP exposure to the immune system is somehow different in SAO compared to non-SAO children and stimulates production of higher quality antibody recognition of this important parasite invasion ligand.

5. CONCLUSIONS AND FUTURE DIRECTIONS

Very early experience with malariotherapy in the United States revealed that high-level resistance to blood-stage infection with *P. vivax* was observed in many, but not all, African Americans (Fig. 2.7). Although not considered at that time, observations of syphilologists of the 1920s initiated efforts to explain this curious occurrence and launched investigations that have revealed the mechanisms that malarial parasites use to infect the RBC. The first critical breakthroughs identifying components of these red cell invasion mechanisms were made by Louis Miller and colleagues in the mid-1970s when the Duffy blood group antigen was identified as the receptor for *P. knowlesi* and *P. vivax*.

As in other fields of biomedical research, invention of the PCR by Kerry Mullis in 1983 transformed many aspects of malaria research. The first published application of PCR demonstrated how sickle cell anaemia could be diagnosed by amplification of a small segment of the β-globin gene (Saiki et al., 1985). Amplification of malarial parasites from human blood samples was performed in the early 1990s to diagnose species with greater sensitivity than conventional blood smear methods (Barker et al., 1992; Barker et al., 1994) and to identify *P. falciparum* strains that were carrying mutations associated with drug resistance (Zolg et al., 1989). As powerful high-throughput multiplex assays for diagnosing malarial infections have become routinely available, perspectives on species complexity of malarial infections have changed significantly. PCR applications have improved our

understanding of the epidemiology of vivax malaria, in particular, where blood smear diagnosis is hampered by lower sensitivity, and in differentiating *P. vivax* from *P. ovale* (which share numerous morphological similarities). Reliable diagnoses of *P. vivax* have brought into question preconceptions that Africans are always fully resistant to *P. vivax* erythrocyte infection and that the parasite is absent from sub-Saharan Africa. PCR diagnostic and genotyping strategies have therefore played significant roles in identifying *P. vivax* infections in Duffy-negative people (Ryan et al., 2006; Cavasini et al., 2007; Ménard et al., 2010; Mendes et al., 2011; Wurtz et al., 2011) and in the performance of the field-based studies that have identified new vivax malaria resistance factors (Cavasini et al., 2007; Kasehagen et al., 2007; Sousa et al., 2007; Louicharoen et al., 2009; Albuquerque et al., 2010; King et al., 2011; Rosanas-Urgell et al., 2012).

Do these recent observations suggest that *P. vivax* is now evolving new capacity to infect human RBCs or has this parasite always had this capacity? Species naturally evolve, particularly when confronted with a selective barrier that threatens their ability to reproduce – Duffy negativity clearly represents this kind of barrier for *P. vivax*. Evidence suggests that the parasite's human red cell invasion mechanism has not been restricted to the Duffy antigen. Clinical observations for decades have reported that Duffy-positive Caucasians return from travels to Duffy-negative Africa with *P. vivax* infections (Phillips-Howard et al., 1990; Gautret et al., 2001; Mendis et al., 2001; Muhlberger et al., 2004; Guerra et al., 2010), and records of African or African-American individuals infected with *P. vivax* (albeit lacking Duffy phenotype data) have appeared periodically (Butler and Sapero, 1947; Hankey et al., 1953; Bray, 1958). The alternative explanation for the sudden increase in *P. vivax*-positive Duffy-negative people is that molecular diagnostic methods now provide clinicians and researchers with tools that are more sensitive than the parasitological methods previously employed. To understand the true epidemiology of vivax malaria in Africa, future population studies should include surveillance for *P. vivax* in molecular diagnostic assays routinely.

With confirmation that *P. vivax* can infect Duffy-negative red cells, it is important to know how the parasite is progressing through the critical steps leading to reticulocyte invasion (Fig. 2.8). What then are the components of the *P. vivax* Duffy-independent invasion mechanism? The electron microscopy that has so vividly captured *P. knowlesi* interactions with Duffy-positive and Duffy-negative red cells has shown that the parasite is able to reorient its apical end in apposition to the red cell membrane of both Duffy-positive and Duffy-negative cells. However, absence of the Duffy antigen limits further junction formation that sets in motion the events required to complete invasion. In the absence of the Duffy antigen, what red cell protein(s) enable the junction formation needed for further downstream events – what is the new invasion receptor? The Duffy antigen has been shown to reside in a cluster of other red cell membrane proteins as part of a protein 4.1R multiprotein complex (Mohandas and Gallagher, 2008). This complex includes Band 3 (link to SAO), glycophorin C and other blood group proteins (Kell, reticulocyte binding homologues (Rh), XK). One wonders, if through its dependence on interaction with the Duffy antigen, whether the parasite's DBP has gained or optimised a molecular connection with other members of the 4.1R complex.

From the parasite's vantage point, an equally important piece to the Duffy-independent puzzle must be identified. Unlike *P. knowlesi*, the DBP in *P. vivax* is found only as a single-copy gene (Carlton et al., 2008). It is possible that new PvDBP polymorphism could enable this protein to interact with alternative receptors; however, studies performed to date have not identified PvDBP variants that are able to bind Duffy-negative cells. Recent cell biology studies indicate that parasite proteins released in succession from the micronemes, rhopteries and dense granules are integral to parasite-host cell recognition, attachment and junction

motility (Carruthers and Sibley, 1997). While AMA1 and RON protein interactions are critical components of the moving junction (Richard et al., 2010; Srinivasan et al., 2011), it is clear that some parasite-host interaction is required to secure initial contact by the parasite's apical end before the junction can be formed. As a number of reticulocyte binding proteins have been localised to the micronemes (Meyer et al., 2009), these proteins would appear to be the likely alternative ligands if PvDBP has been rendered irrelevant in a Duffy-independent invasion mechanism. In *P. falciparum* evidence exists for functional redundancy of erythrocyte binding antigen and Rh, so that inhibition of one pathway is compensated for by the functioning of others (Stubbs et al., 2005; Triglia et al., 2009). Once an invasion pathway can no longer be used because the receptor is absent or the gene for the dominant ligand has been deleted, *P. falciparum* is able to use alternative pathways by re-deploying the expressed suite of ligands (Baum et al., 2005) or by differential gene expression of PfRh genes (Stubbs et al., 2005). It is possible that vivax parasites are able to use alternative invasion pathways that remain cryptic in the presence of the Duffy receptor and become operational in its absence.

Answers to the questions prompted by Duffy-independent infection by *P. vivax* are of critical importance to development of a vivax-specific vaccine. The challenge confronting this mission is a familiar one: *P. vivax* is reluctant to grow in laboratory cultures and this precludes many of the experimental approaches that have been so effective in studying red cell invasion by *P. falciparum* (Cowman and Crabb, 2006). As cellular invasion mechanisms are highly conserved across apicomplexa, new strategies available through parasite genomics and proteomics will be important to mine. It may also be important to keep in mind the important insights gained through studies on *P. knowlesi* (Aikawa et al., 1978).

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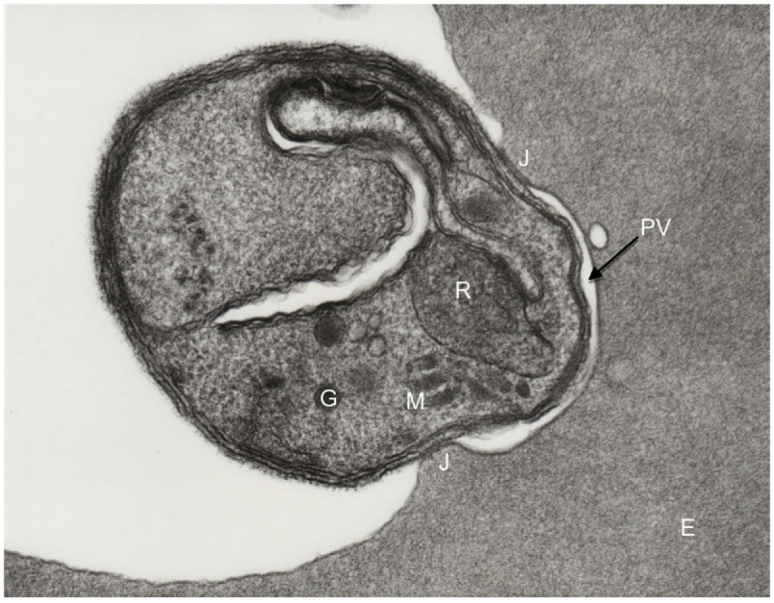


Figure 2.1. *Plasmodium knowlesi* invasion of *Macaca mulatta* red blood cells

A *P. knowlesi* merozoite has commenced the invasion process through formation of gliding junctions involving merozoite and red cell membranes. This enables invagination of the erythrocyte membrane and movement of the parasite into the parasitophorous vacuole. R, rhoptry; M, micronemes; J, gliding junction; PV, parasitophorous vacuole; E, erythrocyte. (Figure from unpublished data, Hisashi Fujioka)

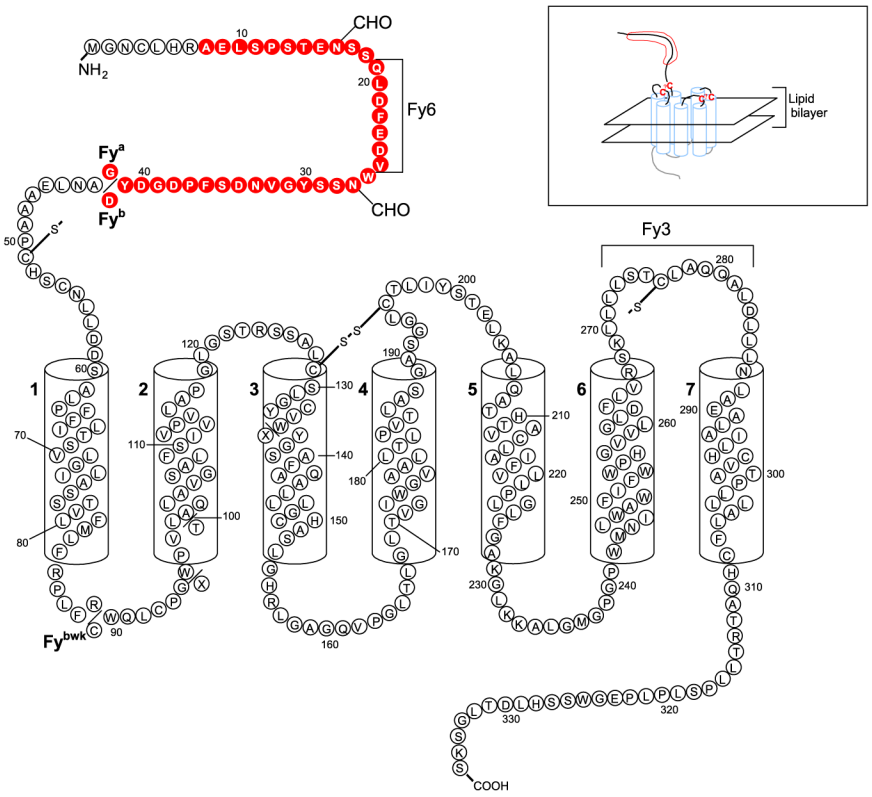


Figure 2.2. The Duffy antigen

The diagram illustrates the primary structure of the 236-amino-acid 36–46-kDa Duffy antigen with seven predicted transmembrane domains and extracellular and intracellular domains. Amino acids comprising the Fy6 and Fy3 antibody-binding domains are marked by brackets. Amino acid sequence polymorphisms are identified at residues 42 (G vs. D; Fy^a vs. Fy^b), 89 (R vs. C; Fy^b vs. Fy^{bweak}) and 100 (A vs. T) and the two premature termination codons (W vs. X) at residue positions 96 and 134. Glycosylation sites are identified at amino acid residues N16 and N27. Disulfide bonds occurring between C129 (extracellular loop 2) and C195 (extracellular loop 3) and between C51 (amino terminal head) and C276 (extracellular loop 3) are predicted to contribute to further tertiary structure within the cell membrane as depicted in the inset. Amino acids predicted to comprise the *P. vivax* binding region are identified in red (Chitnis et al., 1996). Duffy Antigen Function – The Duffy antigen receptor for chemokines (DARC) is a ‘silent’ 7-transmembrane receptor. This results from the absence of a DRYLAIV amino acid motif in the second intracellular loop needed to couple with G-proteins that initiate intracellular signalling cascades (Murphy, 1996). Duffy is one of a few chemokine receptors that bind to inflammatory chemokines, categorised by structural features into two different groups, α (amino acid motif -CC-) and β (amino acid motif –CXC-). On erythrocytes, the Duffy antigen is proposed to act as a sink that binds to excess chemokines and limits inflammation (Darbonne et al., 1991). Reciprocally, Duffy binding of chemokines prevents their diffusion into organs and peripheral tissue space and in this way acts as a reservoir of chemokines in the circulating blood (Fukuma et al., 2003). Duffy is also expressed on a variety of non-erythroid cells including venular endothelial cells; in this context recent studies suggest two potential roles for Duffy. On venular endothelial cells, Duffy has been proposed to act as a chemokine interceptor (internalisation receptor) by internalising and scavenging chemokines (Nibbs et al., 2003). Alternatively, Pruenster et al. have shown that Duffy acts to mediate chemokine transcytosis (Pruenster et al., 2009). In their *in_vitro* system, Duffy-mediated chemokine

transcytosis led to apical retention of intact chemokines and leukocyte migration across Duffy-expressing endothelial cell monolayers. How these complex roles of the Duffy antigen are regulated and influence human health remains to be determined. *(Originally published in* Zimmerman 2004*. The enigma of vivax malaria and erythrocyte Duffy-negativity, in: Dronamraju, K.R., (Ed.), Infectious Disease and Host-Pathogen Evolution.Cambridge University Press, New York, pp 141–172.) (Reproduced with permission from Cambridge University Press and Krishna R. Dronamraju).* (For interpretation of the references to colour in this figure legend, the reader is referred to the online version of this book.)

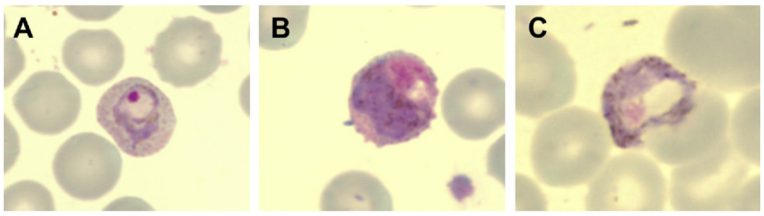


Figure 2.3.

Standard Giemsa-stained thin smear preparations of *P. vivax* infection and development in human Duffy-negative erythrocytes. Panels A and B originated from a 4-year-old female, genotyped as Duffy negative ($FY{\star}B^{ES}/{\star}B^{ES}$), who presented at the Tsiroanomandidy health center with fever (37.8 °C), headache and sweating without previous anti-malarial treatment. Standard blood smear diagnosis revealed a mixed infection with *P. vivax* (parasitaemia = 3040 parasitised red blood cells [pRBC]/μl) and *P. falciparum* (parasitaemia=980 pRBC/μl). PCR-based *Plasmodium* species diagnosis confirmed the blood smear result; *P. malariae* and *P. ovale* were not detected. (A) a *P. vivax* early-stage trophozoite with condensed chromatin, enlarged erythrocyte volume, Schüffner stippling and irregular ring-shaped cytoplasm. (B) a *P. vivax* gametocyte – lavender parasite, larger pink chromatin mass and brown pigment scattered throughout the cytoplasm are characteristics of microgametocytes (male). Panel C originated from a 12- year-old Duffy-negative ($FY{\star}B^{ES}/{\star}B^{ES}$) male, who presented at the Miandrivazo health centre with fever (37.5 °C) and shivering without previous anti-malarial treatment. Standard blood smear diagnosis and light microscopy revealed infection with only *P. vivax* (parasitaemia = 3000 pRBC/μl). PCR-based *Plasmodium* species diagnosis confirmed this blood smear result; *P. falciparum*, *P. malariae* and *P. ovale* were not detected. The parasite featured shows evidence of a *P. vivax* gametocyte – large blue parasite, smaller pink chromatin mass and brown pigment scattered throughout the cytoplasm are characteristics of macrogametocytes (female). *(Adapted from* Ménard et_al, 2010. Plasmodium vivax *clinical malaria is commonly observed in Duffy-negative Malagasy people. Proc. Natl. Acad. Sci. U. S. A. 107, 5967–5971).* (For interpretation of the references to colour in this figure legend, the reader is referred to the online version of this book.)

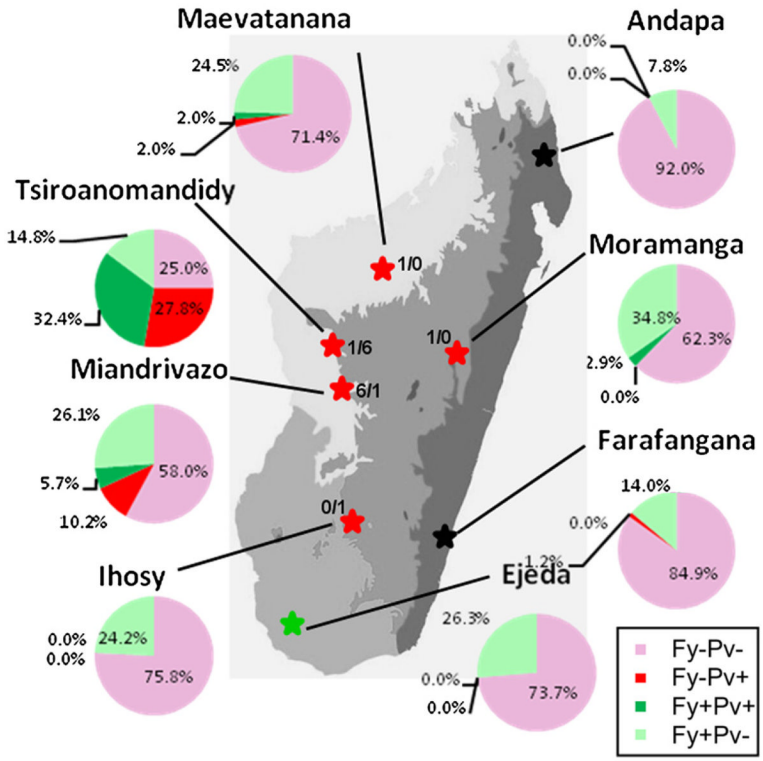


Figure 2.4.

Frequency distribution of *P. vivax* infections and clinical cases identified in Duffy-positive and Duffy-negative Malagasy people. Pie graphs show the prevalence of Duffy-positive (dark/light green) and Duffy-negative (red/pink quadrants) phenotypes in the eight Madagascar study sites. Prevalence of *P. vivax* infection observed in the survey of school-aged children is shown in red and dark green; population subsets not infected with *P. vivax* are pink and light green. Study sites identified by a red star indicate that clinical vivax malaria was observed in Duffy-negative individuals. A green star indicates that vivax malaria was observed in Duffy-positive individuals only (Ejeda). In Ihosy, clinical malaria was observed in one individual with a mixed *P. vivax*/*P. falciparum* infection. *P. vivax* malaria was not observed in Andapa and Farafangana (black star). Malaria transmission strata are identified as tropical (lightest grey), sub-desert (light grey), equatorial (middle grey) and highlands (dark grey). *(Adapted from* Ménard et_al, 2010. Plasmodium vivax *clinical malaria is commonly observed in Duffy-negative Malagasy people. Proc. Natl. Acad. Sci. U. S. A. 107, 5967–5971).* (For interpretation of the references to colour in this figure legend, the reader is referred to the online version of this book.)

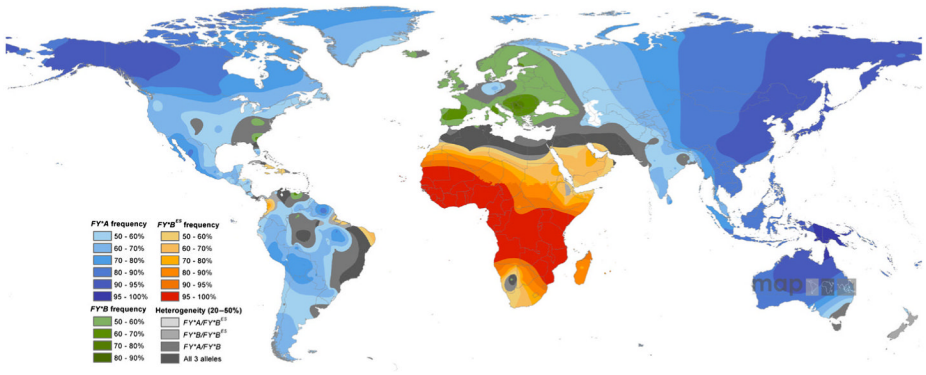
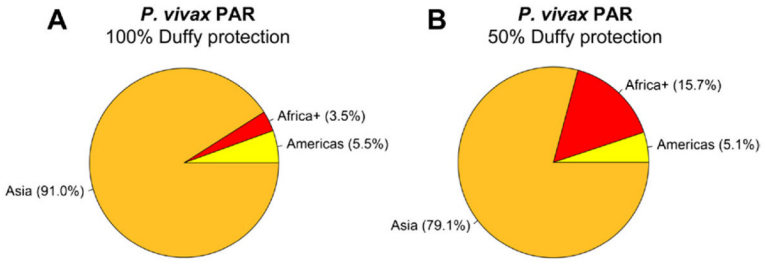


Figure 2.5. Global frequencies of the *FY* alleles

Areas predominated by a single allele (frequency ≥ 50%) are represented by a colour gradient (blue, $FY{\star}A$; green, $FY{\star}B$; red/yellow, $FY{\star}B^{ES}$). Areas of allelic heterogeneity where no single allele predominates, but two or more alleles each have frequencies ≥ 20%, are shown in grey-scale: palest for heterogeneity between the silent $FY{\star}B^{ES}$ allele and either $FY{\star}A$ or $FY{\star}B$ (when co-inherited, these do not generate new phenotypes), and darkest being co-occurrence of all three alleles (and correspondingly the greatest genotypic and phenotypic diversity). Overall percentage surface area of each class is listed in the legend. The probability distribution based on a Bayesian model is summarised as a single statistic: in this case, the median value, as this corresponds best to the input dataset, as previously described (Howes et al., 2011). Median values of the predictions were generated for each allele frequency at a 10 × 10 km resolution on a global grid with GIS software (ArcMap 9.3; ESRI). (For interpretation of the references to colour in this figure legend, the reader is referred to the online version of this book.)



C

Duffy protection	*P. vivax* population at risk in 2010 (millions)			
	Americas	Africa+	CSE Asia	World
100%	137.5	86.4	2 260.9	2 484.8
98%	137.8	101.0	2 261.3	2 500.1
95%	138.4	122.9	2 261.9	2 523.2
90%	139.3	159.4	2 262.9	2 561.6
75%	142.1	269.0	2 265.8	2 676.8
50%	146.7	451.6	2 270.6	2 868.9

Figure 2.6. Estimated change in the *P. vivax* population at risk before (A) and after (B) relaxing the level of resistance conferred by Duffy negativity from 100% to 50%, respectively

Overall increases in the percentage of PvPAR (C). The population-at-risk exclusions are based on the methods developed by Guerra et al. in 2010 (Guerra et al., 2010) and subsequently refined by Gething et al. To define the *P. vivax* population at risk (PvPAR), Gething et al. imposed several layers of exclusion from the countries with endemic *P. vivax* transmission (95 countries). *Annual parasite incidence* (API) data were used to refine the sub-national levels of transmission along administrative boundaries, classifying areas into unstable (<0.1 cases per 1000 population per year), stable transmission (≥0.1 cases per 1000 population per year) and malaria free (Guerra et al., 2010). *Temperature exclusion* based on minimum requirements for parasite sporogony modelled in relation to vector lifespan (Gething et al., 2011). *Aridity mask* to exclude areas too dry to sustain transmission by restricting vector survival and availability of ovipositioning sites. The aridity mask was derived from the bare ground areas in the GlobCover land cover imagery (Guerra et al., 2008). *Medical intelligence* was used to further exclude malaria-free urban areas (modulated with knowledge of the local *Anopheles* vectors) (Guerra et al., 2010). All these methods have been described in greater detail by Gething et al. (2012) and in Chapter 1 in Volume 80 of this special issue. Extensive data collection was necessary for the API exclusions, and individuals who contributed data to this process are acknowledged on the Malaria Atlas Project website (MAP: www.map.ox.ac.uk/). (For a colour version of this figure, the reader is referred to the online version of this book.)

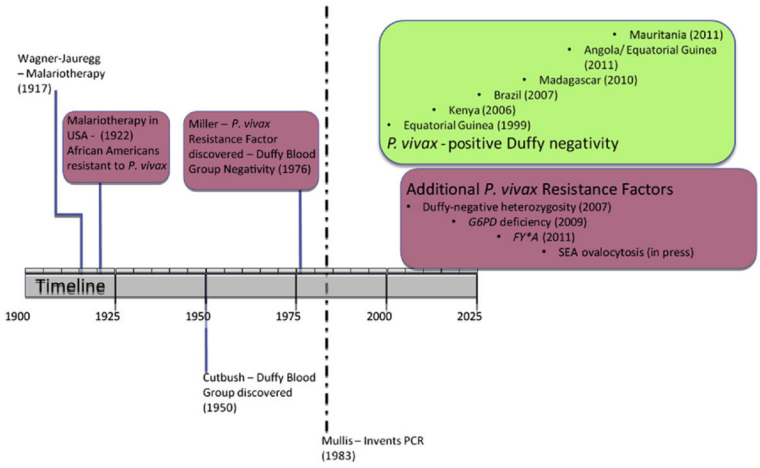


Figure 2.7.
Summary of major events providing insight on resistance to *P. vivax*. (For a colour version of this figure, the reader is referred to the online version of this book.)

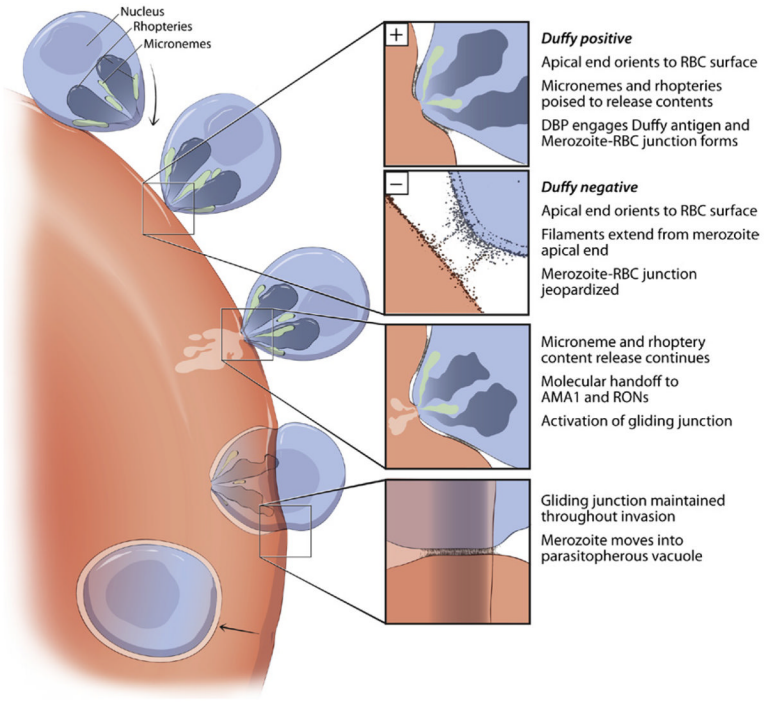


Figure 2.8. Overview of *P. vivax* merozoite interaction with the human red blood cell

Initial attachment occurs between any part of the merozoite (blue) and erythrocyte (red). The merozoite reorients, positioning its apical end for attachment to the red cell membrane. A junction forms between the apical end of the merozoite and the erythrocyte membrane of Duffy-positive cells (first call-out box). In contrast, *P. knowlesi* electron microscopy has shown thin filaments between the merozoite apical end and the Duffy-negative red cell membrane; however, the merozoite is not drawn into contact with the red cell and the junction fails to form. This has implied that junction formation fails to occur between *P. vivax* and the Duffy-negative red cell membrane as well (second call-out box). Once a durable junction has formed between the merozoite and the red cell, micronemes (green) and rhopteries (dark blue) release their contents, the red cell membrane invaginates and the merozoite moves into the parasitophorous vacuole (third call-out box). Movement of the gliding junction is complete once the merozoite is engulfed within the parasitophorous vacuole and the orifice at the red cell membrane is sealed (fourth call-out box). (For interpretation of the references to colour in this figure legend, the reader is referred to the online version of this book.)

Table 2.1

Blood Group Systems

No.	System Name	No. of Antigens	System Symbol	Gene Name(s)	Chromosomal Location	CD	Malaria	Discovered
1	ABO	4	ABO	ABO	9q34.2	–	Yes	1900
2	MNS	46	MNS	GYPA, GYPB, GYPE	4q31.21	CD235	Yes	1927
3	P	1	P1		22q11.2-qter	–	–	1927
4	Rh	50	RH	RHD, RHCE	1p36.11	CD240	–	1940
5	Lutheran	19	LU	LU	19q13.32	CD239	–	1945
6	Kell	31	KEL	KEL	7q34	CD238	–	1946
7	Lewis	6	LE	FUT3	19p13.3	–	–	1946
8	Duffy	6	FY	DARC	1q23.2	CD234	Yes	1950
9	Kidd	3	JK	SLC14A1	18q12.3	–	–	1951
10	Diego	21	DI	SLC4A1	17q21.31	CD233	–	1953
11	Yt (Cartwright)	2	YT	ACHE	7q22.1	–	–	1956
12	Xg	2	XG	XG, MIC2	Xp22.33	CD99	–	1962
13	Scianna	7	SC	ERMAP	1p34.2	–	–	1962
14	Dombrock	6	DO	ART4	12p12.3	CD297	–	1965
15	Colton	3	CO	AQP1	7pl4.3	–	–	1967
16	Landsteiner-Wiener	3	LW	ICAM4	19p13.2	CD242	–	1942
17	Chido/Rodgers	9	CH/RG	C4A, C4B	6p21.3	–	–	1962
18	H	1	H	FUT1	19q13.33	CD173	–	1952
19	Kx (McLeod syndrome)	1	XK	XK	Xp21.1	–	–	1977
20	Gerbich	8	GE	GYPC	2q14.3	CD236	Yes	1960
21	Cromer	15	CROM	CD55	1q32.2	CD55	–	1965
22	Knops	9	KN	CR1	1q32.2	CD35	Yes	1970
23	Indian	4	IN	CD44	11p13	CD44	–	1973
24	Ok	1	OK	BSG	19p13.3	CD147	–	1979
25	Raph	1	RAPH	CD151	11p15.5	CD151	–	–
26	John Milton Hagen	5	JMH	SEMA7A	15q24.1	CD108	–	1978
27	I	1	I	GCNT2	6p24.2	–	–	–
28	Globoside	1	GLOB	B3GALT3	3q26.1	–	–	–
29	Gill	1	GIL	AQP3	9p13.3	–	–	–

No.	System Name	No. of Antigens	System Symbol	Gene Name(s)	Chromosomal Location	CD	Malaria	Discovered
30	Rh-associated glycoprotein	3	RHAG	RHAG	6p21-qter	CD241	–	–

As of March 5, 2012, there are 32 blood group systems, 42 genes and 1312 alleles. For the latest figures, please consult the following website. http://www.ncbi.nlm.nih.gov/projects/gv/rbc/xslcgi.fcgi?cmd=bgmut/summary.

Table 2.2

Working Guidelines for Duffy* blood group nomenclature†

			Phenotype	
Allele	**Antigen**	**Genotype‡**	**Serologic**	**Expression$**
FY★A	Fy^a	*FY★A/FY★A*	Fya+/b−	2×Fy^a, 0×Fy^b
FY★B	Fy^b	*FY★A/FY★A*^{ES}	–	1×Fy^a, 0×Fy^b
FY★X	Fy^{bweak}	*FY★A/FY★B*^{ES}	–	1×Fy^a, 0×Fy^b
FY★A^{ES}	–	*FY★B/FY★B*	Fya−/b+	0×Fy^a, 2×Fy^b
FY★B^{ES}	–	*FY★B/FY★X*	–	0×Fy^a, 1.1×Fy^b
–	–	*FY★B/FY★A*^{ES}	–	0×Fy^a, 1×Fy^b
–	–	*FY★B/FY★B*^{ES}	–	0×Fy^a, 1×Fy^b
–	–	*FY★X/FY★X*	Fya−/b+weak	0×Fy^a, 0.2×Fy^b
–	–	*FY★X/FY★A*^{ES}	–	0×Fy^a, 0.1×Fy^b
–	–	*FY★X/FY★B*^{ES}	–	0×Fy^a, 0.1×Fy^b
–	–	*FY★A/FY★B*	Fya+/Fyb+	1×Fy^a, 1×Fy^b
–	–	*FY★A/FY★X*	–	1×Fy^a, 0.1×Fy^b
–	–	*FY★A*^{ES}/*FY★A*^{ES}	Fya−/Fyb−	0×Fy^a, 0×Fy^b
–	–	*FY★A*^{ES}/*FY★B*^{ES}	–	0×Fy^a, 0×Fy^b
–	–	*FY★B*^{ES}/*FY★B*^{ES}	–	0×Fy^a, 0×Fy^b

*Alternate gene name = Duffy antigen/receptor for chemokines (DARC)

†Consistent with International Society of Blood Transfusion blood group terminology (web address provided below) www.isbtweb.org/working-parties/red-cell-immunogenetics-and-blooct-group-terminology/blood-group-terminology/#c581

‡ES, erythrocyte silent – attributed to the T to C transition at nucleotide −33 in the Duffy gene promote

$Expression phenotypes based on composite of flow cytometry and chemokine binding. (Ménard et al., 2010)