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Deciphering Plasmodium vivax invasion pathway(s) in Duffy-negative patients

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Isabelle Bouyssou. Deciphering Plasmodium vivax invasion pathway(s) in Duffy-negative patients. Parasitology. Sorbonne Université, 2023. English. NNT : 2023SORUS180 . tel-04628214

HAL Id: tel-04628214

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Sorbonne Université

Ecole Doctorale 515 « Complexité du vivant »

Unité de Recherche Paludisme, Génétique et Résistance (Institut Pasteur Paris)

Deciphering *Plasmodium vivax* invasion pathways in Duffy-negative patients

PhD thesis in parasitology

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Directed by Pr. Didier Ménard

Publicly presented and defended on 27.06.2023

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Acknowledgments

Mardi 1^{er} septembre 1998, je tiens l'immense parapluie aux couleurs arc en ciel à bout de bras. Ma mère et moi errons dans les rues de Douala à la recherche de l'école primaire car c'est la rentrée des classes. Je suis trempée jusqu'aux os par les pluies diluviennes et manque de tomber dans le caniveau à chaque pas. Du haut de mes 8 ans, je fulmine intérieurement. On m'a fait croire que vivre au Cameroun serait comme être en vacances toute l'année. Mais là, tout de suite, j'ai du mal à visualiser l'odeur des frangipaniers géants, la saveur des fruits exotiques et la caresse du soleil équatorial. Déjà ce matin il a fallu avaler ces horribles cachets de Nivaquine®. C'est quoi d'abord le paludisme ?

Il ne m'a pas fallu longtemps pour réaliser la chance que j'avais de pouvoir prendre des antipaludiques tous les jours. Mais ce n'est que bien plus tard, en étudiant à l'université, puis en travaillant dans l'industrie pharmaceutique, que cet intérêt tout relatif pour le paludisme s'est transformé d'abord en curiosité, puis en véritable sujet d'étude. De Paris à Londres en passant par Strasbourg, ce doctorat aura été riche en émotions – comprendre : quelques instants de joie pour plusieurs crises de nerfs – et en découvertes. Il me tient donc à cœur de remercier toutes les personnes qui ont contribué, de près ou de loin, à ce projet.

Tout d'abord, je souhaite remercier mon Directeur de thèse, Pr. Didier Ménard, pour m'avoir guidée dans les méandres de la recherche, Dr. Sara El Hoss pour m'avoir encouragée dans ma démarche scientifique et Pr. John Strouboulis pour m'avoir conseillée dans l'obtention d'une bourse de mobilité. Merci également aux autres membres de mon jury de thèse, Pr. Dominique Mazier, Dr. Carlo Severini, Pr. Chetan Chitnis, Dr. Rachida Tahar, Dr. Benoît Malleret et Pr. Olivier Silvie pour avoir accepté la « lourde tâche » de lire, corriger, et commenter ce manuscrit. Je souhaite également remercier l'Agence Nationale de la Recherche, la Fondation pour la Recherche Médicale et l'European Haematology Association pour avoir financé ce projet.

A bien des égards, ce manuscrit est le fruit d'un immense travail d'équipe. Je souhaite donc remercier Dr. Matthieu Schoenhals, Dr. Maria de Fátima Ferreira-da-Cruz, Pr. Sandrine Houzé, Dr. Ali Ould Mohamed Salem Boukhary et Pr. Lemu Golassa pour la collecte et l'envoi d'échantillons, Laurence Ma et Pascal Campagne pour leur support sans failles dans le séquençage et l'analyse génomique des souches de *P. vivax*, Cécile Doderer-Lang pour son aide et son enthousiasme dans la réalisation des essais d'invasion, l'unité de recherche Red Cell

Haematology Laboratory pour son aide dans la caractérisation des érythroblastes Duffy-négatifs et l'unité de recherche Biologie de Plasmodium et Vaccins pour son accueil chaleureux.

Merci à mes parents pour avoir toujours nourri ma curiosité et mes envies de voyage ainsi que ma sœur Sophie pour me rendre visite où que j'aie, et ce même lorsqu'un avis de tempête est annoncé. Ces quatre années de doctorat n'auraient pas été les mêmes sans le soutien de mes amis. J'ai une pensée toute particulière pour Camilla, Carole, Odette, Meri, Natasha et toute la team GBette. Enfin, merci à Clément pour supporter mes bavardages à 03h30 du matin et pour me faire rire au quotidien.

Tuesday, September 1st, 1998: I am resolutely holding the huge colorful umbrella. My mother and I are wandering in the streets of Douala, Cameroon searching for the primary school. I am soaked to the skin by the heavy rain and fall in the gutter at every step. My parents told me that living in Cameroon would be like going on long-term holidays. But I was not fooled: « right now, I cannot really visualize the smell of giant frangipani trees, the taste of exotic fruits and the kiss of equatorial sun. On top of that, I had to swallow these horrific Nivaquine[®] pills for breakfast. What is it for already? Malaria? ».

It did not take me long to realize how lucky I was to take these pills every day. But it is way later, when I was studying at the University, then working in a pharmaceutical industry, that this simple interest for malaria became a scientific wonder, then an actual subject of study. From Paris to London then Strasbourg, this journey has truly been high in emotions – understand: a few instants of joy for a lot of troubles – and discoveries. Therefore, I would like to thank all the persons who contributed, directly or indirectly, to my PhD project.

First of all, I would like to thank my thesis Director, Pr. Didier Ménard, for his precious guidance within the realm of research, Dr. Sara El Hoss for her kind encouragements during experimental work and Pr. John Strouboulis for his endless support in the search of mobility grants. I would like to thank the other members of my thesis jury: Pr. Dominique Mazier, Dr. Carlo Severini, Pr. Chetan Chitnis, Dr. Rachida Tahar, Dr. Benoît Malleret et Pr. Olivier Silvie for accepting the « heavy task » to review, comment, and correct this manuscript. I also wish to thank the Agence Nationale de la Recherche, the Fondation pour la Recherche Médicale and the European Hematology Association for funding this project.

In many ways, this thesis is the result of a great teamwork. I would like to thank Dr. Matthieu Schoenhals, Dr. Maria de Fátima Ferreira-da-Cruz, Pr. Sandrine Houzé, Dr. Ali Ould Mohamed Salem Boukhary and Pr. Lemu Golassa for the collect of *P. vivax* field samples,

Laurence Ma, and Pascal Campagne for their huge support in the sequencing and genomic analysis of these *P. vivax* isolates, Cécile Doderer-Lang for her enthusiasm and help in performing *in vitro* invasion assays, the Red Cell Haematology Laboratory for its help in performing *in vitro* characterization of Duffy-negative erythroblasts and the Biology of Plasmodium and Vaccines research unit for its warm welcome.

Many thanks to my parents for always feeding my curiosity and wanderlust, and to my sister Sophie for visiting me everywhere I go, even when a major storm is announced. These four years of PhD would not have been the same without the support of my friends. My special thoughts go to Camilla, Carole, Odette, Meri, Natasha and the GBette team. I would also like to thank Clément for enduring my long chitchats at 03:30am and for making me laugh every single day.

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Abstract

Vivax malaria is an acute debilitating illness caused by the parasitic protozoan *Plasmodium vivax* and transmitted by female *Anopheles* mosquitoes. It is mainly prevalent in America, South-East Asia, Middle East, Western Pacific, Eastern and Southern Africa but considered rare in Sub-Saharan Africa. Historically, the disease has often been regarded as a benign self-limiting infection. This is due to the observation of low parasitemia in Duffy-positive patients and virtual absence of infections in Duffy-negative individuals. Indeed, previous studies showed that individuals with African and African American origins were naturally resistant to *P. vivax*. Parasitologists thought that this resistance was due to the absence of Duffy Antigen Receptor for Chemokines (DARC) on the surface of their erythrocytes. Subsequently, the identification of the ligand *P. vivax* Duffy Binding Protein (*Pv*DBP) specific to DARC and the evidence that the interaction *Pv*DBP- DARC was crucial for invasion led to a scientific paradigm by which *P. vivax* merozoites exclusively invade Duffy-positive erythrocytes. Consequently, research on *vivax* malaria has long been neglected and many knowledge and tool gaps remain to be filled.

However, since a few years, the stagnating burden of the disease in many countries associated with the increasing report of *P. vivax* infections in Duffy-negative patients raised questions about *P. vivax* invasion pathways. A first hypothesis was that *P. vivax* may have evolved to a new invasion pathway that would overcome Duffy-negativity. Alternatively, a second hypothesis was that Duffy-negative populations may have always been a silent and insidious reservoir of infection which had previously gone unnoticed.

My thesis project aimed at deciphering *Plasmodium vivax* invasion pathways in Duffy-negative patients. A three-phases approach was implemented as following to address the main hypotheses:

1. Bibliographical study: understand the global context and key issues of the presence of *vivax* malaria in Sub-Saharan Africa and review current knowledge on *P. vivax* invasion pathways through the lens of omics technologies. The scientific review « *Plasmodium vivax* blood stage invasion pathways: Contribution of omics technologies in deciphering molecular and cellular mechanisms » has been published in the Comptes-Rendus Biologie of the Académie des Sciences in November 2022.
2. Molecular epidemiological study: understand *vivax* malaria epidemiology in Ethiopia, dissect the genome of *P. vivax* strains circulating in Sub-Saharan Africa and search for

potential molecular signature in genes encoding proteins involved in *P. vivax* invasion pathways using next generation sequencing techniques. A first scientific article « *Vivax* malaria in Duffy-negative patients shows invariably low asexual parasitaemia: Implication towards malaria control in Ethiopia » has been published in Malaria Journal in August 2022. A second article « Potential hidden *Plasmodium vivax* malaria reservoirs from low parasitemia Duffy-negative Ethiopians: molecular evidence » has been in the journal Public Library Of Science Neglected Tropical Diseases in July 2023. A third scientific article is currently being drafted. The manuscripts are presented here.

3. Functional study: characterize Duffy-negative erythroblasts during terminal erythroid differentiation and assess the ability of *P. vivax* merozoites to invade Duffy-negative erythroblasts by developing reproducible *in vitro* functional assays. A scientific article « The DARC side of *P. vivax* in Africa » is currently being drafted. The manuscript is presented here.

Experimental work has mainly been carried out in the Malaria, Genetics and Resistance research unit, Institut Pasteur Paris under the direction of Pr. Didier Ménard. Various collaborations have been established with the research unit Biology of Plasmodium and Vaccines (Institut Pasteur Paris), Institut Pasteur Madagascar, French National Reference Centre for malaria, Addis-Abeba University, Nouackott Al-Aasriya University, Foundation Oswaldo Cruz, and King's College London to successfully complete the thesis project.

Findings of the bibliographical study showed that despite the recent advances of omics technologies, key knowledge and tool gaps remain in *vivax* malaria research. Besides, findings of the molecular epidemiological studies revealed that *P. vivax* strains cluster in distinct geographic clusters. However, these findings did not reveal any association between genetic diversity and adaptation to Duffy-negative human hosts. This suggests that the parasites did not evolve towards an alternative invasion pathway. In fact, findings of the *in vitro* functional study demonstrated that a subset of Duffy-negative erythroblasts can express functional DARC during terminal erythroid differentiation and that *P. vivax* merozoites can invade them. Taken together, these findings contrast with the established scientific paradigm by which *P. vivax* merozoites exclusively invade Duffy-positive erythrocytes and support the assumption that African Duffy-negative populations may be a silent and insidious reservoir of infection, with all the consequences that this entails for the control and eradication of *vivax* malaria in Sub-Saharan Africa.

Key words: *Plasmodium vivax*, malaria, Duffy-negativity, erythropoiesis, invasion, Sub-Saharan Africa

Résumé

Le paludisme à *Plasmodium vivax* est une maladie infectieuse causée par le parasite protozoaire *Plasmodium vivax* et transmise par les moustiques femelles *Anopheles*. Cette maladie est prévalente en Amérique, Asie du Sud-Est, Moyen-Orient, Pacifique Ouest ainsi qu'en Afrique du Nord-Est et Afrique du Sud mais demeure rare en Afrique Sub-saharienne. Historiquement, le paludisme à *P. vivax* a souvent été considéré comme une maladie bénigne et peu dangereuse. En effet, la maladie est caractérisée par de faibles parasitémies chez les patients Duffy-positifs. Par ailleurs, d'anciennes études ont montré que les personnes Duffy-négatives d'origine africaine ou afro-américaine étaient naturellement résistantes. Les parasitologistes pensaient que cette résistance était due à l'absence de récepteurs Duffy Antigen Receptor for Chemokines (DARC) à la surface de leurs érythrocytes. Par la suite, l'identification du ligand *P. vivax* Duffy Binding Protein (PvDBP) spécifique à DARC et la preuve de l'importance de l'interaction PvDBP-DARC dans le mécanisme d'invasion ont conduit à l'établissement d'un paradigme scientifique selon lequel les mérozoïtes de *P. vivax* envahissent seulement les érythrocytes Duffy-positifs. Par conséquent, la recherche sur le paludisme à *P. vivax* a longtemps été négligée à la fois par les parasitologistes et les organismes de financement. A l'heure actuelle, il n'existe que peu d'informations sur la biologie de *P. vivax* et peu d'outils pour le cultiver *in vitro*.

Cependant, depuis quelques d'années, le nombre de cas de paludisme à *P. vivax* stagne dans de nombreux pays et un nombre croissant de cas d'infection chez des patients Duffy-négatifs est reporté en Afrique. Cela a soulevé de vives interrogations quant aux réels mécanismes d'invasion de *P. vivax*. Une première hypothèse proposée est que le parasite a évolué vers un nouveau mécanisme d'invasion outrepassant la Duffy-négativité. Alternativement, une deuxième hypothèse avancée est que les populations Duffy-négatives ont toujours représenté un réservoir silencieux et insidieux d'infection jusque-là passé inaperçu.

Mon projet de thèse intitulé « Voies d'invasion de *Plasmodium vivax* chez les patients Duffy-négatifs » vise donc à décrypter les mécanismes d'invasion de *Plasmodium vivax* chez les patients Duffy-négatifs. Une approche en trois phases a été implémentée afin de répondre aux hypothèses mentionnées :

1. Etude bibliographique : comprendre le contexte historique et scientifique dans lequel s'inscrit le projet, identifier les enjeux clés liés à l'émergence du paludisme à *P. vivax* en Afrique sub-saharienne et étudier les connaissances actuelles sur les mécanismes d'invasion

de *P. vivax* obtenues grâce aux technologies omiques. Une revue scientifique intitulée « *Plasmodium vivax* blood stage invasion pathways : Contribution of omics technologies in deciphering molecular and cellular mechanisms » a été publiée dans les Comptes-Rendus Biologie de l'Académie des Sciences en novembre 2022.

2. Etude d'épidémiologie moléculaire : comprendre l'épidémiologie du paludisme à *P. vivax* en Ethiopie, disséquer le génome d'isolats de terrain de *P. vivax* circulant en Afrique subsaharienne et rechercher une potentielle signature moléculaire au niveau des gènes codant pour des protéines impliquées dans les voies d'invasion de *P. vivax* via des techniques de séquençage avancées. Un premier article scientifique intitulé « *Vivax* malaria in Duffy-negative patients shows invariably low asexual parasitaemia : Implication towards malaria control in Ethiopia » a été publié dans Malaria Journal en août 2022. Un deuxième article scientifique intitulé « Potential hidden *Plasmodium vivax* malaria reservoirs from low parasitemia Duffy-negative Ethiopians: molecular evidence » a été publié dans le journal Public Library Of Science Neglected Tropical Diseases en juillet 2023. Un autre article scientifique, dont les résultats préliminaires seront commentés dans le présent manuscrit, est en cours de rédaction.
3. Etude fonctionnelle : caractériser les érythroblastes Duffy-négatifs pendant leur phase de différenciation érythroïde terminale et évaluer la capacité des mérozoïtes de *P. vivax* à envahir ces érythroblastes Duffy-négatifs grâce au développement d'essais fonctionnels *in vitro*. Un article scientifique intitulé « The DARC side of *P. vivax* in Africa » est en cours de rédaction et sera présenté dans le présent manuscrit.

Les travaux de recherche expérimentale ont été principalement réalisés au sein de l'unité de recherche Paludisme, Génétique et Résistance de l'Institut Pasteur Paris sous la direction du Pr. Didier Ménard. De nombreuses collaborations ont été établies, notamment avec l'unité de recherche Biologie de Plasmodium et Vaccins (Institut Pasteur Paris), l'Institut Pasteur Madagascar, le Centre National de Référence pour le paludisme, l'Université d'Addis-Abeba, l'Université de Nouackott Al-Aasriya, l'Institut Oswaldo Cruz et le laboratoire Red Cell Haematology du King's College London.

Les résultats de l'étude bibliographique ont montré que, malgré les récentes avancées permises par les technologies dites « omiques », la recherche sur le paludisme à *Plasmodium vivax* est entravée par le manque de données scientifiques et de techniques expérimentales. Par ailleurs, les résultats des études d'épidémiologie moléculaire ont révélé que les souches de *P. vivax* se regroupent en clusters géographiques bien définis. Cependant, les résultats n'ont

permis d'identifier aucune association entre la diversité génétique des souches de *P. vivax* et une quelconque adaptation aux hôtes humains Duffy-négatifs. Ce qui suggère que les parasites *P. vivax* n'ont pas évolué vers une voie d'invasion alternative pour infecter les patients Duffy-négatifs. En fait, les résultats de l'étude fonctionnelle ont démontré qu'une sous-population d'érythroblastes Duffy-négatifs sont capables d'exprimer la protéine fonctionnelle DARC pendant leur différenciation érythroïde terminale, et que les mérozoïtes *P. vivax* sont capables de les envahir. Ainsi, l'ensemble de ces travaux de recherche va à l'encontre du paradigme scientifique établi selon lequel les mérozoïtes de *P. vivax* envahissent exclusivement les érythrocytes Duffy-positifs et supporte l'hypothèse que les populations africaines Duffy-négatives représentent un réservoir silencieux et insidieux d'infection. Ce qui entraîne d'importantes conséquences pour le contrôle et l'éradication du paludisme à *P. vivax* en Afrique Sub-saharienne.

Mots clés : *Plasmodium vivax*, paludisme, Duffy-négativité, érythropoïèse, invasion, Afrique Sub-Saharienne

List of abbreviations

ACT: artemisinin-based combination therapy
ARMS: Amplification Refractory Mutation System
BFU-E: burst-forming unit-erythroid progenitors
CFU-E: colony-forming unit-erythroid progenitors
CMP: common myeloid progenitors
CNV: copy number variations
DARC: Duffy antigen receptor for chemokines
DNA: deoxyribonucleic acid
EBI: erythroblastic islands
EPO: erythropoietin
G6PD: glucose-6-phosphate dehydrogenase
GWAS: genome wide association study
HSPC: hematopoietic stem and progenitor cell
MEP: megakaryocytic-erythroid progenitors
PCR: Poly Chain Reaction
Pf. Plasmodium falciparum
Pv. Plasmodium vivax
*Pv*DBP: *P. vivax* Duffy binding protein
*Pv*EBP: *P. vivax* Erythrocyte binding protein
qPCR: quantitative poly chain reaction
RDT: rapid diagnostic test
RNA: ribonucleic acid
SNP: single nucleotide polymorphisms
sWGA: selective whole genome amplification
WGS: whole genome sequencing
WHO: World Health Organization

Introduction

Vivax malaria is an acute debilitating illness caused by the parasitic protozoan *Plasmodium vivax* and transmitted by female *Anopheline* mosquitoes. It is prevalent in America, South-East Asia, Middle East, Western Pacific, and in Eastern and Southern Africa but considered rare in Sub-Saharan Africa¹. Through the years, the assumption that Duffy-negativity was the cause of natural resistance to *vivax* malaria in Sub-Saharan Africa has been supported by findings from experimental infections, demonstrations of uninfected Duffy-negative individuals and microscopically confirmed endemic *P. vivax* strains^{1,2} found in regions where Duffy-negativity was either rare (Northern and Southwestern Africa), less frequent (Horn of Africa) or present but admixed with Duffy-positive populations (Madagascar)¹. However, a growing number of studies has recently reported cases of *vivax* malaria in Duffy-negative patients across South America^{2,3} and Africa⁴⁻¹⁴.

Despite recent efforts, research on *vivax* malaria has long been neglected and its overall burden has probably been underestimated¹⁵. Many key knowledge and tool gaps, such as the understanding of *P. vivax* invasion mechanisms, the development of robust *in vitro* culture systems or the implementation of reliable measures of *P. vivax* clinical burden, remain to be filled¹⁵. In addition, the current stagnating burden of the disease in many countries associated with the increasing number of *P. vivax* infections reported in Duffy-negative African patients raised questions about actual *P. vivax* invasion mechanisms¹⁶. A first hypothesis was that *P. vivax* may have evolved to a new invasion pathway that would overcome Duffy-negativity¹⁷. Alternatively, a second hypothesis was that Duffy-negative populations may have always been a silent and insidious reservoir of infection¹⁸. Overall, these observations also challenge common assumptions about the evolution of host-pathogen interaction and the limit of detection of conventional diagnostic tools¹.

Thus, my thesis project aimed at deciphering *P. vivax* invasion mechanisms in Duffy-negative patients. This was achieved through the implementation of a three-phases approach as following:

1. Bibliographical study: understand the global context and key issues of the presence of *vivax* malaria in Sub-Saharan Africa and review current knowledge on *P. vivax* invasion pathways through the lens of omics technologies.

2. Molecular epidemiological study: understand *vivax* malaria epidemiology in Ethiopia, dissect the genome of *P. vivax* strains circulating in Sub-Saharan Africa and search for potential molecular signature in genes encoding proteins involved in *P. vivax* invasion pathways using next generation sequencing techniques.
3. Functional study: characterize Duffy-negative erythroblasts during terminal erythroid differentiation and assess the ability of *P. vivax* merozoites to invade Duffy-negative erythroblasts by developing reproducible *in vitro* functional assays.

Thesis project

I. Bibliographical study on *vivax* malaria

The first objective of my thesis project was to understand why *vivax* malaria should be considered a complex clinical and public health issue and what do we currently know about *P. vivax* invasion pathways. As recent progress in *vivax* malaria research is just starting to reverse decades of neglect, it was important to dig into history to fully address these questions.

1. Historic and scientific insights on *vivax* malaria

1. Historical context

A medical breakthrough

Malaria is probably one of the oldest diseases in the world. It likely arose in our primate ancestors in Africa and evolved with humans¹⁹. In the Antiquity, many ancient civilizations in Arabia, China, Egypt and Europe described the characteristic periodic fevers of the disease²⁰. It became rapidly clear that the disease was associated to marshes. Various assumptions were evoked to explain the cause, the most common incriminating miasmas rising from the swamps²¹. In fact, our scientific understanding of malaria started in the late 19th century with the early beginnings of microbiology²¹. In 1880, Alphonse Laveran found microscopic *Plasmodium* parasites in the blood of a patient²². Around 1890, Patrick Manson, who had previously worked on another blood parasite, postulated that mosquitoes may act as a vector to transmit malaria. Under his influence, Ronald Ross undertook experimental investigations²³. In 1897, he first discovered *P. falciparum* oocysts in a female *Anopheline* mosquito, then all stages of *P. relictum* in *Culicinae* mosquitoes²¹. In 1898, Giovanni Battista Grassi, Amico Bignami, and Giuseppe Bastianelli described the developmental stages of malaria parasites in female *Anopheline* mosquitoes²⁴⁻²⁶. In 1948, Henry Shortt and Cyril Garnham proved that a phase of division in the liver preceded the phase of development in the blood²⁷. Finally, in 1982, Wojciech Krotoski showed that *P. vivax* liver stages could remain dormant for several months²⁸.

An old scientific paradigm

Between 1917 and 1950, malaria therapy was used to treat many conditions, including neurosyphilis²⁹. It is in this context that clinical and field trials reported for the first time that African and African American individuals were highly resistant to *vivax* malaria³⁰⁻³³. Between

1950 and 2005, the identification of Duffy blood groups^{34,35} and the demonstration of the molecular origin of Duffy-negativity as a single point mutation in the sequence of the GATA1-promoter region of the *ackr1* gene^{36,37} established that the natural resistance to *vivax* malaria was related to the absence of Duffy antigen receptors for chemokines (DARC) on the surface of human erythrocytes³⁸. Seminal works and *in vitro* studies subsequently confirmed that DARC was required as a receptor for *P. vivax* merozoites to invade erythrocytes^{38,39}. In parallel, an *in vitro* study demonstrated that *P. vivax* Duffy binding protein (*PvDBP*) was the specific ligand for DARC and that the interaction *PvDBP*-DARC was crucial for invasion⁴⁰. Taken together, these findings led to a scientific paradigm by which *P. vivax* exclusively invades Duffy-positive erythrocytes through the interaction *PvDBP*-DARC. Thereupon, *vivax* malaria was assumed to be seldom transmitted or even absent in regions where Duffy-negativity is predominant such as Sub-Saharan Africa.

New evidence of *P. vivax* infections in Duffy-negative patients

In the early 20th century, rapid economic, environmental, and social changes led to the decline of malaria in Europe and the United States. In the 1970s, the eradication of malaria in Europe contributed to a disinterest in malaria research⁴¹. In particular, *vivax* malaria has long been considered a benign self-limiting infection and neglected by parasitologists. This was partly due to the observation of low parasitaemia in Duffy-positive patients and virtual absence of infections in Duffy-negative individuals⁴². However, new evidence showed that *P. vivax* infection and transmission occur in Sub-Saharan Africa. As early as 1943, Cyril Garnham described an outbreak of *P. vivax* in Kenya¹². In the 2000's, reports of *P. vivax* infections in Duffy-negative individuals throughout South America^{2,3} and Africa^{10,12,43} triggered awareness of a potential threat. Between 2010 and 2019, many surveys revealed additional cases of *P. vivax* infections in Duffy-negative patients by PCR^{5-9,11,43-49}. Since 2019, further investigations have confirmed *P. vivax* endemic transmission in Sub-Saharan Africa^{4,50-55}.

2. Overview of the current situation

Epidemiology

According to the latest WHO's malaria report, populations at risk are mainly children under age five, pregnant women and migrants⁵⁶. Between 2000 and 2021, the global burden of *vivax* malaria decreased from 20.5 million cases to 4.9 million cases⁵⁶. Within this period, a significant decline of the burden was recorded in America, Southeast Asia, and Western Pacific.

However, since a few years, this burden seems to stagnate and even increase in some regions affected by political and economic instability such as Papua New Guinea, Sudan and Yemen¹⁶. In Africa, the growing number of cases of *vivax* malaria reported in Duffy-negative patients and travellers highlight the existence of a parasite reservoir occurring in asymptomatic and low-density infections⁵⁷. But estimates should be interpreted with caution as they are based on clinical cases of *vivax* malaria and restricted to a limited number of blood surveys¹.



Figure 1: Global burden of *vivax* malaria. In 2020, the global burden of *vivax* malaria was 4.9 million cases. The disease was prevalent in America, South-East Asia, Middle East, Western Pacific, and North-East Africa. Figure from <https://data.malariaatlas.org>.

Pathophysiology

In Duffy-positive patients, the common clinical symptoms are fever, chills, and nausea. Severe illness is dominated by profound anemia⁵⁸ and thrombocytopenia along with leukopenia and neutropenia⁵⁹. Respiratory, hepatic, renal, and circulatory dysfunctions can also be observed^{60,61}. In addition, chronic recurring febrile illness occurs when *P. vivax* hypnozoites emerge weeks, months, or years after primary infection⁶². In Duffy-negative patients, there is no evidence of mitigation but *P. vivax* infections are believed to be more benign¹.

Diagnosis

Clinical symptoms alone do not permit accurate diagnosis of *P. vivax* infections. Any suspected case requires collection of venous blood and analysis by light microscopy, rapid diagnosis tests (RDTs), or PCR methods⁵⁶. But as *P. vivax* infections are characterized by

asynchrony and low parasitaemia in peripheral blood, these conventional methods may contribute to missed diagnoses¹.

Treatment

The WHO recommends mass drug administration to reduce *P. vivax* transmission. According to the guidelines, the first line treatment is chloroquine or artemisinin-based combination therapies (ACTs) in combination with an 8-aminoquinoline, primaquine or tafenoquine⁵⁶. Chloroquine and artemisinin are efficient drugs to eliminate blood stage parasites⁶³. Primaquine and tafenoquine are specific drugs to eliminate latent hypnozoites and prevent relapses^{63,64}. However, chloroquine-resistant, multidrug-resistant, and tolerant-primaquine *P. vivax* strains are currently emerging⁶³. Besides, primaquine and tafenoquine cannot be administered to children, pregnant women and patients who present a severe deficiency in glucose-6-phosphate dehydrogenase (G6PD) because of their adverse effects⁶⁵.

Prevention

Prevention of *vivax* malaria includes the use of insecticide-treated nets and insecticides to prevent primary *P. vivax* infections and interrupt transmission. But their effectiveness depends on distribution, coverage, adherence, maintenance, vector biting patterns and levels of insecticide resistance⁶⁵. The only suppressive chemoprophylactic strategy that specifically targets *P. vivax* is a weekly chloroquine dose given during pregnancy and lactation. But it is seldom used, probably because of insufficient cost-effectiveness⁶⁵.

3. *P. vivax* pathogenicity

Infection

P. vivax displays singular biological features that give rise to a complex pathogenicity and have important clinical consequences. Within the human host, its hepatic cycle results in the production of latent hypnozoites leading to asymptomatic infections and relapses⁶⁶. Its erythrocytic cycle is usually characterized by asynchrony and low parasitemia (<10,000 parasites per µl of blood) but can lead to severe illness⁶⁷. Once inside the reticulocytes, the parasite induces morphological alterations such as the deformation of their membrane, depletion of certain proteins including CD71⁶⁸, expulsion of intracellular content, and formation of Schüffner's dots that may facilitate evasion from host immune system⁶⁹. If *P. vivax* preference for immature CD71+ reticulocytes is assumed to self-limit its expansion in the

peripheral blood, recent evidence indicates that the parasite also exhibits a strong tropism for extravascular tissues in deep organs such as the bone marrow^{70,71} and the spleen⁷². These organs are active sites of erythropoiesis or extramedullary erythropoiesis, providing the immature CD71+ reticulocytes required for the parasite propagation. Besides, *P. vivax* trophozoites are motile. While motility seems futile in an organism passively flowing within the vascular canals, it may present an advantage to access the extravascular niches¹.

Transmission

P. vivax has a high transmission potential due to early, continuous production of gametocytes, high infectivity, and development through short sporogonic cycles at low temperatures (18-25°C) in the mosquito vector⁷³. Hence, even though endemic transmission occurs predominantly across tropical latitudes, the parasite adapts to a wide range of habitats and climatic conditions throughout America, South-East Asia, Middle East, Western Pacific, and North-East Africa¹. For a long time, the predominance of Duffy-negative populations has been considered the basis of its virtual absence in Western, Central, and part of Eastern Africa¹. But, as explained previously, recent surveys showed that endemic transmission occurs at low prevalence and density of parasitaemia in Sub-Saharan Africa⁷⁴.

4. Human host biology

Duffy blood groups

As mentioned previously, the Duffy blood classification system is based on the presence or absence of DARC, also known as Fy antigen, on the surface of erythrocytes, vasculature endothelial cells, alveolar epithelial cells, collecting tubules, and Purkinje cells⁷⁵. There are six different antigens arising from variations in the *ackr1* gene encoding DARC. The two major codominant alleles are Fy^a (Fy01) and Fy^b (Fy02) that result from a single nucleotide polymorphism (SNP) at position 125 (125G>A) and express the antigens Fya and Fyb. The antigens Fy03, Fy04, Fy05 and Fy06 are minor variations of Fya and Fyb epitopes⁷⁶. In addition, the antigen Fyx corresponds to a variation in which the allele Fy^x expresses only weakly the antigen Fyb^{77,78}. This yields four different phenotypes: Fy(a+b+), Fy(a+b-), Fy(a-b+) and Fy(a-b-)⁷⁹. There are two different genetic backgrounds giving rise to the Duffy-negative phenotype Fy(a-b-). The first one corresponds to a mutation in the promoter region GATA-1 of the *ackr1* gene, abolishing the expression of DARC in erythrocytes but not in epithelial cells³⁷. The second one corresponds to a point mutation introducing a premature stop

codon into the coding sequence of the *ackr1* gene and abolishing the expression of DARC in all the cells⁸⁰. In Sub-Saharan Africa, the Duffy-negative phenotype Fy(a-b-) is predominant and often occurs with homozygosity for the Fy^b allele carrying a point mutation abolishing DARC only in erythrocytes⁸¹.

Human erythropoiesis

In human adults, the bone marrow is a semi-solid tissue found in the cancellous portions of bones. It is constituted of reticular connective tissue stroma, sinusoidal capillaries, and erythroblastic islands (EBI)⁸². EBIs are composed of one or two central macrophages surrounded by erythroid cells at various stages of maturation. These macrophages play a crucial role in erythropoiesis acting as an anchor to the erythroblasts and providing cellular interactions required for proliferation and differentiation. Thus, EBIs are specialized niches in which erythropoiesis, the process of blood production, occurs⁸³. Erythropoiesis starts with an engagement phase during which the multipotent hematopoietic stem cells (HSCs) give rise to the common myeloid progenitors (CMPs). These CMPs generate bipotent megakaryocytic-erythroid progenitors (MEPs) that have potential to form erythroid or megakaryocytic cells. Under the regulation of the humoral cytokine erythropoietin (EPO), these MEPs differentiate into burst-forming unit-erythroid progenitors (BFU-Es) that further differentiate into colony-forming unit-erythroid progenitors (CFU-Es) then pro-erythroblasts⁸⁴. During a terminal differentiation phase, the pro-erythroblasts sequentially mature into early basophilic, late basophilic, polychromatic, and orthochromatic erythroblasts. The orthochromatic erythroblasts eventually enucleate to produce reticulocytes⁸⁵. Reticulocytes exit the bone marrow and reach the bloodstream where they mature into erythrocytes. The erythrocytes circulate in the bloodstream for a period of 120 days and are then eliminated by macrophages in the reticuloendothelial system⁸⁶. Erythropoiesis is tightly regulated by several elements of the bone marrow environment such as growth factors, transcription factors and cytokines. In certain pathological circumstances, extramedullary erythropoiesis can also occur in response to hematopoietic stress. However, the mechanisms are not yet well understood⁸⁷.

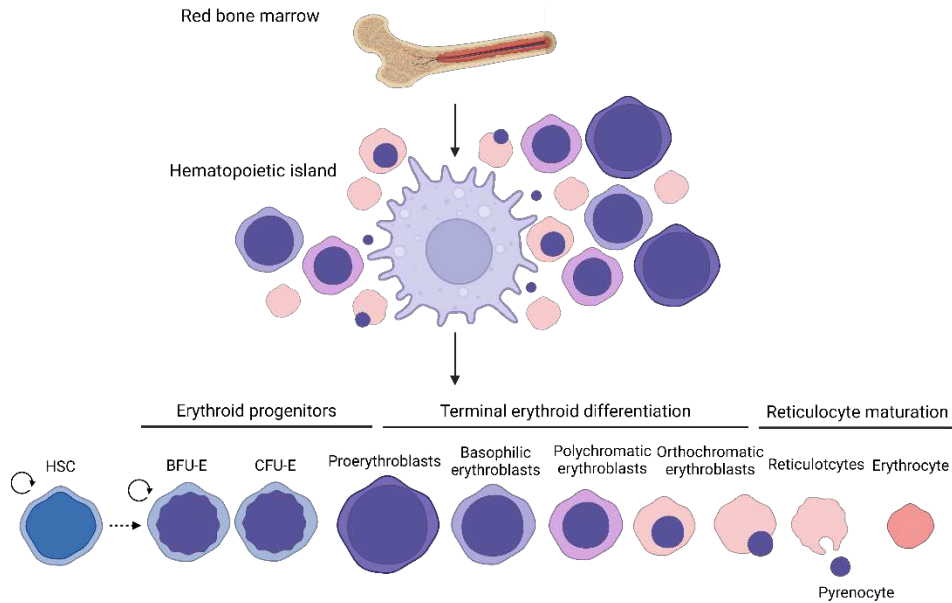


Figure 2: Human erythropoiesis. The bone marrow contains erythroblastic islands which are the specific niches for erythropoiesis. HSCs give rise to CMPs that generate MEPs. MEPs differentiate into BFU-Es that further differentiate into CFU-Es then proerythroblasts. Proerythroblasts sequentially mature into basophilic erythroblasts, polychromatic erythroblasts, and orthochromatic erythroblasts. The orthochromatic erythroblasts eventually enucleate to form reticulocytes. Reticulocytes migrate to the blood stream and mature into erythrocytes. Figure created with BioRender.com

2. *P. vivax* invasion mechanisms through the lens of omics technologies

Research on *vivax* malaria has been hampered by low prioritization from researchers, policy makers, and funding agencies as well as limited access to biological material and lack of robust *in vitro* culture systems. Consequently, there is an important knowledge and tool gap that drastically restricts our understanding of *P. vivax* biology⁴². Based on this observation, we decided to scrutinize the intriguing question of how *P. vivax* invades Duffy-negative reticulocytes through the lens of omics technologies. Omics technologies cluster various disciplines and aim at characterizing and quantifying biological molecules that translate into the structure, function, and dynamics of an organism⁸⁸. Their introduction with novel and accessible techniques such as third generation sequencing and RNA sequencing at single cell level, two-dimensional electrophoresis, liquid chromatography, and mass spectrometry, has progressively improved our understanding of *P. vivax* genetics, transcripts, and proteins. Thus, the aim of our scientific review « *Plasmodium vivax* blood stage invasion pathways: contribution of omics technologies in deciphering molecular and cellular mechanisms » was to provide broad insights into *P. vivax* invasion mechanisms generated by genomics,

transcriptomics, and proteomics and to illustrate the importance of integrated multi-omics studies. Interestingly, we found that the critical question for *vivax* malaria research cut across several disciplines. While genomic technologies provide important information on *P. vivax* genetic diversity, they may not be sufficient to understand their functional consequences. While transcriptomic technologies help in the characterization of *P. vivax* stage-specific transcripts, data analysis is still limited by small sample sizes and paucity in constantly expressed genes. And while proteomic technologies succeed in deciphering the various interactions between *P. vivax* invasion-related ligands and cognate receptors, they fail in providing a general picture of *P. vivax* invasion mechanisms. We thus concluded that there is an urgent need for the development of integrated, complementary multi-omics studies and *in vitro* functional assays to better understand *P. vivax* biology and support the future implementation of prevention, control, and eradication strategies worldwide. The scientific review « *Plasmodium vivax* blood stage invasion pathways: contribution of omics technologies in deciphering molecular and cellular mechanisms » has been published in the « Comptes-rendus Biologies de l'Académie des Sciences » in November 2022 and is presented below.



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Comptes Rendus

Biologies

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Volume 345, issue 2 (2022), p. 91-133

Published online: 24 November 2022

Issue date: 8 December 2022

<https://doi.org/10.5802/crbio.95>

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Centre Mersenne pour l'édition scientifique ouverte*
www.centre-mersenne.org
e-ISSN : 1768-3238



Review Article / Article de revue

Plasmodium vivax blood stage invasion pathways: Contribution of omics technologies in deciphering molecular and cellular mechanisms

*Voies d'invasion de Plasmodium vivax au stade
intra-érythrocytaire : Contribution des technologies
dites « omiques » au décryptage des mécanismes moléculaires
et cellulaires*

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Abstract. *Vivax* malaria is an infectious disease caused by *Plasmodium vivax*, a parasitic protozoan transmitted by female Anopheline mosquitoes. Historically, *vivax* malaria has often been regarded as a benign self-limiting infection due to the observation of low parasitemia in Duffy-positive patients in endemic transmission areas and the virtual absence of infections in Duffy-negative individuals in Sub Saharan Africa. However, the latest estimates show that the burden of the disease is not decreasing in many countries and cases of *vivax* infections in Duffy-negative individuals are increasingly reported throughout Africa. This raised questions about the accuracy of diagnostics and the evolution of interactions between humans and parasites. For a long time, our knowledge on *P. vivax* biology has been hampered due to the limited access to biological material and the lack of robust *in vitro* culture methods. Consequently, little is currently known about *P. vivax* blood stage invasion mechanisms. The introduction of omics technologies with novel and accessible techniques such as third generation sequencing and RNA sequencing at single cell level, two-dimensional electrophoresis, liquid chromatography, and mass spectrometry, has progressively improved our understanding of *P. vivax* genetics, transcripts, and proteins. This review aims to provide broad insights into *P. vivax* invasion mechanisms generated by genomics, transcriptomics, and proteomics and to illustrate the importance of integrated multi-omics studies.

Résumé. Le paludisme à *Plasmodium vivax* est une maladie infectieuse causée par un parasite protozoaire *Plasmodium vivax*, transmis par les moustiques *Anophèle* femelles. Historiquement, le paludisme à *P. vivax* a souvent été considéré comme une infection bénigne en raison de l'observation d'une faible parasitémie chez les patients Duffy-positifs dans les zones d'endémie et de la quasi-absence d'infections chez les individus Duffy-négatifs vivant majoritairement en Afrique subsaharienne. Cependant, les dernières estimations montrent que le poids de la maladie ne diminue pas dans de nombreux pays et que des cas d'infections à *P. vivax* chez des individus Duffy-négatifs sont de plus en plus souvent observés en Afrique. Cela soulève des interrogations sur la précision des diagnostics et l'évolution des interactions hôte-parasite. Pendant longtemps, nos connaissances sur la biologie de *P. vivax* ont été entravées par un accès limité au matériel biologique et un manque de méthodes robustes pour la culture *in vitro*. Par conséquent, nous n'avons encore que peu d'informations concernant les mécanismes d'invasion des stades sanguins de *P. vivax*. L'introduction des technologies dites « omiques », avec le développement de techniques innovantes et abordables telles que le séquençage d'ADN de troisième génération, le séquençage ARN à l'échelle de la cellule « single-cell », l'électrophorèse bidimensionnelle, la chromatographie liquide et la spectrométrie de masse, a progressivement amélioré notre compréhension des gènes, des transcrits et des protéines de *P. vivax*. Cette revue a non seulement pour but de fournir un aperçu général des mécanismes d'invasion de *P. vivax* acquis grâce aux techniques génomiques, transcriptomiques et protéomiques mais également d'illustrer l'importance de la complémentarité de ces approches.

Keywords. Malaria, *P. vivax*, Invasion, Duffy negativity, Omics.

Mots-clés. Paludisme, *P. vivax*, Invasion, Duffy-négativité, Technologies omiques.

Note. Didier Ménard is the 2021 laureate of the Prix de Mme Jules Martin, née Louise Basset by the Académie des sciences. / Didier Ménard est lauréat 2021 du Prix de Mme Jules Martin, née Louise Basset par l'Académie des sciences.

Published online: 24 November 2022, Issue date: 8 December 2022

La version française de l'article est disponible à la suite de la version anglaise

1. Introduction

Malaria is an acute debilitating illness that is caused by parasitic protozoans from the genus *Plasmodium* that are transmitted by *Anopheles* mosquitoes. According to the latest report of the World Health Organization (WHO), the disease accounted for 241 million cases and 627,000 deaths worldwide in 2020. The estimated number of malaria deaths stood at 627,000 in 2020 (an increase of 69,000 deaths over the previous year). Sub Saharan African countries continue

to carry a disproportionately high share of the global malaria burden, with 95% of all malaria cases and 96% of deaths. Children under 5 years of age accounted for about 80% of all malaria deaths in this region [1].

Among the species responsible for human infection, *Plasmodium vivax* is the most geographically widespread and the leading cause of disease outside Sub Saharan Africa, in Southeast Asia, South America and Northeast Africa [2]. *P. vivax* has a com-

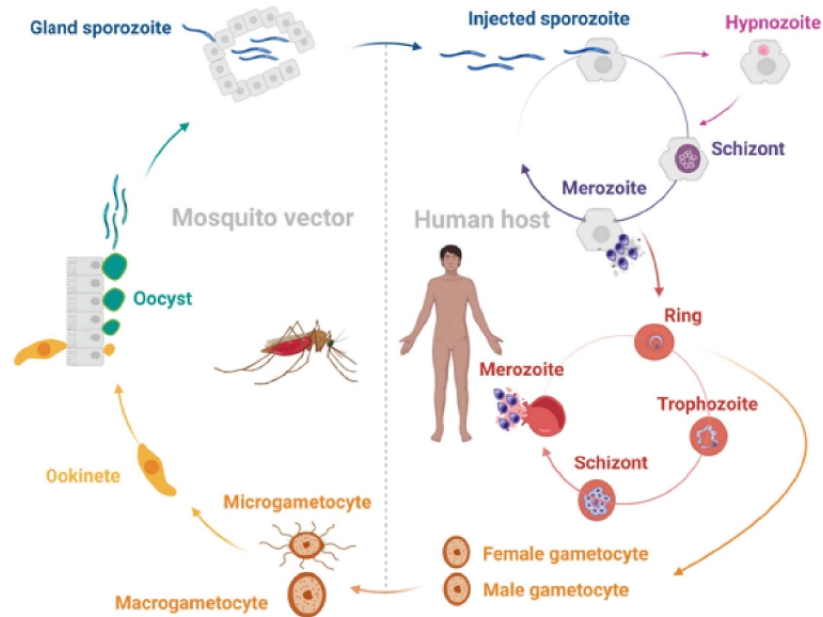


Figure 1. *P. vivax* life cycle. The life cycle of *P. vivax* involves the cyclical infection of a Anopheline mosquito vector and a human host. When a malaria-infected mosquito takes a blood meal, it injects sporozoites into the human host. They migrate through the dermis to the liver and invade hepatocytes. They mature into tissue schizonts and establish a stock of latent hypnozoites. When schizonts rupture, they release merozoites that migrate to the bloodstream and invade reticulocytes. They develop into rings, trophozoites and blood-stage schizonts. When the schizonts rupture, they lyse the infected reticulocytes and release new merozoites. A small proportion of the parasite population differentiates into male and female gametocytes. When a mosquito takes a new blood meal, it ingests gametocytes. In the midgut, the microgametocytes and macrogametocytes fertilize, undertake sexual division, and transform into ookinetes that mature into oocysts. When the oocysts rupture, they release sporozoites that migrate to the salivary glands to perpetuate the life cycle. Figure created with BioRender.com.

plex life cycle consisting of the cyclical infection of a human host and a female *Anopheline* mosquito vector. Following the bite of an infected mosquito, sporozoites migrate through the dermis into capillaries, reach the liver and invade hepatocytes [3]. While most sporozoites undertake an asexual maturation into schizonts, some of them diverge to form quiescent hypnozoites. These hypnozoites can emerge weeks or months after the primary infection and give rise to relapsing episodes of malaria [4]. When schizonts eventually rupture, they release merozoites that migrate into the bloodstream and invade reticulocytes. They develop into rings, trophozoites and

then schizonts [2]. Again, schizonts rupture and release new merozoites out of the infected reticulocytes to invade healthy ones. This results in a continuous round of asexual multiplications increasing the number of parasites [5]. Alternatively, some parasites differentiate into male or female gametocytes. When they infect new mosquitoes, they undergo fertilization inside the midgut and produce ookinetes that mature into oocysts. These oocysts release sporozoites that migrate to salivary glands and can be inoculated into new hosts by female mosquitoes during a bloodmeal. Thereby, *P. vivax* life cycle is perpetuated (Figure 1) [6].

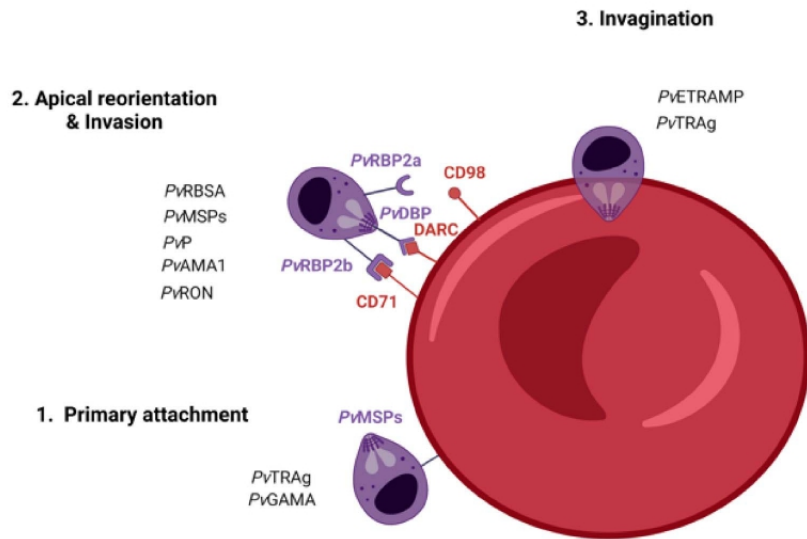


Figure 2. *P. vivax* invasion mechanisms within Duffy-positive reticulocytes. (1) Primary attachment & Reorientation—when *P. vivax* merozoites are released in the blood circulation, the primary attachment involves **PvMSPs**. Other proteins such as **PvTRAg56.2** and **PvGAMA** may play a role in the adhesion and stabilization of the parasite. (2) Ligand binding & Receptors—the **PvDBP** is gradually coupled to **DARC** (region II) to create a stable heterotetramer consisting of two **PvDBP** molecules and two **DARC** molecules. The recognition and selection of reticulocytes for invasion are mediated by **PvRBPs**, notably through the interactions **PvRBP2b-CD71** and **PvRBP2a-CD98**. In absence of biological evidence and based on observations during red blood cell invasion by *P. falciparum* or *P. knowlesi* merozoites, we suppose that many *P. vivax* proteins including **PvMSPs**, **PvRBSA** and other proteins may play a role to ensure *P. vivax* apical reorientation. (3) Tight junction & Engagement—*P. vivax* merozoites penetrate the reticulocytes via glideosome activity. Proteins such as **PvAMA1** and **PvRON** might lead either directly or indirectly to the formation of a tight and irreversible junction and mediate invasion. Several *P. vivax* surface proteins may be rapidly degraded whereas others such as **PvTRAg36.6** and **PvETRAPM** may be expressed to form a parasitophorous vacuole and support the development of the parasite. Of note, the *P. vivax* merozoite invasion mechanisms described here are so far largely limited to a comparison with *P. falciparum* and *P. knowlesi* invasion homologs and a handful of known *P. vivax* invasion gene families. The role of *P. vivax* ligands present in purple bold font have been confirmed, while the others present in black font are only suspected. Figure created with BioRender.com.

While it may be cryptic, *P. vivax* is the most widely distributed cause of malaria across the globe [7]. Indeed, its ability to develop at low temperatures (18–25 °C) provides potential for infectivity and transmission in both tropical and temperate areas [8]. Interestingly, the intra-erythrocytic cycle of *P. vivax* is characterized by low parasitemia and asynchrony, which contribute to missed diagnoses [9]. Low parasitemia, commonly observed in *P. vivax*

infection, is probably due to the unique tropism of *P. vivax* merozoites for reticulocytes and the specific physiological and/or metabolic needs of the parasite for blood-stage growth and survival [2]. In absence of biological evidence, a model for invasion by *P. vivax* has been proposed, based on observations during red blood cell invasion by *P. falciparum* or *P. knowlesi* merozoites. It suggests that during the invasion process, *P. vivax* expresses adhesins to bind receptors on

the surface of reticulocytes and establish an initial and reversible attachment. This results in the deformation of the reticulocyte membrane and the apical reorientation of merozoites. Then, they activate organelles and release invasion-related proteins to form a tight and irreversible junction [10]. The merozoites are propelled by an actin–myosin motor and the junction moves from the apical to posterior pole. That forms the parasitophorous vacuole in which the parasites will develop after the invasion pore and reticulocyte membrane are resealed [11, 12] (Figure 2 and Table 1 for details).

For many years, scientists have sought to identify the key host–parasite interactions leading to the invasion of human reticulocytes by *P. vivax*. Between 1920 and 1960, clinical and field trials carried out with individuals of African or African American ancestry demonstrated that they were highly resistant to *vivax* malaria when challenged with human blood or mosquitoes infected with limited numbers of *P. vivax* strains [13–16]. Following the identification of the Duffy blood groups [17, 18], it was established that the natural resistance to *vivax* infection was related to the absence of Duffy antigen receptors for chemokines (DARC) on the surface of human erythrocytes [19]. The molecular basis of Duffy-negativity was later demonstrated as a single point mutation in the GATA1 (tct>cct) sequence located in the 5′ promoter region of the *ackr1* gene [20,21]. Seminal works with controlled experimental infections of volunteers through sporozoite challenges [19] and *in vitro* studies using *P. knowlesi* (a zoonotic malaria parasite closely related to *P. vivax*) as a model [22] subsequently confirmed that DARC was required as a receptor for *P. vivax* merozoites to invade erythrocytes. In parallel, the parasite ligand that specifically binds DARC was identified as *P. vivax* Duffy binding protein (*PvDBP*) [23]. Hence, the scientific paradigm by which *P. vivax* exclusively invades Duffy-positive erythrocytes through the interaction between *PvDBP* and DARC was established and *vivax* malaria was assumed to be seldom transmitted or even absent in regions where Duffy-negativity is predominant such as Sub Saharan Africa [19]. However, over the last two decades, many studies have reported cases of *P. vivax* infection within Duffy-negative individuals from Sub Saharan countries such as Angola [24], Benin [25], Cameroon [26, 27], Equatorial Guinea [24, 28], Ethiopia [29], Kenya [30],

Madagascar [31], Mali [32], Mauritania [33] and Sudan [34]. Although such unexpected observations are still scarce, they raise questions about the actual invasion mechanisms used by *P. vivax* to infect Duffy-negative individuals. They also challenge common assumptions such as the central role played by the *PvDBP*-DARC interaction in blood-stage infections, the limit of detection of current diagnostic tools and the evolution of host–pathogen interactions [35]. At present, we do not know whether these cases represent a pre-existing phenomenon that was overlooked in the past or a newly evolved DARC-independent invasion pathway that could lead to an expansion of *vivax* malaria within Duffy-negative populations [36].

Because the collection of field samples from remote endemic areas is difficult, time demanding and expensive, research has long been restricted to direct sampling from patient donors or the study of closely related *Plasmodium* species such as *P. knowlesi* [37, 38] or *P. cynomolgi* [39]. Furthermore, the development of reproducible long-term *in vitro* culture methods is still impaired by *P. vivax* low parasitemia and strong tropism for reticulocytes [40].

Nevertheless, recent omics technologies have revolutionized *vivax* malaria research with the development of innovative and affordable techniques enabling a more comprehensive characterization of *P. vivax* genes, transcripts, and proteins. This review aims to provide broad insights into *P. vivax* invasion mechanisms enabled by genomics, transcriptomics, and proteomics and to illustrate the importance of integrated multi-omics studies.

2. *P. vivax* molecular mechanisms of invasion

In 2004, the *P. vivax* genome sequencing project provided the first reference sequence *P. vivax* Salvador-1 (Sal-1) using a whole genome shotgun strategy [41, 42]. In the last twenty years, a few additional field and clinical *P. vivax* isolates have been sequenced through microarray, high-depth sequencing, or whole genome sequencing. All these sequences are publicly available on online database such as PlasmoDB (<https://plasmodb.org/plasmo/app>) and are regularly annotated. Overall, the analysis of these *P. vivax* sequences has contributed to the identification of potential invasion-related genes.

Table 1. List of *P. vivax* invasion-related ligands characterized by omics technologies

Step	Pv ligands	Accession numbers	Role (confirmed/potential)	Erythrocyte receptor	Vaccine candidates (blood stage)	Evidence from		
						Genomics	Proteomics	
Primary attachment & reorientation	MSP1	PVP01_0728900	Confirmed		Yes	<i>pvmSP1</i> is highly polymorphic (SNPs) [43]	Among all genes, <i>pvmSP1</i> is one of the most expressed gene in <i>P. vivax</i> schizonts [44]	Based on the study of homologous PfMSPs in <i>P. falciparum</i> , it has been hypothesized that <i>PvmSP1</i> may recognize a cogent receptor on the surface of reticulocytes [45]
	MSP7	PVP01_1219500, PVP01_1219700, PVP01_1219400, PVP01_1219600, PVP01_1220300, PVP01_1220300, PVP01_1220400, PVP01_1219900	Potential			<i>pvmSP7</i> is highly polymorphic (SNPs) [46]. <i>pvmSP3</i> presented copy number variations ranging from 1–4 copies	<i>pvmSP7</i> transcript peaks during the late trophozoite-schizont transition [47]. <i>pvmSP3</i> transcripts were detected in very late schizonts [5]. Among all genes, <i>pvmSP9</i> is one of the most expressed gene in <i>P. vivax</i> schizonts [44]	Based on homology with <i>PfMSP7</i> , <i>PvmSP7</i> may be a specific ligand for the receptor P-selectin [48]
	GAMA	PVP01_0505600	Potential			<i>pvgama</i> displays a size polymorphism [49]		Functional assays: <i>PvGAMA</i> may play a role in the adhesion of <i>P. vivax</i> merozoites to reticulocytes [49] and binds to Band3 and ANK1 [50]

(continued on next page)

Table 1. (continued)

Step	Pv ligands	Accession numbers	Role (confirmed/potential)	Erythrocyte receptor	Vaccine candidates (blood stage)	Genomics	Evidence from Transcriptomics	Proteomics
	MSP1P	PVP01_0728800	Potential					<i>Pv</i> MSP1P may play an important role in the adhesion of <i>P. vivax</i> merozoites to reticulocytes through interaction with its C-terminal region [43]
	TRAg56.2	PVX_088850	Potential			The <i>pvtrag</i> gene family is seldom polymorphic [51]	Single cell RNA sequencing; different expression levels for different members of the <i>pvtrag</i> gene family [5]	Immunoprecipitation and mass spectrometry: <i>Pv</i> TRAg56.2 seems to co-localize with <i>Pv</i> MSP1 and interact with <i>Pv</i> MSP7 to play a stabilizing role [52]
Ligand bindings	<i>DBP</i>	PVP01_0623800	Confirmed	DARC	Yes	<i>pvdbp</i> is highly polymorphic (SNPs, CNVs) [53]	<i>pvdbp</i> gene amplification was associated with an increase in messenger RNA (mRNA) level, suggesting a potential increase in <i>Pv</i> DBP protein level [54]	Binding and functional assays
	<i>EBP</i>	PVP01_0102300	Potential			<i>pvebp</i> is highly polymorphic but less than <i>pvdbp</i> (SNPs, CNVs) [55]	<i>pvebp</i> transcripts were found to be highly expressed during <i>P. vivax</i> schizont stage [55, 56]	<i>Pv</i> EBP1 binds preferentially immature Duffy-positive reticulocytes, but a minimal binding was observed in immature Duffy-negative reticulocytes [57, 58]

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Table 1. (continued)

Step	Pv ligands	Accession numbers	Role (confirmed/potential)	Erythrocyte receptor	Vaccine candidates (blood stage)	Evidence from		
						Genomics	Transcriptomics	Proteomics
	<i>RBP2b</i>	PVP01_0800700	Confirmed	CD71		<i>pvrp2a</i> has conserved regions but is relatively polymorphic [59,60]	<i>Ex vivo</i> culture system: the interaction <i>Pv</i> RBP2b-CD71 plays a crucial role in the recognition of reticulocytes [61]	
	<i>RBP2a</i>	PVP01_1402400	Confirmed	CD98		<i>pvrp2a</i> has conserved regions but is relatively polymorphic [59,60]	Binding assays: CD98 is the cognate receptor of <i>Pv</i> RBP2a and the interaction <i>Pv</i> RBP2a-CD98 is involved in the recognition of reticulocytes [62]	
	<i>RBSA</i>	PVP01_0004240	Potential			<i>pvrbsa</i> presents less genetic diversity than other <i>P. vivax</i> merozoite surface genes [63]	<i>Pv</i> RBSA specifically binds to Duffy-positive reticulocytes, but its cognate receptor is unknown [63]	
	<i>P12</i>	PVP01_1136400	Potential				The <i>Pv</i> P protein family is expressed on the surface of <i>P. vivax</i> merozoites and may play a role in the invasion of reticulocytes [64]	
Tight junction formation	<i>AMA1</i>	PVP01_0934200	Potential		Yes		Functional assays: conserved functions for <i>Pf</i> AMA1 and <i>Pv</i> AMA1 [65]. May be involved with <i>Pv</i> RON2 in the formation of the tight junction [66]	

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Table 1. (continued)

Step	Pv ligands	Accession numbers	Role (confirmed/potential)	Erythrocyte receptor	Vaccine candidates (blood stage)	Genomics	Transcriptomics	Proteomics
	<i>RON</i>	PVP01_0916600	Potential			Identification of <i>pvrpn4</i> by homology to <i>pfrpn4</i> and <i>pkron4</i> in <i>P. vivax</i> VCG-1 [67]	<i>pvrpn4</i> presents a similar transcription pattern to the one reported for <i>pfrpn4</i> [67], <i>pvrpn5</i> transcripts were found in schizonts [66]	Indirect immunofluorescence and immunoassays: <i>PvRON4</i> may be expressed in <i>P. vivax</i> VCG-1 apical pole and may participate in the formation of a tight junction [67], <i>PvRON5</i> has a marked binding preference for reticulocytes and is thus suspected to be involved in the invasion process [66]
	<i>ETRAPM</i>	PVP01_0532300	Potential					<i>PvETRAPM</i> could be involved in the formation of parasitophorous vacuole
	<i>TRAg36.6</i>	PVX_112690	Potential			DNA sequencing: the <i>pvrtrag</i> gene family is seldom polymorphic [51]	Single cell RNA sequencing: different expression levels for different members of the <i>pvrtrag</i> gene family [5]	Immunoprecipitation and mass spectrometry: <i>PvTRAg36.6</i> seems to colocalize with <i>PvETRAPM</i> to form and/or maintain the parasitophorous vacuole membrane [52]
Unknown function	<i>MSA180</i>	PVP01_0814200	Potential				Among all genes, <i>pvrmsa180</i> is one of the most expressed gene in <i>P. vivax</i> schizonts [44]	

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Table 1. (continued)

Step	Pv ligands	Accession numbers	Role (confirmed/potential)	Erythrocyte receptor	Vaccine candidates (blood stage)	Genomics	Evidence from Transcriptomics	Proteomics
	<i>P92</i>	PVP01_1107900	Potential				Among all genes, <i>pvrpama</i> is one of the most expressed gene in <i>P. vivax</i> schizonts [44]	
	<i>RAMA</i>	PVP01_0107500	Potential					
	<i>RBP1a</i>	PVP01_0701200	Potential			DNA sequencing: genetic diversity of <i>pvrp1a</i> seems to be low [68]	<i>pvrp1</i> transcripts were found to be highly expressed during <i>P. vivax</i> schizont stage [55, 56]	Native <i>PvRBP1</i> and <i>PvRBP2</i> may function as an adhesive protein complex [69]
	<i>RBP1b</i>	PVP01_0701100	Potential			DNA sequencing: genetic diversity of <i>pvrp1b</i> seems to be low [68]	<i>pvrp1</i> transcripts were found to be highly expressed during <i>P. vivax</i> schizont stage [55, 56]	Native <i>PvRBP1</i> and <i>PvRBP2</i> may function as an adhesive protein complex [69]
	<i>RBP2c</i>	PVP01_0534300	Potential			DNA sequencing: <i>pvrp2c</i> is highly polymorphic [51, 59, 70]		
	<i>RBP1p1 (partial)</i>	?	Potential			DNA sequencing: identification of the partial gene <i>pvrp1p1</i>		
	<i>RBP2p1 (partial)</i>	PVP01_0534400	Potential			DNA sequencing: identification of the partial gene <i>pvrp2p1</i>		
	<i>RBP2p2 (partial)</i>	PVX_101590	Potential			DNA sequencing: identification of the partial gene <i>pvrp2p2</i>		

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Table I. (continued)

Step	Pv ligands	Accession numbers	Role (confirmed/potential)	Erythrocyte receptor	Vaccine candidates (blood stage)	Genomics	Evidence from Transcriptomics	Proteomics
	<i>RBP2d (pseudo)</i>	PVP01_1471400	Potential			DNA sequencing: identification of the pseudogene <i>purbhp2d</i>		
	<i>RBP2e (pseudo)</i>	PVP01_0700500	Potential			DNA sequencing: identification of the pseudogene <i>purbhp2e</i>		
	<i>RBP3 (pseudo)</i>	PVP01_1469400	Potential			DNA sequencing: identification of the pseudogene <i>purbhp3</i>		
	<i>RhopH3</i>	PVP01_0703800	Potential				Among all genes, <i>purhoph3</i> is one of the most expressed gene in <i>P. vivax</i> schizonts [44]	
	<i>RON5</i>	KP026121	Potential				The <i>pvron5</i> transcript is found in schizonts in <i>P. vivax</i> VCG-1 [66]	Binding, Western blot and immunofluorescence assays: <i>PvRON5</i> is expressed in <i>P. vivax</i> apical pole and preferentially binds reticulocytes [66]

Accession numbers starting from PVP01 are from *PvP01* reference strain while accession numbers starting from PVX are from Sal-I reference strain (<https://plasmodb.org/plasmo/app>). Accession numbers starting from KP are from VCG-1 reference strain (<https://www.ncbi.nlm.nih.gov>).

2.1. The crucial role of *pvdbp* gene in the invasion of Duffy-positive reticulocytes

The *P. vivax* Duffy Binding Protein (*pvdbp*) gene encodes the invasion-related ligand *P. vivax* Duffy Binding Protein (*PvDBP*) [71]. The *pvdbp* gene displays coding sequences for a N-terminal signal peptide domain, a conserved cysteine-rich Duffy binding domain referred to as region II, another conserved cysteine-rich domain referred to as region VI, followed by a transmembrane domain and a C-terminal cytoplasmic tail [72]. Based on current high throughput sequencing data, our appreciation of genetic diversity is less and less limited by the nature and/or density of markers studied along the genome. Notably, the recent publication of more than two hundred *P. vivax* genomes showed that the *pvdbp* gene sequences were highly polymorphic irrespective of their geographic origin. Interestingly, many non-synonymous single nucleotide polymorphisms (SNPs) were found in the DARC binding domain region II (*PvDBPII*) [53]. The diversity and localization of these SNPs suggest that the *pvdbp* gene may be under differential selective pressure due to antibody responses and its function in receptor-binding and invasion [51].

In addition, copy number variations (CNVs) at the *pvdbp* locus were found to be one of the most common gene expansions to occur across multiple alleles worldwide [73]. While the detection of CNVs relied, in the past, on labour intensive and costly techniques such as *in situ* hybridization, recent advances in high throughput sequencing enabled their direct detection based on sequencing data. Notably, whole genome sequencing approaches revealed two distinct duplications in parasites from Madagascar and Cambodia. In Madagascar, *P. vivax* isolates from Duffy-positive and Duffy-negative patients exhibited a common duplication with two copies of *pvdbp* next to each other [74]. In Cambodia, a different duplication of the *pvdbp* gene was commonly detected in *P. vivax* isolates from Duffy-positive patients. Furthermore, the proportion of *P. vivax* isolates carrying the duplication was found to be significantly higher in homozygous Duffy-positive patients than in heterozygous Duffy-positive patients who are expected to exhibit a lower expression of DARC on the surface of their reticulocytes. However, the latter analysis could not completely rule out that patients could

carry multiple *P. vivax* clones while the estimation of CNVs by quantitative PCR can only reflect the major clone [53]. More recently, a geographical survey demonstrated that these two distinct duplications of *pvdbp* were circulating in Asia, South America, and East Africa. While Duffy negativity is prevalent in some regions, duplications of the *pvdbp* gene were also largely found in regions where Duffy negativity was almost absent [51]. In contrast, a concurrent genomic analysis showed that no duplication of *pvdbp* was found in one hundred eighty-two *P. vivax* isolates from eleven endemic countries. However, it was noticed that this discrepancy was probably due to the primers used for CNV validation by PCR [75].

Despite the specificity and distribution of the duplications of *pvdbp*, there was no evidence that *P. vivax* genomes bypassed the barrier of Duffy negativity through this genetic variation. Larger expansions of *pvdbp* were also detected worldwide. They clustered separately but presented similar patterns. High haplotype diversity was also observed between the copies while the 5' and 3' flanking sequences displayed moderate diversity [76]. In the Asia-Pacific region, sequencing of more than two hundred *P. vivax* isolates from Duffy-positive patients revealed the common presence of two to three copies of the *pvdbp* gene [73]. In Ethiopia, sequences of two *P. vivax* isolates from Duffy-negative patients exhibited the presence of three and eight copies of the *pvdbp* gene [57]. Later on, another common expansion of the *pvdbp* gene, quite similar to those previously described in Madagascar and Cambodia but ranging from three to five copies, was also detected [51]. These findings have therefore allowed to hypothesize that the expansion of *pvdbp* may allow for mutation arising in the second copy. Such expansions may facilitate the binding to an alternative erythrocytic receptor with a lower affinity for *PvDBP* ligand [57]. However, there is no evidence of localized-selected CNVs that would enhance invasion efficiency and induce selective pressure. Since the invasion of Duffy-positive erythrocytes depends on the interaction between *PvDBP* and DARC, it is likely that the expansions of *pvdbp* may be driven by genetic variations in DARC or inhibitory antibody responses to *PvDBPII* [75]. Nonetheless, it is unclear whether these expansions do provide an enhanced ability to the parasite to invade Duffy-negative erythrocytes.

2.2. The potential role of *pvebp* gene in an alternative invasion pathway

The *P. vivax* Erythrocyte Binding Protein (*pvebp*) gene was discovered through *de novo* assembly of one *P. vivax* isolate from Cambodia. It encodes the putative *P. vivax* Erythrocyte Binding Protein (*PvEBP*) ligand and is present in most *P. vivax* genomes. The organization of the *pvebp* gene is similar to the *pvdgp* gene with an N-terminal signal peptide, a Duffy binding like domain referred to as region II, another cysteine-rich domain referred to as region VI followed by a transmembrane domain and cytoplasmic tail [55]. Despite these similarities, *pvebp* appears to cluster separately from all known *Plasmodium* Duffy binding protein like genes. DNA sequencing of ten *P. vivax* genomes from Asia, South Pacific, Africa, Central and South America showed that *pvebp* is polymorphic though less diverse than *pvdgp*. Indeed, the *pvebp* gene displayed only 11 polymorphic sites in 2412 positions while the *pvdgp* gene displayed 32 polymorphic sites in 3213 positions for the same samples. Most of the SNPs detected were non-synonymous [55]. Furthermore, the genomic analysis of two hundred nineteen *P. vivax* isolates from Duffy-positive patients in Cambodia and Madagascar confirmed that *pvebp* was less diverse than *pvdgp* (12 vs. 33 alleles observed from the same isolates) but presented only non-synonymous mutations indicating the existence of a strong selective pressure. Indeed, eleven non-synonymous point mutations were found in *pvebp* sequences and led to twelve different alleles. Some SNPs were observed in both countries whereas others were specific to Cambodia and Madagascar. These SNPs were distributed across the Duffy binding like domain [53]. Just as for *pvdgp*, the diversity and distribution of SNPs in *pvebp* reflected the likely existence of a selective pressure in the *pvebp* gene though it seemed to operate at a lower level than in the *pvdgp* gene [58].

The genomic analysis of *P. vivax* isolates from Cambodia, Madagascar and Ethiopia also revealed the existence of copy number variations in the *pvebp* gene. In Madagascar, *pvebp* expansions were observed, ranging from two to five copies. A bimodal distribution of parasites with a specific population presenting more than three copies was found. However, no significant difference in susceptibility to *vivax* infection was observed between

homozygous and heterozygous Duffy-positive individuals. In Cambodia, *pvebp* expansions ranged from one to two copies. The proportion of *P. vivax* isolates with multiple gene copies was more important in high transmission areas [53]. Overall, a high proportion of *P. vivax* isolates with multiple gene copies was found in Madagascar where Duffy-negativity and Duffy-positivity coexist as compared to Cambodia where Duffy-negativity is virtually absent. In contrast, no *pvebp* expansion was found in Ethiopia where Duffy-negativity is also frequent [51]. Considering the similarities observed between the *pvdgp* and *pvebp* genes, the polymorphism and expansion of *pvebp* may correlate with the expression of a new ligand interacting with erythrocyte membrane proteins other than DARC and therefore contribute to an alternative invasion pathway [55]. However, no evidence that such variations confer any abilities to infect Duffy-negative individuals have been provided so far.

2.3. The role of *pvrpb* and *pvrbsa* genes in reticulocyte selectivity

The *P. vivax* Reticulocyte Binding Protein (*pvrpb*) gene family was identified based on the annotation of adhesive ligands of *P. vivax* genome [69]. Genome sequencing of several *P. vivax* isolates identified eleven members in the *pvrpb* gene family including five full-length genes (*pvrpb1a*, *pvrpb1b*, *pvrpb2a*, *pvrpb2b* and *pvrpb2c*), three partial genes (*pvrpb1p1*, *pvrpb2p1* and *pvrpb2p2*) and three pseudogenes (*pvrpb2d*, *pvrpb2e* and *pvrpb3*) [77]. The *pvrpb* genes are constituted of two exons with one exon encoding a signal peptide at the N-terminal region and another exon encoding a transmembrane domain at the C-terminal region [56]. Recent investigations reported that their genetic diversity seems to be generally low, except for the *pvrpb2* genes [68]. The analysis of *P. vivax* isolates from China, Thailand and Ethiopia confirmed that *pvrpb2c* was highly polymorphic [51, 59, 70]. Although *pvrpb2a* and *pvrpb2b* presented highly conserved regions, the genomic analysis of *P. vivax* isolates from Papua New Guinea and Thailand showed that they were also relatively polymorphic [59, 60]. As observed for the *pvdgp* and *pvebp* genes, non-synonymous SNPs clustered around three main sites including the reticulocyte

binding domain. This may reflect the functional importance of this binding site and the existence of a selective pressure [60].

Furthermore, CNVs affecting both *pvrbp2a* and *pvrbp2b* were observed in two *P. vivax* isolates from Thailand. While a strain presented duplication of both genes (*pvrbp2a* and *pvrbp2b*), the other strain had two copies of *pvrbp2b* only [59]. These findings suggest that the *pvrbp* genes may encode ligands involved in the recognition of target cells. The *P. vivax* reticulocyte binding surface antigen (*pvrbsa*) gene has also been identified as a gene potentially involved in the recognition of reticulocytes though it displays less genetic diversity than other *P. vivax* merozoite surface genes [63].

2.4. The potential role of *pvmosp* genes in primary attachment

The *P. vivax* merozoite surface protein (*pvmosp*) gene family encodes multiple antigenic determinants that are suspected to mediate the primary attachment between *P. vivax* merozoites and human reticulocytes [11]. These *pvmosp* genes are highly polymorphic. In particular, *pvmosp1* [43], *pvmosp3* [43, 78] and *pvmosp7* [46] exhibit numerous non-synonymous SNPs indicating that they may be under selective immune pressure [51]. Analysis of *P. vivax* isolates from Ethiopia revealed that *pvmosp3* presented copy number variations ranging from one to four copies. Interestingly, the expansions of *pvdhp* and *pvmosp3* were often observed as tandem copies. The functional relevance of this expansion of copy numbers is still not known.

2.5. The other genes and their potential role in *P. vivax* invasion pathway

Other *P. vivax* genes are suspected to be involved in the invasion process. For instance, the *P. vivax* tryptophan-rich antigen (*pvtrag*) gene family is thought to encode thirty-six proteins that can bind to human erythrocytes [79]. The genomic analysis of *P. vivax* strains from Ethiopia revealed that the *pvtrag* gene family seems to bear only a few genetic polymorphisms [51]. Similarly, the *P. vivax* glycosylphosphatidylinositol-anchored micronemal antigen (*pugama*) gene encodes the *P. vivax*

glycosylphosphatidylinositol-anchored micronemal antigen (*PvGAMA*) that binds erythrocytes regardless of Duffy antigen status [43]. This protein may play a role in the adhesion of *P. vivax* merozoites to its target cells [49]. Finally, the *P. vivax* rhoptry neck protein (*pvrpn*) gene family encodes for *P. vivax* rhoptry neck proteins (*PvRON*) that may participate in the formation of the tight junction as demonstrated for the *P. falciparum* homologs [67].

Genomic technologies clearly contributed to refine *vivax* malaria research by reducing the cost of sequencing as well as improving the quality and availability of sequences. However, only a few hundred *P. vivax* genomes have been sequenced so far. Field and clinical isolates present low parasitemia and high human DNA contamination that still make the acquisition of sufficient amount of high-quality parasite DNA difficult. Yet, the available genomic sequences have enabled the identification of a few potential invasion-related genes. Single nucleotide polymorphisms and copy number variations found in these genes may lead to structural differences and result in selective pressures, but the functional consequences of these variations remain unknown. Analysis of *P. vivax* genomes should be combined with the identification of their transcriptomic profiles and characterization of their putative proteins to detect specific signatures, which may provide insights into function. But the lack of *in vitro* culture methods for *P. vivax* makes functional analysis difficult.

3. Transcription of *P. vivax* invasion-related genes

Constant advances of high throughput sequencing associated with the recent ability to mature one cycle *ex vivo* isolates have opened the way to transcriptomic studies. The profiling of *P. vivax* blood stage transcriptome through microarray and RNA sequencing (bulk and at single cell level) is progressively unveiling new experimental abilities and elucidating the transcription, function, and regulation of invasion-related genes.

3.1. Stage-specific expression of *P. vivax* invasion-related genes

Generating high quality transcriptomes across multiple *P. vivax* isolates is essential to explore the changes

in gene expression throughout the intraerythrocytic cycle and leading to invasion. Recently, the single cell transcriptomics profiling in *P. vivax* isolates from bloodstream infections of *Aotus* and *Saimiri* monkeys revealed that most of the genes had a stage-specific expression and were strongly over-expressed, in contrast to housekeeping genes [5]. Given that most clinical infections were asynchronous, the analysis of *P. vivax* transcriptomes identified distinct populations. Rings and early trophozoites mainly expressed genes involved in transcription and translation whereas late trophozoites and schizonts possibly expressed invasion-related genes [5]. For instance, the *pvebp* and *pvrbp1* transcripts were found to be highly expressed during *P. vivax* schizont stage [55, 56]. Similarly, the *pvmosp3* transcripts were detected in very late schizonts [5] and the *pvmosp7* transcript generally peaked during the late trophozoite-schizont transition, when merozoites are being formed [47]. In the primate model-adapted *P. vivax* strain (VCG-1), the *pvrn5* transcripts were found in schizonts [66]. Moreover, RNA sequencing experiments conducted with *ex vivo* *P. vivax* isolates from Brazil showed a wide variation in the expression of genes that were suspected to be involved either in the host-parasite interaction, reticulocyte selectivity or invasion. In particular, *pvmosp1*, *pvmosp9*, *P. vivax* merozoite surface antigen 180 (*pvmosa180*), *P. vivax* rhoptry-associated membrane antigen (*pvrma*) and *P. vivax* rhoptry H3 (*pvrhoph3*) were the most expressed among all genes [44]. The expression of invasion-related genes in late schizonts implies the key importance of merozoite development and reticulocyte invasion for *P. vivax* survival.

3.2. Selective expression of *P. vivax* invasion-related genes

The current *P. vivax* genome contains many gene families that were defined based on sequence homology and conservation of amino acid domains. Single-cell RNA sequencing analyses demonstrated that selective gene expression may be due to its specific role or additional functions. While most of the *pvmosp* genes are expressed during late developmental stages, *pvmosp3* was found to be exclusively expressed in very late schizonts suggesting its role in the invasion process. More surprisingly,

a *pvmosp7* like gene appeared to be exclusively expressed in gametocytes implying a functional difference [5]. Comparable results were reported using clinical *P. vivax* isolates from Thailand. While the expression of most of the *pvmosp7* genes was associated with schizonts, the *pvmosp7a* and *pvmosp7f* genes maintained high expression levels throughout the entire intraerythrocytic cycle. Such findings suggest that these paralogs may have additional functions besides their established role on the surface of merozoites [47]. Similarly, different expression levels were observed among members of the *pvrtrg* gene family but their significance is unknown [5]. Despite their sequence homology, members of a gene family may also have different functions at specific time points [5]. Finally, a directional and amplification-free transcriptome (DAFT) sequencing of four *P. vivax* isolates from Cambodia revealed that transcription may be a selective process where the parasites would select particular members of a gene family in response to the host [80]. The authors showed that genes that were the most variably expressed among isolates physically clustered along chromosomal regions. For instance, the *pvmosp3* locus presented many paralogs which were likely to be of functionally redundant thus increasing antigenic diversity and enhancing immune evasion during the invasion process. Within clusters, gene expression appears to arise as if parasites were choosing the gene version to be expressed within a given host [80]. Overall, these findings reflect a developmental regulation and functional differentiation within specific gene families.

3.3. Amplification of *P. vivax* invasion-related genes

In *Plasmodium* species, gene amplification may provide a simple way to increase gene expression and thereby changing invasion phenotypes. Previous genomic studies demonstrated that some *P. vivax* strains had amplification of invasion-related genes. Notably, the *pvdhp* gene is highly variable in the number of gene copies. For instance, transcriptomic profiling of schizonts from Cambodian *P. vivax* isolates revealed that the *pvdhp* gene amplification was associated with an increase in messenger RNA (mRNA) level, suggesting a potential increase in *Pv* DBP protein level [54]. Given that *Pv*DBP protein

plays a crucial role in the invasion of Duffy-positive reticulocytes, it was hypothesized that the parasites capable of infecting Duffy-negative reticulocytes may have been selected for their ability to use multiple copies of *pvdbp* in order to bind an alternative receptor [69]. However, *P. vivax* strains with multiple copies of *pvdbp* have also been commonly detected in regions where Duffy-negativity is rare. On another note, even though *pvebp* presents many similarities with *pvdbp*, there is no evidence that its amplification leads to increased mRNA and/or protein levels [53]. Thus, the functional consequences of invasion-related gene expansion are uncertain and require further investigations with a high number of *P. vivax* isolates.

3.4. A complex and dynamic regulation system

Transcriptomic data from individual parasites suggested that *P. vivax* exhibited a tightly regulated transcriptional cascade during its intraerythrocytic cycle [81]. Investigations showed that co-expressed genes presented conserved motifs, and potential sites for regulatory binding proteins [82]. For instance, long 5' and 3' untranslated transcribed regions (UTRs) were observed in genes suspected to be involved in the invasion and the formation of parasitophorous vacuole [80]. In addition, variations of 5'-UTRs seemed to lead to a differential selection of transcription start sites (TSS) reflecting the dispersed patterns of transcription that could arise [83]. The presence of bidirectional promoters to regulate the expression of adjacent gene pairs has also been frequently observed. Lastly, post-transcriptional modifications such as chromatin structure, non-coding transcripts and alternative splicing may play an important role in regulation, but the mechanisms remain unclear. Notably, the putative spatial relationship between genes which are variably expressed suggests that chromatin structure and access may be important for gene regulation [83]. Thus, *P. vivax* has an elusive and complex regulatory network that may govern invasion-related genes and thereby allow the parasite to thrive within infected human reticulocytes.

Transcriptomics provides precious insights into the dynamics of *P. vivax* invasion-related genes. It is likely that *P. vivax* schizonts selectively express specific genes to initiate the invasion process. However,

the small sample sizes, lack of reliable *in vitro* culture methods and paucity of constantly expressed genes complicate the identification of new transcripts and regulatory motifs. In the future, single-cell profiling and long read sequencing of *P. vivax* isolates from geographically distinct regions should be combined with proteomic approaches to assess the full diversity of invasion-related ligand expression.

4. *P. vivax* cellular invasion mechanisms

Proteomics data for *P. vivax* infections are minimal due to the limited access to biological material and lack of robust *in vitro* culture system. The list of proteins expressed in *P. vivax* merozoites has thus not been clearly defined. This has generated critical knowledge gaps in the understanding of *P. vivax* invasion mechanisms. Given that the basic structure of merozoites is similar across *Plasmodium* species, most of the researchers based the investigation of *P. vivax* invasion mechanisms on the hypothesis that the parasite may use the same steps despite having different cell tropism and engaging different receptor-ligand interactions.

4.1. Primary attachment

The invasion process may be initiated by the reversible attachment of *P. vivax* merozoites to reticulocytes. This binding may be mediated by *P. vivax* Merozoite Surface Proteins (*PvMSPs*), which may form large complexes constituted of glycoposphatidylinositol anchored proteins and transmembrane proteins covering the surface of *P. vivax* merozoites. Based on the study of homologous *PfMSPs* in *P. falciparum*, it has been hypothesized that *PvMSP1* may recognize a cognate receptor on the surface of reticulocytes. The C-terminal sub-unit (*MSP-1₄₂*) could be cleaved and most of the complex may be shed, leaving only a small C-terminal sub-unit (*MSP-1₁₉*) on the surface. Similarly, its paralog *PvMSP1P* may play an important role in the adhesion of *P. vivax* merozoites to reticulocytes through interaction with its C-terminal region [43]. Furthermore, it has been hypothesized that *PvMSP7* may be a specific ligand for the receptor P-selectin which acts as a cell adhesion molecule [48] and that *PvMSP1* and *PvMSP7* could bind together to form a multi-protein complex that enhances invasion efficiency [47].

Other ligands may be involved in this step, but their ability to trigger attachment or abrogate recognition by the host immune system are unknown. For instance, the *Pv*TRAG56.2 protein seems to co-localize with *Pv*MSP1 on the merozoite surface and interact with *Pv*MSP7 to play a stabilizing role [52]. In the same vein, the micronemal *Pv*GAMA protein may bind to specific receptors on the surface of reticulocytes and thus play a role in the adhesion of *P. vivax* merozoites to their target host cells [65]. Indeed, the proteomic analysis of ankyrin 1 (ANK1) and Band 3 by various biochemical approaches, including liquid chromatography-mass spectrometry, showed that the fragment 2 of the membrane binding domain of ANK1 and the extracellular loop 5 of Band 3 bind to *Pf*GAMA and *Pv*GAMA. In addition, anti-Band 3-P5 antibodies block the binding of *Pf*GAMA to erythrocytes [50]. Furthermore, field and laboratory studies showed that *P. vivax* exposure induce robust IgG responses to *Pv*GAMA in natural exposure [84, 85] and that anti *Pv*GAMA antibodies inhibit *in vitro* *P. knowlesi* invasion and growth [86], although knockout of the *pkgama* gene had a minimal effect on *P. knowlesi* growth. Thus, the primary attachment of *P. vivax* merozoites to reticulocytes or erythrocytes involves specific and reversible interactions to guarantee an efficient encounter.

4.2. Apical reorientation

Following primary attachment, *P. vivax* may undergo apical reorientation which triggers various ligand-receptor interactions. As *P. vivax* merozoites have a strong tropism for reticulocytes (0.2–2% of the total erythrocyte population), the chances to encounter a cell permissive to invasion are low. Thus, it was hypothesized that a host cell selection step occurs during apical reorientation to prevent the merozoites from invading non-target cells [87]. Based on the study of homologous proteins in other *Plasmodium* species, the *Pv*RBP protein family is believed to mediate *P. vivax* tropism for reticulocytes. Previous works showed that native *Pv*RBP1 and *Pv*RBP2 may function as an adhesive protein complex [69] and *Pv*RBP2a and *Pv*RBP2b can bind reticulocytes independently of their Duffy phenotype [60]. In 2015, an *ex vivo* study confirmed that *P. vivax* merozoites preferentially target immature

reticulocytes expressing the marker CD71 [61]. In 2018, another study showed that CD71 was the cognate receptor for the ligand *Pv*RBP2b [88]. Analyses by cryo-electron microscopy, immunoprecipitation and fluorescence resonance energy transfer confirmed that the binding of *Pv*RBP2b to CD71 is specific and directly proportional to the level of CD71 expression [89]. Finally, functional studies identified the marker CD98, expressed on the surface of immature reticulocytes, as the cognate receptor of *Pv*RBP2a [62]. Both interactions *Pv*RBP2b—CD71 and *Pv*RBP2a—CD98 seem to drive the strict tropism of *P. vivax* for reticulocytes. Their action may be independent or combined, suggesting the potential existence of alternative invasion pathways. Apart from the *Pv*RBP proteins, the *P. vivax* reticulocyte binding surface antigenic adhesin (*Pv*RBSA) may also play a role in the recognition of reticulocytes. A recent study showed that the recombinant *Pv*RBSA (*rPv*RBSA) binds specifically to Duffy-positive reticulocytes. But its cognate receptor is still unknown [63]. In addition to their adhesive function, *Pv*MSP proteins may also help in the recognition of reticulocytes. Indeed, recent investigations showed that peptides from *Pv*MSP1 have higher binding activity to reticulocytes than erythrocytes [45]. Although the mechanisms remain unclear, the recognition of immature reticulocytes by *P. vivax* merozoites seems to be governed by *Pv*RBP2a and/or *Pv*RBP2b, potentially in conjunction with *Pv*RBSA and *Pv*MSPs. The *Pv*RBP proteins may also trigger subsequent steps of invasion such as the modulation of erythrocyte cytoskeleton, assembly of the tight junction or signalling for the secretion of rophtry proteins.

4.2.1. Invasion

The apical reorientation is followed by the formation of a tight and irreversible junction between *P. vivax* merozoites and reticulocytes. Within Duffy-positive individuals, in absence of evidence, we do not know whether the *P. vivax* Duffy Binding Protein (*Pv*DBP) localizes to the tight junction between merozoites surface and the receptor Duffy Antigen Receptor for Chemokines (DARC). Indeed, for *P. falciparum*, *Pf*EBA175 (a *P. falciparum* erythrocyte-binding ligand) binds Glycophorin A to trigger release of *Pf*RH5 and other rophtry proteins, but *Pf*EBA175 itself does not participate in junction

formation. The *Pv*DBP binding site is monomeric in the absence of DARC but dimerizes upon receptor engagement and forms a homodimer with the N-terminal extracellular domain of DARC [60]. The formation of the tight junction may also be facilitated by the secretion of the ligand *P. vivax* Apical Membrane Antigen 1 (*Pv*AMA1) to the merozoite surface, the injection of *P. vivax* rhoptry neck protein (*Pv*RON) complexes to the reticulocyte surface and their binding. Notably, it has been shown that *Pv*RON5 has a marked binding preference for reticulocytes and is thus suspected to be involved in the invasion process [66]. Finally, the expression of various members of the *P. vivax* 6-cysteine protein (*Pv*P) family on the merozoite surface is also thought to play a role in this step [64].

Within Duffy-negative individuals, the mechanisms and factors involved in the formation of the tight junction are unknown, but several hypotheses are currently discussed (Figure 3). In Madagascar, the evidence of *vivax* infections in Duffy-negative patients correlated with the existence of a common *pvd*bp gene duplication. This supported the hypothesis that the *pvd*bp gene amplification may enhance the binding of the *Pv*DBP protein to another receptor with lower affinity. However, such an alternative receptor has not been identified yet. Further *P. vivax* strains presenting multiple *pvd*bp copies are frequently found in endemic zones where Duffy-negativity is rare [53]. A second hypothesis is that the *Pv*EBP ligand may work as an alternate ligand for the invasion of Duffy-negative reticulocytes. Indeed, *Pv*EBP appears to share structural similarities with *Pv*DBP such as a Duffy binding site and a C-terminal domain with conserved cysteine positions, and likely similar functions [55]. Based on these structural similarities, a recombinant receptor binding site for *Pv*EBP (*rPv*EBP_{II}) was produced. Results of binding assays showed that *Pv*EBP_{II} binds preferentially immature Duffy-positive reticulocytes, but a minimal binding was observed in immature Duffy-negative reticulocytes. In contrast, no binding was observed for mature erythrocytes [57, 58]. Recently, a functional study performed on *P. vivax* isolates from Ethiopia confirmed that *Pv*EBP_{II} can bind both Duffy-positive and Duffy-negative reticulocytes though at a lower frequency than *Pv*DBP. This finding indicates that *Pv*EBP_{II} may not have the same binding capacities

as *Pv*DBP_{II} despite their structural similarities [51]. As the cognate receptor of *Pv*EBP is still unknown, it is unclear whether this potential alternate ligand plays a role in the invasion of Duffy-negative reticulocytes. Finally, it was recently alleged that Duffy-negative reticulocytes may express low and/or transient levels of DARC on the surface of their reticulocytes. Preliminary data suggest that DARC may be expressed on the surface of erythroid precursor cells from Duffy-negative individuals but require to be confirmed [90].

4.3. Invagination

Once *P. vivax* merozoites are irreversibly committed to invasion, they can penetrate the reticulocytes via glideosome activity and seal themselves within the parasitophorous vacuole. Although these mechanisms remain unclear, it seems that *Pv*TRAg36.6 and *P. vivax* early transcribed membrane protein (*Pv*ETRAMP) colocalize in the apical region of the merozoites to form and/or maintain the parasitophorous vacuole membrane [52].

While proteomic technologies are not yet fully exploited, they seem essential to answer long-standing questions about *P. vivax* invasion mechanisms. The constant improvement of techniques (two-dimensional gel electrophoresis, mass spectrometry, yeast two-hybrids screens, etc.), high-throughput approach, computational prediction programs and data processing capabilities offers great promises to study the expression, interaction, and modification of *P. vivax* invasion-related ligands. However, proteomic data alone may be insufficient to establish a fine analysis of the invasion process and propose testable models. They require to be integrated into a more global and fundamental knowledge of *P. vivax* biology [91].

5. Conclusion

For a long time, *vivax* malaria has been considered a benign infection. Consequently, research on *P. vivax* received lower priority from researchers, policy makers and funding agencies. In addition, the limited access to biological material and lack of robust *in vitro* culture systems hampered the development of functional studies.

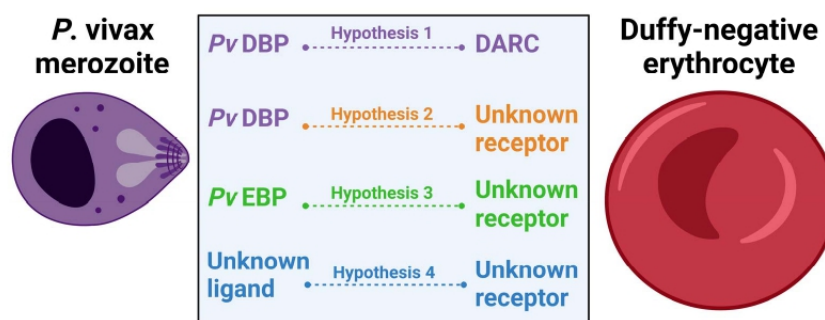


Figure 3. Hypothetical *P. vivax* invasion mechanisms within Duffy-negative reticulocytes. Hypothesis 1. *P. vivax* merozoites invade Duffy-negative reticulocytes through the interaction between the ligand PvDBP and the receptor DARC implying that Duffy-negative reticulocytes may express DARC at low and/or transient level. Hypothesis 2. *P. vivax* merozoites invade Duffy-negative reticulocytes through the interaction between the ligand PvDBP and an unknown receptor expressed on the surface of Duffy-negative reticulocytes. Hypothesis 3. *P. vivax* merozoites invade Duffy-negative reticulocytes through the interaction between the ligand PvEBP and an unknown receptor expressed on the surface of Duffy-negative reticulocytes. Hypothesis 4. *P. vivax* merozoites invade Duffy-negative reticulocytes through the interaction between an unknown ligand expressed on the surface of *P. vivax* merozoites and an unknown receptor expressed on the surface of Duffy-negative reticulocytes. Figure created with BioRender.com.

To address this issue, scientists have considered evidence from homologous protein families in other *Plasmodium* species and produced recombinant proteins to study *P. vivax* invasion-related ligands. But these methods have not fully provided comprehensive information on the actual functions of native ligands. In fact, the critical question for *vivax* malaria research cut across several disciplines. Indeed, genomics technologies shed some light on *P. vivax* genetic diversity. But they may not be sufficient to understand the functional consequences of these variations. While transcriptomic technologies are powerful tools to characterize *P. vivax* stage-specific and highly regulated transcripts, data analysis is limited by the small sample sizes and paucity in constantly expressed genes. However, recent applications of single-cell technologies have enabled to profile both liver-stage parasites and host cells to explore host-parasite interactions [92] and this technology could be applied to study host factors in the reticulocyte invasion pathway. In addition to omics studies, various *in vivo* models have been utilized for studying *P. vivax* invasion pathways. Non-human primate models (NHP) have been widely used in the past, despite

their inherent limitations such as ethical concerns and the variations of host susceptibility to infection with *P. vivax* field isolates, necessitating adaption before replication, likely modifying the biological features of the adapted strains [55]. More recently, efforts have been made to develop humanized mouse systems as models to replicate *in vivo* *P. vivax* infections [93]. Although encouraging, this highly fragile and expensive system still remains in its infancy and needs further refinement to allow continuous maintenance of asexual blood stage infection and develop genetically modified *P. vivax* parasites [94].

In this context, proteomic technologies focus on deciphering the various interactions between *P. vivax* invasion-related ligands and cognate receptors. Nevertheless, they fail to provide a general picture of *P. vivax* invasion mechanisms, especially explaining how *P. vivax* is capable to invade Duffy-negative reticulocytes. Thus, the development of integrated and complementary multi-omics studies together with *in vitro* invasion studies should allow us to better understand the biology of this parasite and support the future implementation of prevention, control, and eradication strategies worldwide.

Conflicts of interest

Authors have no conflict of interest to declare.

Acknowledgements

This review was supported by a grant overseen by the French National Research Agency (VPeRs, ANR-18-

CE15-0026), by the Institut Pasteur, Paris, the Laboratoire d'Excellence (LabEx) "French Parasitology Alliance for Health Care" (ANR-11-15 LABX-0024-PARAFRAP) and the University of Strasbourg through the Programme IdEX 2022 (Attractivité — Dotation d'accueil des professeurs d'université recrutés en 2021).

Version française

1. Introduction

Le paludisme est une maladie débilitante aiguë causée par des parasites protozoaires du genre *Plasmodium* qui sont transmis par des moustiques *Anopheles* femelles. Selon le dernier rapport de l'Organisation mondiale de la santé (OMS), cette maladie a été responsable de 241 millions de cas et de 627,000 décès dans le monde en 2020. Le nombre estimé de décès dus au paludisme s'élevait à 627,000 en 2020 (soit une augmentation de 69,000 décès par rapport à l'année précédente). Les pays d'Afrique subsaharienne continuent de supporter une part disproportionnée de la charge mondiale du paludisme, avec 95% de tous les cas de paludisme et 96% des décès. Les enfants de moins de 5 ans représentent environ 80% de tous les décès dus au paludisme dans cette région [1].

Parmi les espèces responsables de l'infection humaine, *Plasmodium vivax* est la plus répandue géographiquement et la principale cause de maladie en dehors de l'Afrique subsaharienne, en Asie du Sud-Est, en Amérique du Sud et en Afrique du Nord-Est [2]. *P. vivax* présente un cycle de vie complexe consistant en l'infection cyclique d'un hôte humain et d'un moustique vecteur anophèle femelle. Après la piqûre d'un moustique infecté, les sporozoïtes migrent à travers le derme dans les capillaires, atteignent le foie et envahissent les hépatocytes [3]. Alors que la plupart des sporozoïtes entreprennent une maturation asexuée en schizontes, certains d'entre eux divergent pour former des formes quiescentes, les hypnozoïtes. Ces hypnozoïtes sont capables d'émerger des semaines voire des mois après la primo-infection pour donner lieu à des épisodes récurrents de paludisme [4]. Lorsque les schizontes finissent par se rompre, ils

libèrent des mérozoïtes qui migrent dans la circulation sanguine et envahissent les réticulocytes. Ils se transforment en anneaux, en trophozoïtes, puis en schizontes [2]. Les schizontes se rompent et libèrent de nouveaux mérozoïtes qui vont envahir les réticulocytes sains. Il en résulte un cycle continu de multiplications asexuées, ce qui augmente progressivement le nombre de parasites dans la circulation sanguine [5]. Par ailleurs, certains parasites se différencient en gamétocytes mâles ou femelles. Lorsqu'un moustique prélève son repas sanguin chez un hôte infecté, les gamétocytes entreprennent une fécondation à l'intérieur de l'intestin moyen du moustique et produisent des ookinètes qui se transforment en oocystes. Ces oocystes libèrent ensuite des sporozoïtes qui migrent vers les glandes salivaires pour être inoculés chez de nouveaux hôtes par les moustiques femelles lors d'un repas sanguin. Ainsi, le cycle de vie de *P. vivax* se perpétue (Figure 1) [6].

Bien que cryptique, *P. vivax* est la cause de paludisme la plus répandue dans le monde [7]. En effet, sa capacité à se développer à basse température (18–25 °C) lui confère un potentiel d'infectivité et de transmission dans les zones tropicales et tempérées [8]. Par ailleurs, son cycle intra-érythrocytaire est caractérisé par une faible parasitémie et une asynchronie, ce qui contribue probablement à des erreurs de diagnostic (faux négatifs) [9]. En effet, la faible parasitémie, couramment observée dans les infections à *P. vivax*, est probablement due au fort tropisme des mérozoïtes de *P. vivax* pour les réticulocytes et aux besoins physiologiques et/ou métaboliques spécifiques des stades sanguins du parasite pour sa croissance et sa survie [2]. En l'absence de solides preuves biologiques, un modèle d'invasion par *P. vivax* a été proposé, sur la base des observations

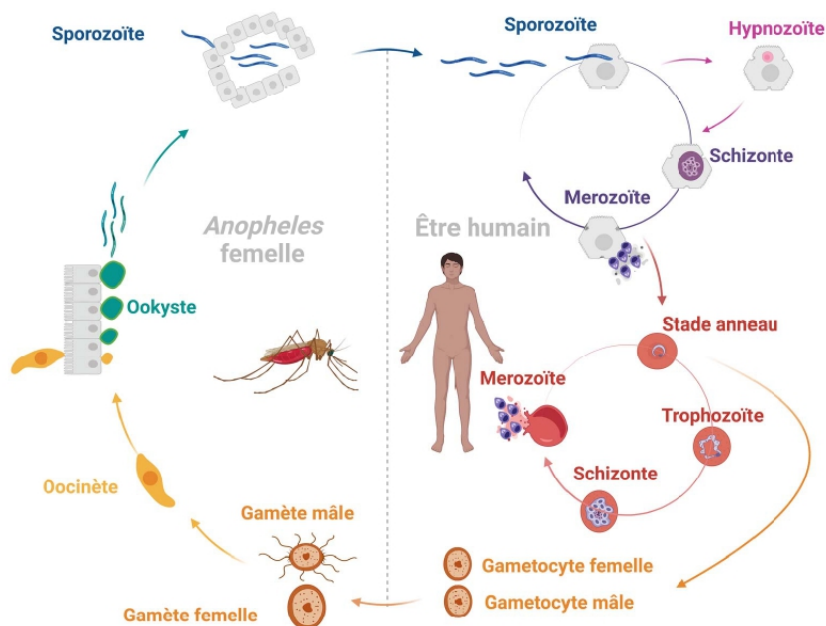


FIGURE 1. Cycle de vie de *P. vivax*. Le cycle de vie de *P. vivax* implique l'infection cyclique d'un moustique vecteur Anophèle et d'un hôte humain. Lorsqu'un moustique infecté par *P. vivax* prend un repas sanguin, il injecte des sporozoïtes dans l'hôte humain. Ces sporozoïtes migrent à travers le derme jusqu'au foie et envahissent les hépatocytes. Ils mûrissent en schizontes tissulaires et établissent un stock d'hypnozoïtes latents. Lorsque les schizontes se rompent, ils libèrent des mérozoïtes qui migrent vers la circulation sanguine et envahissent les réticulocytes. Ils se développent en anneaux, en trophozoïtes et en schizontes intra-érythrocytaires. Lorsque les schizontes se rompent, ils lysent les réticulocytes infectés et libèrent de nouveaux mérozoïtes. Une petite proportion de la population parasitaire se différencie en gamétocytes mâles et femelles. Lorsqu'un moustique prend un nouveau repas sanguin, il ingère des gamétocytes. Dans l'intestin moyen, les microgamétocytes et les macrogamétocytes se fécondent, entreprennent une division sexuelle et se transforment en ookinètes qui mûrissent en oocystes. Lorsque les oocystes se rompent, ils libèrent des sporozoïtes qui migrent vers les glandes salivaires pour perpétuer le cycle de vie. Figure créée avec BioRender.com.

faites lors de l'invasion d'érythrocytes humains par les mérozoïtes de *P. falciparum* ou *P. knowlesi*. Ce modèle suggère qu'au cours du processus d'invasion, *P. vivax* exprime des adhésines qui se lieraient à des récepteurs présents à la surface des réticulocytes et établiraient une fixation initiale et réversible. Cela entraînerait la déformation de la membrane des réticulocytes et la réorientation apicale des mérozoïtes. Ensuite, ils activeraient des organelles et li-

béreraient des protéines pour former une jonction serrée et irréversible [10]. Les mérozoïtes seraient propulsés par un moteur actine-myosine et la jonction se déplacerait du pôle apical au pôle postérieur. Cela formerait la vacuole parasitophore dans laquelle les parasites se développeraient après la fermeture du pore d'invasion et de la membrane réticulocyttaire [11, 12] (Figure 2 et Tableau 1 pour les détails).

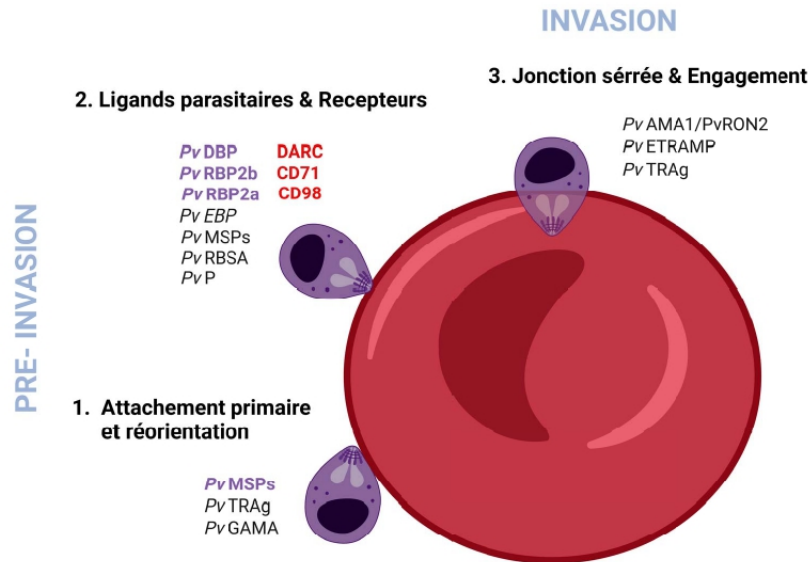


FIGURE 2. Mécanismes d'invasion de *P. vivax* dans les réticulocytes Duffy-positifs. (1) Attachement primaire et réorientation — Lorsque les mérozoïtes de *P. vivax* sont libérés dans la circulation sanguine, la fixation primaire implique les protéines PvMSPs. D'autres protéines telles que *Pv*TRAg56.2 et *Pv*GAMA pourraient jouer un rôle dans l'adhésion et la stabilisation du parasite. (2) Liaison au ligand et récepteurs — Le ligand *Pv*DBP est progressivement couplé au récepteur DARC (région II) pour créer un hétérotétramère stable constitué de deux molécules de *Pv*DBP et de deux molécules de DARC. La reconnaissance et la sélection des réticulocytes pour l'invasion sont médiées par les protéines *Pv*RBPs, notamment par les interactions *Pv*RBP2b-CD71 et *Pv*RBP2a-CD98. En l'absence de preuves biologiques et sur la base des observations faites pendant l'invasion des érythrocytes par les mérozoïtes de *P. falciparum* ou de *P. knowlesi*, nous supposons que de nombreuses protéines de *P. vivax*, y compris les *Pv*MSPs, les *Pv*RBSAs et autres, pourraient jouer un rôle dans la réorientation apicale de *P. vivax*. (3) Jonction serrée et engagement — Les mérozoïtes de *P. vivax* pénètrent dans les réticulocytes via l'activité du glidésome. Des protéines telles que *Pv*AMA1 et *Pv*RON pourraient conduire directement ou indirectement à la formation d'une jonction serrée et irréversible et servir de médiateurs à l'invasion. Plusieurs protéines de surface de *P. vivax* pourraient être rapidement dégradées alors que d'autres, comme *Pv*TRAg36.6 et *Pv*ETRAMP, pourraient être exprimées pour former une vacuole parasitophore et favoriser le développement du parasite. Il est important de noter que les mécanismes d'invasion des mérozoïtes de *P. vivax* décrits ici sont jusqu'à présent largement limités à une comparaison avec les homologues d'invasion de *P. falciparum* et *P. knowlesi* et une poignée de familles de gènes d'invasion de *P. vivax* connues. Le rôle des ligands de *P. vivax* présents en caractères gras violets a été confirmé, tandis que les autres présents en caractères noirs sont seulement suspectés. Figure créée avec BioRender.com.

TABLEAU 1. Liste des ligands liés à l'invasion de *P. vivax* caractérisés par les technologies omiques

Étape	Ligands parasitaires d'accésion	Numéro	Rôle (confirmer/potentiel)	Recepteur érythrocytaire (stade sanguin)	Candidats vaccins (stade sanguin)	Preuves biologiques obtenues par des études	
						Genomiques	Transcriptomiques / Proteomiques
Primary attachment & reorientation	<i>MSP1</i>	PVP01_0728900	Confirmer	Oui		<i>pvmSP1</i> est fortement polymorphe (mutations) [43]	Parmi tous les gènes, <i>pvmSP1</i> est un des plus exprimés au stade schizonte [44] Par homologie avec les protéines PfMSPs chez <i>P. falciparum</i> , il a été émis comme hypothèse que PfMSP1 pourrait reconnaître un récepteur situé à la surface des réticulocytes [45]
	<i>MSP7</i>	PVP01_1219500, PVP01_1219700, PVP01_1219400, PVP01_1219600, PVP01_1220300, PVP01_1220300, PVP01_1220400, PVP01_1219900	Potentiel			<i>pvmSP7</i> est fortement polymorphe (mutations) [46]. <i>pvmSP3</i> présente un nombre de copie amplifiée allant de 1 à 4 copies	Les ARN messagers de <i>pvmSP7</i> transcript sont les plus élevés au stade trophozoïte âgé-schizonte [47]. Les ARN messagers de <i>pvmSP3</i> sont détectés au stade schizonte âgé [5]. Parmi tous les gènes, <i>pvmSP9</i> est un des plus exprimés au stade schizonte [44]
	<i>GAMA</i>	PVP01_0505600	Potentiel			<i>pvgama</i> présente un polymorphisme de taille [49]	Etudes fonctionnelles: <i>PvGAMA</i> pourrait jouer un rôle dans l'adhésion des mérozoïtes de <i>P. vivax</i> aux réticulocytes [49] et se lie aux protéines Band3 et ANK1 [50]

(suite à la page suivante)

TABLEAU I. (Suite)

Etape	Ligands parasitaires	Numéro d'accension	Rôle (confirmer/potentiel)	Recepteur erythrocytaire	Candidats vaccins (stage sanguin)	Preuves biologiques obtenues par des études		
						Genomiques	Transcriptomiques	Proteomiques
	<i>MSP1P</i>	PVP01_0728800	Potentiel					<i>PfMSP1P</i> pourrait jouer un rôle dans l'adhésion des mérozoïtes de <i>P. vivax</i> aux réticulocytes grâce à une interaction avec sa région C terminale [43]
	<i>TRAg56.2</i>	PVX_088850	Potentiel			la famille des gènes <i>pvtrag</i> est peu polymorphe [51]	Séquencage à l'échelle cellulaire: des niveaux d'expression des ARN messagers différents ont été observés entre les membres des gènes de la famille <i>pvtrag</i> [5]	Immunoprecipitation et spectrométrie de masse: <i>PfTRAg56.2</i> semble se co-localiser avec <i>PfMSP1</i> et interagir avec <i>PfMSP7</i> et jouer un rôle stabilisateur [52]
Ligand bindings	<i>DBP</i>	PVP01_0623800	Confirmer	DARC	Oui	<i>pvdbp</i> est fortement polymorphe (mutations et variation du nombre de copie de gène) [53]	L'amplification du gène <i>pvdbp</i> serait associée à une augmentation de l'expression des ARN messagers, suggérant une augmentation de la protéine <i>PfVDBP</i> [54]	Etudes de liaison et études fonctionnelles
	<i>EBP</i>	PVP01_0102300	Potentiel			<i>pvebp</i> est fortement polymorphe mais moins que <i>pvdbp</i> (mutations et variation du nombre de copie de gène) [55]	Les ARN messagers de <i>pvebp</i> sont fortement exprimés au stade schizonte [55,56]	<i>PfEBP1</i> se lie préférentiellement aux réticulocytes Duffy-positifs immatures. La liaison avec les réticulocytes Duffy-négatifs immatures est minime [57,58].

(suite à la page suivante)

TABLEAU I. (Suite)

Etape	Ligands parasitaires	Numéro d'accèsion	Rôle (confirmer/potentiel)	Recepteur érythrocytaire	Candidats vaccins (stade sanguin)	Preuves biologiques obtenues par des études		
						Genomiques	Transcriptomiques	Proteomiques
<i>RBP2b</i>	PVP01_0800700	Confirmer	CD71	Malgré l'existence de régions conservées, <i>pvrp2a</i> est relativement polymorphe [59, 60]	Culture <i>ex vivo</i> : l'interaction <i>PvrRBP2b</i> -CD71 joue une rôle crucial dans la reconnaissance des réticulocytes [61]			
<i>RBP2a</i>	PVP01_1402400	Confirmer	CD98	Malgré l'existence de régions conservées, <i>pvrp2a</i> est relativement polymorphe [59, 60]	Etudes de liaison: CD98 est le récepteur de la protéine <i>PvrRBP2a</i> de <i>P. vivax</i> et l'interaction entre <i>PvrRBP2a</i> -CD98 est impliquée dans la reconnaissance des réticulocytes [62]			
<i>RBSA</i>	PVP01_0004240	Potentiel		<i>pvrbsa</i> présente une diversité génétique réduite par rapport aux autres gènes codant pour des protéines de surface des mérozoïtes de <i>P. vivax</i> [63]	<i>PvrRBSA</i> se lie spécifiquement aux réticulocytes Duffy-positifs, mais son récepteur n'est pas connu [63]			
<i>PI2</i>	PVP01_1136400	Potentiel			Les protéines de la famille Pvp sont exprimées à la surface des mérozoïtes de <i>P. vivax</i> et ils pourraient jouer un rôle dans l'invasion des réticulocytes [64]			

(suite à la page suivante)

TABLEAU 1. (Suite)

Etape	Ligands parasitaires	Numéro d'accension	Rôle (confirmer/potentiel)	Recepteur érythrocytaire	Candidats vaccins (stage sanguin)	Preuves biologiques obtenues par des études		
						Genomiques	Transcriptomiques	Proteomiques
Tight junction formation	AMAI	PVP01_0934200	Potentiel		Oui			Etudes fonctionnelles: La fonction de <i>PvAMAI</i> semble similaire à celle de <i>PfAMAI</i> [65] et <i>PvAMAI</i> pourrait être impliquée avec RON2 dans la formation de la jonction serrée [66]
	RON	PVP01_0916600	Potentiel				<i>pvrn4</i> présente un profil de transcription similaire à celui de <i>pfron4</i> [67]. <i>pvrn5</i> est exprimé au stade schizonte [66]	Immunofluorescence indirecte et immunosaisys: <i>PvRON4</i> serait exprimée au niveau du pôle apical de <i>P. vivax</i> (souche VCG-1) et pourrait participer à la formation de la jonction serrée [67]. <i>PvRON5</i> présente une préférence de liaison avec les réticulocytes et est donc fortement suspecté d'être impliqué dans le processus d'invasion des réticulocytes [66].
	ETTRAMP	PVP01_0532300	Potentiel					ETTRAMP serait impliqué dans la formation de la vacuole parasitophore, (suite à la page suivante)

TABLEAU I. (Suite)

Etape	Ligands parasitaires	Numéro d'accèsion	Rôle (confirmer/potentiel)	Recepteur érythrocytaire	Candidats vaccins (stade sanguin)	Preuves biologiques obtenues par des études		
						Genomiques	Transcriptomiques	Proteomiques
	<i>TRAg36.6</i>	PVX_112690	Potentiel			Séquencage de l'ADN: la famille de gène <i>putrag</i> est rarement polymorphe [51]	Séquencage à l'échelle cellulaire: des niveaux d'expression des ARN messagers différents ont été observés entre les membres des gènes de la famille <i>putrag</i> [5]	Immunoprecipitation et spectrométrie de masse: <i>PvTRAg36.6</i> semble se co-localiser avec <i>PvETRAMP</i> pour former et/ou maintenir l'intégrité de la membrane de la vacuole parasitophore [52]
Unknown function	<i>MSA180</i>	PVP01_0814200	Potentiel				Parmi tous les gènes, <i>pmsa180</i> est un des plus exprimés au stade schizonte [44]	
	<i>P92</i>	PVP01_1107900	Potentiel					
	<i>RAMA</i>	PVP01_0107500	Potentiel				Parmi tous les gènes, <i>pvrana</i> est un des plus exprimés au stade schizonte [44]	
	<i>RBPIa</i>	PVP01_0701200	Potentiel			Séquencage de l'ADN: la diversité génétique de <i>pvrp1a</i> semble réduite [68]	Les ARN messagers de <i>pvrp1</i> sont fortement exprimés au stade schizonte [55,56].	Les protéines <i>PvRBPI</i> et <i>PvRBPI2</i> natives pourraient être impliquées dans un complexe protéique d'adhésion [69]
	<i>RBPIb</i>	PVP01_0701100	Potentiel			Séquencage de l'ADN: la diversité génétique de <i>pvrp1b</i> semble réduite [68]	Les ARN messagers de <i>pvrp1</i> sont fortement exprimés au stade schizonte [55,56].	Les protéines <i>PvRBPI</i> et <i>PvRBPI2</i> natives pourraient être impliquées dans un complexe protéique d'adhésion [69]

(suite à la page suivante)

TABLEAU 1. (Suite)

Etape	Ligands parasitaires	Numéro d'accèsion	Rôle (confirmer/potentiel)	Recepteur érythrocytaire	Candidats vaccins (stade sanguin)	Preuves biologiques obtenues par des études	
						Genomiques	Proteomiques
	<i>RBP2c</i>	PVP01_0534300	Potentiel			Séquençage de l'ADN: <i>pvrbp2c</i> est fortement polymorphe [51,59,70]	
	<i>RBP1p1 (partial)</i>	-	Potentiel			Séquençage de l'ADN: identification partielle du gène <i>pvrbp1p1</i>	
	<i>RBP2p1 (partial)</i>	PVP01_0534400	Potentiel			Séquençage de l'ADN: identification partielle du gène <i>pvrbp2p1</i>	
	<i>RBP2p2 (partial)</i>	PVX_101590	Potentiel			Séquençage de l'ADN: identification partielle du gène <i>pvrbp2p2</i> .	
	<i>RBP2d (pseudo)</i>	PVP01_1471400	Potentiel			Séquençage de l'ADN: identification du pseudogène <i>pvrbp2d</i> .	
	<i>RBP2e (pseudo)</i>	PVP01_0700500	Potentiel			Séquençage de l'ADN: identification du pseudogène <i>pvrbp2e</i> .	

(suite à la page suivante)

TABLEAU 1. (Suite)

Etape	Ligands parasitaires	Numéro d'accension	Rôle (confirmer/potentiel)	Recepteur érythrocytaire vaccins (stade sanguin)	Candidats	Preuves biologiques obtenues par des études		
						Genomiques	Transcriptomiques	Proteomiques
	<i>RBP3 (pseudo)</i>	PVP01_1469400	Potentiel			Séquencage de l'ADN: identification du pseudogène <i>purbp3</i> .		
	<i>RhopH3</i>	PVP01_0703800	Potentiel			Parmi tous les gènes, <i>pvrhopH3</i> est un des plus exprimé au stade schizonte [44]		
	<i>RON5</i>		Potentiel			Les ARN messagers de <i>pvrON5</i> sont fortement exprimés au stade schizonte dans la souche VCG-1 [66]		Etudes de liaison, Western blot et tests d'immunofluorescence: <i>PvRON5</i> serait exprimée au niveau du pôle apical de <i>P. vivax</i> (souche VCG-1) and présente une préférence de liaison avec les réticulocytes [66]

Les numéros d'accension commençant par PVP01 proviennent de la souche de référence *PvP01*, tandis que les numéros d'accension commençant par PVX proviennent de la souche de référence Sal-1 (<https://plasmodb.org/plasmo/app>). Les numéros d'accension commençant par KP proviennent de la souche de référence VCG-1 (<https://www.ncbi.nlm.nih.gov>).

Pendant longtemps, les scientifiques ont cherché à identifier les principales interactions hôte-parasite conduisant à l'invasion des réticulocytes humains par *P. vivax*. Entre 1920 et 1960, des essais cliniques et de terrain ont été menés sur des individus d'origine africaine ou afro-américaine. Ces essais ont démontré que ces individus étaient hautement résistants au paludisme à *P. vivax* lorsqu'ils étaient exposés à du sang humain ou à des moustiques infectés par un nombre limité de souches de *P. vivax* [13–16]. Après l'identification des groupes sanguins Duffy [17, 18], il a été établi que la résistance naturelle à l'infection à *P. vivax* était liée à l'absence de la protéine Duffy Antigen Receptor for Chemokines (DARC) à la surface des érythrocytes humains [19]. La base moléculaire de l'absence d'expression de l'antigène Duffy à la surface des globules rouges a ensuite été démontrée comme étant due une mutation ponctuelle unique dans la séquence *GATA1* (tct>cct) située dans la région promotrice 5' du gène *ackr1* [20, 21]. Des travaux novateurs portant sur des infections expérimentales contrôlées de volontaires à l'aide de sporozoïtes de *P. vivax* [19] et des études *in vitro* utilisant *P. knowlesi* (un parasite responsable du paludisme zoonotique étroitement apparenté à *P. vivax*) comme modèle [22] ont ensuite confirmé que l'interaction entre DARC et les mérozoïtes de *P. vivax* était cruciale pour permettre l'invasion des réticulocytes. Parallèlement, le ligand du parasite qui se lie spécifiquement à DARC a été identifié comme étant la protéine *P. vivax* Duffy Binding Protein (*PvDBP*) [23]. Ainsi, le paradigme scientifique selon lequel *P. vivax* envahit exclusivement les érythrocytes Duffy-positifs par le biais de l'interaction entre *PvDBP* et DARC était établi. Il a également été supposé que le paludisme à *P. vivax* était rarement transmis ou même absent dans les régions où la proportion d'individus Duffy-négatifs est prédominante comme en Afrique subsaharienne [19]. Cependant, au cours des deux dernières décennies, de nombreuses études ont rapporté des cas d'infection à *P. vivax* chez des individus Duffy-négatifs dans des pays subsahariens comme l'Angola [24], le Bénin [25], le Cameroun [26, 27], la Guinée équatoriale [24, 28], l'Éthiopie [29], le Kenya [30], Madagascar [31], le Mali [32], la Mauritanie [33] et le Soudan [34]. Bien que ces observations soient encore parcellaires, elles soulèvent des interrogations quant aux mécanismes d'invasion utilisés par *P. vivax* pour infecter les individus

Duffy-négatifs. Elles remettent également en question certaines hypothèses telles que le rôle central et unique joué par l'interaction *PvDBP*-DARC dans l'invasion des réticulocytes, la limite de détection des outils de diagnostic actuels et l'évolution des interactions hôte-pathogène [35]. A l'heure actuelle, nous ne savons toujours pas si ces cas représentent un phénomène préexistant qui a été négligé dans le passé ou une nouvelle voie d'invasion indépendante de DARC, qui pourrait potentiellement conduire à une expansion du paludisme à *P. vivax* au sein des populations Duffy-négatives [36].

La collecte d'échantillons de terrain dans des zones endémiques éloignées étant difficile, longue et coûteuse, la recherche s'est longtemps limitée à l'échantillonnage direct de patients donneurs ou à l'étude d'espèces de *Plasmodium* étroitement apparentées telles que *P. knowlesi* [37, 38] ou *P. cynomolgi* [39]. En outre, le développement de méthodes de culture *in vitro* reproductibles est encore entravé par la faible parasitémie de *P. vivax* et son fort tropisme pour les réticulocytes humains [40].

Néanmoins, les récentes technologies dites « omiques » ont révolutionné la recherche sur le paludisme à *P. vivax* avec le développement de techniques innovantes et abordables permettant une caractérisation plus complète des gènes, des transcrits et des protéines de *P. vivax*. Cette revue a non seulement pour but de fournir un aperçu général des mécanismes d'invasion de *P. vivax* grâce aux techniques génomiques, transcriptomiques et protéomiques mais également d'illustrer l'importance de la complémentarité de ces approches.

2. Mécanismes moléculaires d'invasion de *P. vivax*

En 2004, le projet de séquençage du génome de *P. vivax* a fourni la première séquence de référence *P. vivax* Salvador-1 (Sal-1) en utilisant une stratégie de séquençage du génome entier [41, 42]. Au cours des vingt dernières années, plusieurs autres isolats de terrain et cliniques de *P. vivax* ont été séquençés par microarray, séquençage profond ou séquençage du génome entier. Toutes ces séquences sont disponibles publiquement sur des bases de données en ligne telles que PlasmoDB (<https://plasmodb.org/plasmo/app>) et sont régulièrement annotées. Globalement, l'analyse de ces séquences de *P. vivax* a

contribué à l'identification de gènes potentiellement liés à l'invasion.

2.1. Le rôle crucial du gène *pvdbp* dans l'invasion des réticulocytes Duffy-positifs

Le gène *P. vivax* Duffy binding protein (*pvdbp*) code pour le ligand *PvDBP* [71]. Il présente des séquences de codage pour un domaine peptidique N-terminal, un domaine d'attachement Duffy riche en cystéines (*PvDBPII*), un autre domaine riche en cystéines (*PvDBPVI*), un domaine transmembranaire et une queue cytoplasmique C-terminale [72]. Sur la base des données actuelles de séquençage à haut débit, notre appréciation de la diversité génétique est de moins en moins limitée par la nature et/ou la densité des marqueurs génomiques étudiés. Notamment, la publication récente de plus de deux cents génomes de *P. vivax* a montré que les séquences du gène *pvdbp* étaient hautement polymorphes, quelle que soit leur origine géographique. Il est intéressant de noter que de nombreux polymorphismes nucléotidiques simples (SNP) non synonymes ont été trouvés dans le domaine *PvDBPII* [53]. La diversité et la localisation de ces SNPs suggèrent que le gène *pvdbp* pourrait subir une pression sélective différentielle en raison des réponses des anticorps et de sa fonction dans la liaison aux récepteurs et l'invasion [51].

En outre, les variations du nombre de copies (CNV) au niveau du locus *pvdbp* se sont révélées être l'une des expansions génétiques les plus courantes, avec des allèles multiples [73]. Alors que la détection des CNVs reposait dans le passé, sur des techniques coûteuses et nécessitant une main-d'œuvre importante comme l'hybridation *in situ*, les progrès récents du séquençage à haut débit ont permis leur détection directe à partir des données de séquençage. Notamment, les approches de séquençage du génome entier ont révélé deux duplications distinctes chez les souches *P. vivax* circulant à Madagascar et au Cambodge. À Madagascar, les isolats de *P. vivax* provenant de patients Duffy-positifs et Duffy-négatifs présentaient une duplication commune avec deux copies de *pvdbp* l'une à côté de l'autre [74]. Au Cambodge, une duplication différente du gène *pvdbp* a été couramment détectée dans les isolats de *P. vivax* provenant de patients Duffy-positifs. De plus, la proportion d'isolats de *P. vivax* portant la duplication s'est avérée être significativement plus élevée chez

les patients homozygotes Duffy-positifs que chez les patients hétérozygotes Duffy-positifs qui devraient présenter une plus faible expression de DARC à la surface de leurs réticulocytes. Cependant, cette dernière analyse n'a pas permis d'exclure complètement que les patients puissent être porteurs de plusieurs clones de *P. vivax*, alors que l'estimation des CNVs par PCR quantitative ne reflète que le clone principal [53]. Récemment, une étude géographique a démontré que les deux duplications distinctes du gène *pvdbp* circulaient en Asie, en Amérique du Sud et en Afrique de l'Est. Alors que la Duffy-négativité est prévalente dans certaines régions, les duplications du gène *pvdbp* ont également été largement trouvées dans des régions où la Duffy-négativité était presque absente [51]. En revanche, une analyse génomique simultanée a montré qu'aucune duplication de *pvdbp* n'a été trouvée dans cent quatre-vingt-deux isolats de *P. vivax* provenant de onze pays endémiques. Cependant, il a été remarqué que cette divergence était probablement due aux amorces utilisées pour la validation des CNVs par PCR [75].

Malgré la spécificité et la distribution des duplications du gène *pvdbp*, rien n'indique que les génomes de *P. vivax* aient contourné la barrière de la Duffy-négativité grâce à cette variation génétique. Des expansions plus importantes de *pvdbp* ont également été détectées dans le monde entier. Elles se regroupent séparément mais présentent des schémas similaires. Une grande diversité d'haplotypes a également été observée entre les copies, tandis que les séquences flanquantes 5' et 3' présentaient une diversité modérée [76]. Dans la région Asie-Pacifique, le séquençage de plus de deux cents isolats de *P. vivax* provenant de patients Duffy-positifs a révélé la présence commune de deux à trois copies du gène *pvdbp* [73]. En Ethiopie, les séquences de deux isolats de *P. vivax* provenant de patients Duffy-négatifs ont montré la présence de trois et huit copies du gène *pvdbp* [57]. Par la suite, une autre expansion commune du gène *pvdbp*, assez similaire à celles précédemment décrites à Madagascar et au Cambodge mais allant de trois à cinq copies, a également été détectée [51]. Ces résultats suggèrent que l'expansion du gène *pvdbp* pourrait permettre la survenue de mutations dans la deuxième copie du gène. De telles expansions pourraient faciliter la liaison à un autre récepteur érythrocytaire ayant une affinité plus faible pour le ligand *PvDBP* [57]. Cependant, il n'y a

aucune preuve de l'existence de CNVs localisés et sélectionnés qui amélioreraient l'efficacité de l'invasion et induiraient une pression sélective. Étant donné que l'invasion des érythrocytes Duffy-positifs dépend de l'interaction entre *PvDBP* et *DARC*, il est probable que les expansions du gène *pvdbp* soient dues à des variations génétiques de *DARC* ou à des réponses à des anticorps inhibant l'attachement au domaine *PvDBPII* [75]. Néanmoins, il ne semble pas que ces expansions donnent au parasite une capacité accrue pour envahir les érythrocytes Duffy-négatifs.

2.2. Le potentiel rôle du gène *pvebp* dans une voie d'invasion alternative

Le gène *P. vivax* erythrocyte binding protein (*pvebp*) a été découvert grâce à l'assemblage *de novo* d'un isolat de *P. vivax* du Cambodge. Il code pour un ligand putatif de la protéine *P. vivax* Erythrocyte Binding Protein (*PvEBP*) et est présent dans la plupart des génomes de *P. vivax*. L'organisation du gène *pvebp* est similaire à celle du gène *pvdbp* avec un peptide signal N-terminal, un domaine d'attachement Duffy *PvEBPII* semblable au domaine *PvDBPII*, un autre domaine riche en cystéines *PvEBPVI*, un domaine transmembranaire et une queue cytoplasmique C-terminale [55]. Malgré ces similitudes, le gène *pvebp* semble se regrouper séparément de tous les gènes possédant un domaine d'attachement Duffy-binding like. Le séquençage de l'ADN de dix génomes de *P. vivax* provenant d'Asie, du Pacifique Sud, d'Afrique, d'Amérique centrale et d'Amérique du Sud a montré que le gène *pvebp* est polymorphe bien que moins divers que le gène *pvdbp*. En effet, le gène *pvebp* ne présente que 11 sites polymorphes sur 2412 positions alors que le gène *pvdbp* présente 32 sites polymorphes sur 3213 positions pour les mêmes échantillons. La plupart des SNPs détectés étaient non synonymes [55]. De plus, l'analyse génomique de deux cent dix-neuf isolats de *P. vivax* provenant de patients Duffy-positifs au Cambodge et à Madagascar a confirmé que le gène *pvebp* était moins diversifié que le gène *pvdbp* (12 vs. 33 allèles observés à partir des mêmes isolats) et ne présentait que des mutations non synonymes indiquant l'existence d'une forte pression sélective. En effet, onze mutations ponctuelles non synonymes ont été trouvées dans les séquences *pvebp* et ont conduit à douze allèles différents. Certains SNPs ont été observés dans

les deux pays alors que d'autres étaient spécifiques au Cambodge et à Madagascar. Ces SNPs étaient distribués dans le domaine d'attachement [53]. Tout comme pour le gène *pvdbp*, la diversité et la distribution des SNPs dans le gène *pvebp* reflètent l'existence probable d'une pression sélective, bien qu'elle semble opérer à un niveau plus faible [58].

L'analyse génomique des isolats de *P. vivax* du Cambodge, de Madagascar et d'Éthiopie a également révélé l'existence de variations du nombre de copies dans le gène *pvebp*. À Madagascar, des expansions du gène *pvebp* ont été observées, allant de deux à cinq copies. Une distribution bimodale des parasites avec une population spécifique présentant plus de trois copies a été trouvée. Cependant, aucune différence significative de susceptibilité à l'infection à *P. vivax* n'a été observée entre les individus homozygotes et hétérozygotes Duffy-positifs. Au Cambodge, les expansions de *pvebp* variaient d'une à deux copies. La proportion d'isolats de *P. vivax* présentant des copies multiples du gène était plus importante dans les zones de forte transmission [53]. Dans l'ensemble, une proportion élevée d'isolats de *P. vivax* avec des copies de gènes multiples a été trouvée à Madagascar où la Duffy-négativité et la Duffy-positivité coexistent par rapport au Cambodge où la Duffy-négativité est pratiquement absente. En revanche, aucune expansion de *pvebp* n'a été trouvée en Éthiopie où la Duffy-négativité est également fréquente [51]. Compte tenu des similitudes observées entre les gènes *pvdbp* et *pvebp*, le polymorphisme et l'expansion du gène *pvebp* pourraient être corrélés à l'expression d'un nouveau ligand interagissant avec des protéines membranaires érythrocytaires autres que *DARC* et contribuer ainsi à une voie d'invasion alternative [55]. Cependant, aucune preuve que ces variations confèrent des capacités à infecter des individus Duffy-négatifs n'a été fournie jusqu'à présent.

2.3. Le rôle des gènes *pvrpbp* et *pvrbsa* dans la sélectivité des réticulocytes

La famille de gènes *P. vivax* reticulocyte binding protein (*pvrpbp*) a été identifiée sur la base de l'annotation des ligands adhésifs du génome de *P. vivax* [69]. Le séquençage du génome de plusieurs isolats de *P. vivax* a identifié onze membres de la famille *pvrpbp*, dont cinq gènes complets (*pvrpbp1a*, *pvrpbp1b*, *pvrpbp2a*, *pvrpbp2b* et *pvrpbp2c*), trois gènes partiels

(*pvrpb1p1*, *pvrpb2p1* et *pvrpb2p2*) et trois pseudo-gènes (*pvrpb2d*, *pvrpb2e* et *pvrpb3*) [77]. Les gènes *pvrpb* sont constitués de deux exons, l'un codant pour un peptide signal dans la région N-terminale et l'autre pour un domaine transmembranaire dans la région C-terminale [56]. Des études récentes ont montré que leur diversité génétique semble être généralement faible, sauf pour les gènes *pvrpb2* [68]. L'analyse des isolats de *P. vivax* de Chine, de Thaïlande et d'Éthiopie a confirmé que le gène *pvrpb2c* était hautement polymorphe [51, 59, 70]. Bien que *pvrpb2a* et *pvrpb2b* présentent des régions hautement conservées, l'analyse génomique des isolats de *P. vivax* de Papouasie-Nouvelle-Guinée et de Thaïlande a montré qu'ils étaient également relativement polymorphes [59,60]. Comme observé pour les gènes *pvdbp* et *pvebp*, les SNPs non synonymes se sont regroupés autour de trois sites principaux, dont le domaine de liaison aux réticulocytes. Cela peut refléter l'importance fonctionnelle de ce site de liaison et l'existence d'une pression sélective [60].

De plus, des CNVs affectant à la fois *pvrpb2a* et *pvrpb2b* ont été observés dans deux isolats de *P. vivax* de Thaïlande. Alors qu'une souche présentait une duplication des deux gènes (*pvrpb2a* et *pvrpb2b*), l'autre souche avait deux copies de *pvrpb2b* seulement [59]. Ces résultats suggèrent que les gènes *pvrpb* peuvent coder des ligands impliqués dans la reconnaissance de cellules cibles. Le gène *P. vivax* *reticulocyte binding surface antigen* (*pvrbsa*) a également été identifié comme un gène potentiellement impliqué dans la reconnaissance des réticulocytes, bien qu'il présente moins de diversité génétique que les autres gènes de surface des mérozoïtes de *P. vivax* [63].

2.4. Le rôle potentiel des gènes *pvmsp* dans l'attachement primaire

La famille de gènes *P. vivax* *merozoïte surface protein* (*pvmsp*) code pour de multiples déterminants antigéniques qui sont suspectés être les médiateurs de l'attachement primaire entre les mérozoïtes de *P. vivax* et les réticulocytes humains [11]. Ces gènes *pvmsp* sont hautement polymorphes. En particulier, *pvmsp1* [43], *pvmsp3* [43, 78] et *pvmsp7* [46] présentent de nombreux SNPs non synonymes indiquant qu'ils peuvent être soumis à une pression immunitaire sélective [51]. L'analyse des isolats de

P. vivax d'Éthiopie a révélé que *pvmsp3* présentait des CNVs allant d'une à quatre copies. Par ailleurs, il est intéressant de noter que les expansions de *pvdbp* et *pvmsp3* ont souvent été observées en tandem. La pertinence fonctionnelle de cette expansion du nombre de copies n'est pas encore connue.

2.5. Les autres gènes et leur rôle potentiel dans la voie d'invasion de *P. vivax*

D'autres gènes de *P. vivax* sont suspectés d'être impliqués dans le processus d'invasion. Par exemple, la famille des gènes *P. vivax* tryptophan rich antigen (*pvtrag*) coderait pour trente-six protéines qui peuvent se lier aux érythrocytes humains [79]. L'analyse génomique de souches de *P. vivax* en Éthiopie a révélé que la famille de gènes *pvtrag* ne semble porter que quelques polymorphismes génétiques [51]. De même, le gène *P. vivax* *glycosylphosphatidylinositol-anchored micronemal antigen* (*pvgama*) code pour l'antigène micronémique ancré dans le glycosylphosphatidylinositol de *P. vivax* (*PvGAMA*) qui se lie aux érythrocytes indépendamment du statut Duffy [43]. Cette protéine peut jouer un rôle dans l'adhésion des mérozoïtes de *P. vivax* à leurs cellules cibles [49]. Enfin, la famille de gènes *P. vivax* *rhostry neck protein* (*pvrn*) code pour les protéines *P. vivax* *Rhostry Neck* (*PvRON*) qui pourraient participer à la formation de la jonction serrée comme cela a été démontré pour les homologues de *P. falciparum* [67].

Les technologies génomiques ont clairement contribué à affiner la recherche sur le paludisme à *P. vivax* en réduisant le coût du séquençage ainsi qu'en améliorant la qualité et la disponibilité des séquences. Cependant, seules quelques centaines de génomes de *P. vivax* ont été séquencés jusqu'à présent. Les isolats de terrain et cliniques présentent une faible parasitémie et une forte contamination par l'ADN humain, ce qui rend toujours difficile l'acquisition d'une quantité suffisante d'ADN parasitaire de bonne qualité. Pourtant, les séquences génomiques disponibles à ce jour ont permis l'identification de quelques gènes potentiellement liés à l'invasion. Les polymorphismes de nucléotides simples (SNPs) et les variations du nombre de copies (CNVs) trouvés dans ces gènes peuvent conduire à des différences structurelles et entraîner des pressions sélectives. Cependant, les conséquences fonctionnelles de ces variations restent inconnues.

L'analyse des génomes de *P. vivax* devrait être combinée à l'identification de leurs profils transcriptomiques et à la caractérisation de leurs protéines putatives afin de détecter des signatures spécifiques, susceptibles de fournir des informations sur leur fonction. Mais l'absence de méthodes robustes permettant de cultiver *in vitro* *P. vivax* rend l'analyse fonctionnelle difficile.

3. Transcription des gènes d'invasion de *P. vivax*

Les progrès constants du séquençage à haut débit associés à la capacité récente de maturer des isolats de *P. vivax* par culture *ex vivo* pendant un cycle ont ouvert la voie aux études transcriptomiques. Le profil transcriptomique des stades sanguins de *P. vivax* via l'utilisation de microarrays et d'un séquençage ARN (à l'échelle de la population ou de l'individu) dévoile progressivement de nouvelles capacités expérimentales et permet d'élucider la transcription, la fonction et la régulation des gènes liés à l'invasion.

3.1. Expression spécifique des gènes liés à l'invasion

La génération de transcriptomes de haute qualité à travers l'utilisation de multiples isolats de *P. vivax* est essentielle pour explorer les changements dans l'expression des gènes tout au long du cycle intra-érythrocytaire. Récemment, les profils transcriptomiques d'isolats de *P. vivax* d'infections sanguines provenant de singes *Aotus* et *Saimiri* ont révélé que la plupart des gènes de *P. vivax* avaient une expression spécifique à un stade parasitaire et étaient fortement surexprimés, contrairement aux gènes domestiques [5]. Étant donné que la plupart des infections cliniques étaient asynchrones, l'analyse des transcriptomes de *P. vivax* a permis d'identifier des populations distinctes. Les anneaux et les trophozoïtes précoces exprimaient principalement des gènes impliqués dans la transcription et la traduction, tandis que les trophozoïtes tardifs et les schizontes exprimaient des gènes potentiellement liés à l'invasion [5]. Par exemple, les transcrits *pvebp* et *pvrpb1* ont été trouvés fortement exprimés pendant le stade schizonte de *P. vivax* [55, 56]. De même, les transcrits *pvmisp3* ont été détectés dans les schizontes très tardifs [5] et le transcrit *pvmisp7* a généralement atteint

un pic pendant la transition trophozoïte tardif vers schizonte, lorsque les mérozoïtes sont formés [47]. Dans la souche de *P. vivax* adaptée au modèle primate (VCG-1), les transcrits *pvrpn5* ont été trouvés dans les schizontes [66]. De plus, des expériences de séquençage ARN menées avec des isolats de *P. vivax* cultivés *ex vivo* provenant du Brésil ont montré une grande variation dans l'expression des gènes suspectés d'être impliqués dans l'interaction hôte-parasite, la sélectivité des réticulocytes ou l'invasion. En particulier, *pvmisp1*, *pvmisp9*, *P. vivax merozoite surface antigen 180* (*pvmisa180*), *P. vivax rhoptry-associated membrane antigen* (*pvrma*) et *P. vivax rhoptry H3* (*pvrhoph3*) étaient les plus exprimés parmi tous les gènes [44]. L'expression des gènes liés à l'invasion dans les schizontes tardifs implique l'importance du développement des mérozoïtes et de l'invasion des réticulocytes pour la survie de *P. vivax*.

3.2. Expression sélective des gènes liés à l'invasion de *P. vivax*

Le génome actuel de *P. vivax* contient de nombreuses familles de gènes qui ont été définies sur la base de l'homologie de séquence et de la conservation des domaines d'acides aminés. Les analyses de séquençage RNA à l'échelle de la cellule (single cell) ont démontré que l'expression sélective des gènes peut être due à leur rôle spécifique ou à des fonctions supplémentaires. Alors que la plupart des gènes *pvmisp* sont exprimés au cours des stades de développement tardifs, on a constaté que *pvmisp3* était exclusivement exprimé dans les schizontes très tardifs, ce qui suggère son rôle dans le processus d'invasion. Étonnamment, un gène similaire à *pvmisp7* s'est avéré être exprimé exclusivement dans les gamétocytes, ce qui implique une différence fonctionnelle [5]. Des résultats comparables ont été rapportés en utilisant des isolats cliniques de *P. vivax* provenant de Thaïlande. Alors que l'expression de la plupart des gènes *pvmisp7* était associée aux schizontes, les gènes *pvmisp7a* et *pvmisp7f* ont maintenu des niveaux d'expression élevés pendant tout le cycle intra-érythrocytaire. Ces résultats suggèrent que ces paralogues peuvent avoir des fonctions supplémentaires en plus de leur rôle établi à la surface des mérozoïtes [47].

De même, des niveaux d'expression différents ont été observés parmi les membres de la famille

de gènes *pvtrag*, mais leur signification est inconnue [5]. Malgré leur homologie de séquence, les membres d'une famille de gènes peuvent également avoir des fonctions différentes à des moments spécifiques [5]. Enfin, un séquençage du transcriptome sans amplification et directionnel (*DAFT*) de quatre isolats de *P. vivax* du Cambodge a révélé que la transcription peut être un processus sélectif dans lequel les parasites sélectionneraient des membres particuliers d'une famille de gènes en réponse à l'hôte [80]. Les auteurs ont montré que les gènes dont l'expression variait le plus entre les isolats étaient physiquement regroupés le long de régions chromosomiques. Par exemple, le locus *pvmsp3* présente de nombreux paralogues qui étaient susceptibles d'être fonctionnellement redondants, augmentant ainsi la diversité antigénique et améliorant l'évasion immunitaire pendant le processus d'invasion. Au sein des clusters, l'expression des gènes semble se produire comme si les parasites choisissaient la version du gène à exprimer chez un hôte donné [80]. Globalement, ces résultats reflètent une régulation du développement et une différenciation fonctionnelle au sein de familles de gènes spécifiques.

3.3. Amplification des gènes liés à l'invasion de *P. vivax*

Chez les espèces de *Plasmodium*, l'amplification des gènes peut constituer un moyen simple d'augmenter le niveau d'expression des gènes et de modifier ainsi les phénotypes d'invasion. Des études génomiques antérieures ont démontré que certaines souches de *P. vivax* présentaient une amplification des gènes liés à l'invasion. Notamment, le gène *pvdhp* est très variable dans le nombre de copies du gène. Par exemple, le profil transcriptomique de schizontes d'isolats cambodgiens de *P. vivax* a révélé que l'amplification du gène *pvdhp* était associée à une augmentation de l'expression d'ARN messager (ARNm), ce qui suggère une augmentation potentielle du niveau de la protéine PvDBP [54]. Étant donné que la protéine PvDBP joue un rôle crucial dans l'invasion des réticulocytes Duffy-positifs, on a émis l'hypothèse que les parasites capables d'infecter les réticulocytes Duffy-négatifs ont pu être sélectionnés pour leur capacité à utiliser des copies multiples de *pvdhp* afin de se lier à un récepteur alternatif [69]. Cependant, des souches de *P. vivax* avec des copies

multiples de *pvdhp* ont également été couramment détectées dans des régions où la Duffy-négativité est rare. Par ailleurs, même si le gène *pvebp* présente de nombreuses similitudes avec le gène *pvdhp*, rien ne prouve que son amplification entraîne une augmentation des niveaux d'ARNm et/ou de protéines [53]. Ainsi, les conséquences fonctionnelles de l'expansion des gènes liés à l'invasion sont incertaines et nécessitent des investigations supplémentaires avec un nombre élevé d'isolats de *P. vivax*.

3.4. Un système de régulation complexe et dynamique

Les données transcriptomiques des parasites suggèrent que *P. vivax* présente une cascade transcriptionnelle étroitement régulée pendant son cycle intra-érythrocytaire [81]. Des études ont montré que les gènes co-exprimés présentaient des motifs conservés et des sites potentiels pour des protéines de liaison régulatrices [82]. Par exemple, de longues régions transcrites non traduites (UTR) 5' et 3' ont été observées dans des gènes suspectés être impliqués dans l'invasion et la formation de la vacuole parasitophore [80]. De plus, les variations des 5'-UTRs semblent conduire à une sélection différentielle des sites de début de transcription (TSS) reflétant la variation du niveau d'expression des gènes [83]. La présence de promoteurs bidirectionnels pour réguler l'expression de paires de gènes adjacents a également été fréquemment observée. Enfin, les modifications post-transcriptionnelles telles que la structure de la chromatine, les transcrits non codants et l'épissage alternatif peuvent jouer un rôle important dans la régulation, mais les mécanismes restent encore mal compris. Notamment, la relation spatiale présumée entre les gènes qui sont exprimés de manière variable suggère que la structure et l'accès à la chromatine peuvent être importants pour la régulation des gènes [83]. Ainsi, *P. vivax* dispose d'un réseau de régulation insaisissable et complexe qui peut régir l'expression des gènes liés à l'invasion et permettre ainsi au parasite de se développer dans les réticulocytes humains infectés.

La transcriptomique fournit des informations précieuses sur la dynamique des gènes liés à l'invasion de *P. vivax*. Il est probable que les schizontes de *P. vivax* expriment sélectivement des gènes spécifiques pour initier le processus d'invasion. Cependant, la

petite taille des échantillons, le manque de méthodes de culture *in vitro* fiables et la dynamique d'expression des gènes de ménage compliquent l'identification de nouveaux transcrits et de motifs régulateurs. À l'avenir, l'exploration des profils transcriptomiques à l'échelle cellulaire et le séquençage à longue lecture d'isolats de *P. vivax* provenant de régions géographiquement distinctes devraient être combinés à des approches protéomiques pour évaluer toute la diversité de l'expression des ligands liés à l'invasion.

4. Mécanismes cellulaires d'invasion de *P. vivax*

Les données protéomiques concernant les infections à *P. vivax* sont minimales en raison de l'accès limité au matériel biologique et au manque de système robuste permettant de cultiver *in vitro* le parasite. La liste des protéines exprimées par les mérozoïtes de *P. vivax* n'a donc toujours pas été clairement définie. Cela a généré des lacunes importantes dans la compréhension des mécanismes d'invasion de *P. vivax*. Étant donné que la structure de base des mérozoïtes est similaire chez toutes les espèces de *Plasmodium*, la plupart des chercheurs ont basé l'étude des mécanismes d'invasion de *P. vivax* sur l'hypothèse que le parasite établit les mêmes étapes malgré un tropisme cellulaire différent et des interactions récepteur-ligand différentes.

4.1. Attachement primaire

Le processus d'invasion semble être initié par l'attachement réversible des mérozoïtes de *P. vivax* aux réticulocytes. Cette fixation peut être médiée par les protéines de surface des mérozoïtes de *P. vivax* (*PvMSP*), qui peuvent former de grands complexes constitués de protéines ancrées au glycosylphosphatidylinositol et de protéines transmembranaires couvrant la surface des mérozoïtes de *P. vivax*. Sur la base de l'étude des protéines de surface des mérozoïtes de *P. falciparum* (*PfMSP*) homologues aux *PvMSPs* de *P. vivax*, on a émis l'hypothèse que la protéine *PvMSP1* pourrait reconnaître un récepteur à la surface des réticulocytes humains. La sous-unité C-terminale (MSP-142) de *PvMSP1* pourrait être clivée et la plus grande partie du complexe pourrait être éliminée, laissant seulement une petite sous-unité C-terminale (MSP-119) à la surface.

De même, son paralogue *PvMSP1P* pourrait jouer un rôle important dans l'adhésion des mérozoïtes de *P. vivax* aux réticulocytes par interaction avec sa région C-terminale [43]. De plus, on a émis l'hypothèse que la protéine *PvMSP7* pourrait être un ligand spécifique au récepteur P-sélectine qui agit comme une molécule d'adhésion cellulaire [48]. De même, la protéine *PvMSP1* et la protéine *PvMSP7* pourraient se lier ensemble pour former un complexe afin d'augmenter l'efficacité de l'invasion [47].

D'autres ligands pourraient être impliqués dans cette étape, mais leur capacité à déclencher l'attachement ou à abroger la reconnaissance par le système immunitaire de l'hôte est inconnue. Par exemple, la protéine *PvTRAg56.2* semble se localiser avec la protéine *PvMSP1* à la surface du mérozoïte et interagir avec la protéine *PvMSP7* pour jouer un rôle stabilisateur [52]. De la même façon, la protéine micronémale *PvGAMA* pourrait se lier à des récepteurs spécifiques à la surface des réticulocytes et ainsi jouer un rôle dans l'adhésion des mérozoïtes de *P. vivax* à leurs cellules hôtes cibles [65]. En effet, l'analyse protéomique de la protéine ankyrine 1 (ANK1) et de la protéine Band 3 par diverses approches biochimiques incluant la chromatographie liquide-spectrométrie de masse, a montré que le fragment 2 du domaine de liaison à la membrane de l'ANK1 et la boucle extracellulaire 5 de Band 3 se lient à la protéine *PfGAMA* et à la protéine *PvGAMA*. En outre, les anticorps anti-Band 3-P5 bloquent la liaison *PfGAMA* aux érythrocytes [50]. Par ailleurs, des études sur le terrain et en laboratoire ont montré que l'exposition à *P. vivax* induit des réponses IgG robustes à *PvGAMA* dans le cadre d'une exposition naturelle [84, 85] et que les anticorps anti-*PvGAMA* inhibent l'invasion et la croissance *in vitro* de *P. knowlesi* [86], bien que le knock-out du gène *pkgama* ait un effet minime sur la croissance de *P. knowlesi*. Ainsi, l'attachement primaire des mérozoïtes de *P. vivax* aux réticulocytes ou aux érythrocytes implique des interactions spécifiques et réversibles permettant la réorientation apicale.

4.2. Réorientation apicale

Après la fixation primaire, il se pourrait que *P. vivax* subisse une réorientation apicale qui déclenche diverses interactions ligand-récepteur. Comme les mérozoïtes de *P. vivax* ont un fort tropisme pour les

réticulocytes (qui représentent entre 0,2% et 2% de la population érythrocytaire totale), les chances de rencontrer une cellule permissive à l'invasion sont faibles. On a donc émis l'hypothèse qu'une étape de sélection de la cellule hôte se produisait pendant la réorientation apicale pour empêcher les mérozoïtes d'envahir des cellules non ciblées [87]. D'après l'étude de protéines homologues chez d'autres espèces de *Plasmodium*, on pense que la famille de protéines *PvRBP* est responsable du tropisme de *P. vivax* pour les réticulocytes. Des travaux récents ont montré que les protéines *PvRBP1* et *PvRBP2* natives peuvent fonctionner comme un complexe protéique adhésif [69] et que les protéines *PvRBP2a* et *PvRBP2b* peuvent se lier aux réticulocytes indépendamment de leur phénotype Duffy [60]. En 2015, une étude *ex vivo* a confirmé que les mérozoïtes de *P. vivax* ciblent préférentiellement les réticulocytes immatures exprimant le marqueur CD71 [61]. En 2018, une autre étude a montré que CD71 était le récepteur du ligand *PvRBP2b* [88]. Des analyses par microscopie cryo-électronique, immunoprécipitation et transfert d'énergie par résonance de fluorescence ont confirmé que la liaison de *PvRBP2b* à CD71 était spécifique et directement proportionnelle au niveau d'expression de CD71 [89]. Enfin, des études fonctionnelles ont identifié le marqueur CD98, exprimé à la surface des réticulocytes immatures, comme étant le principal récepteur du *PvRBP2a* [62]. Les deux interactions *PvRBP2b* - CD71 et *PvRBP2a* - CD98 semblent être à l'origine du tropisme strict de *P. vivax* pour les réticulocytes. Leur action pourrait être indépendante ou combinée, ce qui suggère l'existence potentielle de voies d'invasion alternatives. Outre les protéines *PvRBP*, l'adhésine antigénique de surface liant les réticulocytes de *P. vivax* (*PvRBSA*) pourrait également jouer un rôle dans la reconnaissance des réticulocytes. Une étude récente a montré que la protéine *PvRBSA* recombinante (*rPvRBSA*) se lie spécifiquement aux réticulocytes Duffy-positifs. Mais son récepteur est encore inconnu [63]. En plus de leur fonction adhésive, les protéines *PvMSPs* peuvent également contribuer à la reconnaissance des réticulocytes. En effet, des études récentes ont montré que les peptides de la protéine *PvMSP1* ont une activité de liaison plus élevée avec les réticulocytes qu'avec les érythrocytes [45]. Bien que les mécanismes ne soient pas encore clairs, la reconnaissance des réticulocytes

immatures par les mérozoïtes de *P. vivax* semble être gouvernée par *PvRBP2a* et/ou *PvRBP2b*, potentiellement en conjonction avec la protéine *PvRBSA* et les protéines *PvMSPs*. Les protéines *PvRBP*s pourraient également déclencher des étapes ultérieures de l'invasion, telles que la modulation du cytosquelette érythrocytaire, l'assemblage de la jonction serrée ou la signalisation de la sécrétion de protéines *PvRhops*.

4.3. Invasion

La réorientation apicale pourrait être suivie par la formation d'une jonction serrée et irréversible entre les mérozoïtes de *P. vivax* et les réticulocytes. Chez les individus Duffy-positifs, en l'absence de preuves, nous ne savons pas si la protéine *PvDBP* se localise à la jonction serrée entre la surface des mérozoïtes et le récepteur DARC. En effet, pour *P. falciparum*, *PfEBA175* (un ligand de liaison aux érythrocytes de *P. falciparum*) se lie à la Glycophorine A pour déclencher la libération de *PfRH5* et d'autres protéines *PfRhops*. Mais *PfEBA175* lui-même ne participe pas à la formation de la jonction. Le site de liaison de *PvDBP* est monomérique en l'absence de DARC mais se dimérise lors de l'engagement du récepteur et forme un homodimère avec le domaine extracellulaire N-terminal de DARC [60]. La formation de la jonction serrée pourrait également être facilitée par la sécrétion du ligand *P. vivax* Apical Membrane Antigen 1 (*PvAMA1*) à la surface du mérozoïte, l'injection de complexes de la protéine *PvRON* à la surface des réticulocytes et leur interaction. Il a notamment été démontré que *PvRON5* a une préférence marquée pour les réticulocytes et est donc soupçonné d'être impliqué dans le processus d'invasion [66]. Enfin, l'expression de divers membres de la famille de la protéine vivax 6-cystéine (*PvP*) à la surface du mérozoïte jouerait également un rôle dans cette étape [64].

Chez les individus Duffy-négatifs, les mécanismes et les facteurs impliqués dans la formation de la jonction serrée restent inconnus, mais plusieurs hypothèses sont actuellement discutées (Figure 3). À Madagascar, la mise en évidence d'infections à *P. vivax* chez des patients Duffy-négatifs a été corrélée à l'existence d'une duplication commune du gène *pvdhp*. Ceci a conforté l'hypothèse selon laquelle l'amplification du gène *pvdhp* pourrait favoriser la liaison de la protéine *PvDBP* à un autre récepteur de moindre affinité. Cependant, un tel récepteur alternatif n'a pas encore été identifié. De plus, les

souches de *P. vivax* présentant de multiples copies de *pvdbp* sont fréquemment trouvées dans les zones endémiques où la Duffy-négativité est rare [53]. Une deuxième hypothèse est que le ligand *PvEBP* pourrait fonctionner comme un ligand alternatif pour l'invasion des réticulocytes Duffy-négatifs. En effet, le ligand *PvEBP* semble partager des similitudes structurales avec le ligand *PvDBP*, comme un site d'attachement Duffy-binding like et un domaine C-terminal avec des positions de cystéine conservées, et probablement des fonctions similaires [55]. Sur la base de ces similarités structurales, un site de liaison au récepteur recombinant pour *PvEBP* (*rPvEBP*) a été produit. Les résultats des essais de liaison ont montré que *PvEBP* se lie préférentiellement aux réticulocytes immatures Duffy-positifs, mais une liaison minimale a été observée dans les réticulocytes immatures Duffy-négatifs. En revanche, aucune liaison n'a été observée pour les érythrocytes matures [57, 58]. Récemment, une étude fonctionnelle réalisée sur des isolats de *P. vivax* provenant d'Éthiopie a confirmé que *PvEBP* peut se lier aux réticulocytes Duffy-positifs et Duffy-négatifs, mais à une fréquence plus faible que *PvDBP*. Cette découverte indique que le domaine *PvEBP* n'a peut-être pas les mêmes capacités de liaison que le domaine *PvDBP* malgré leurs similitudes structurales [51]. Le récepteur principal de *PvEBP* étant encore inconnu, on ne sait pas si ce ligand alternatif potentiel joue un rôle dans l'invasion des réticulocytes Duffy-négatifs. Enfin, il a été récemment évoqué que les réticulocytes Duffy-négatifs peuvent exprimer transitoirement des niveaux faibles de DARC à la surface de leurs réticulocytes. Des données préliminaires suggèrent que DARC pourrait être exprimé à la surface des cellules précurseurs érythroïdes des individus Duffy-négatifs, mais cela doit encore être confirmé [90].

4.4. Invagination

Une fois que les mérozoïtes de *P. vivax* sont engagés de manière irréversible dans le processus d'invasion, ils pourraient pénétrer dans les réticulocytes via l'activité du glidosome et se sceller dans la vacuole parasitophore. Bien que ces mécanismes ne soient pas clairs, il semble que la protéine *PvTRAG36.6* et la protéine *P. vivax* Early Transcribed Membrane Protein (*PvETAMP*) soient colocalisés dans la région apicale des mérozoïtes pour former et/ou maintenir la membrane de la vacuole parasitophore [52].

Bien que les technologies protéomiques ne soient pas encore pleinement exploitées, elles semblent essentielles pour répondre à des questions concernant les mécanismes d'invasion de *P. vivax*. L'amélioration constante des techniques (électrophorèse sur gel bidimensionnelle, spectrométrie de masse, criblage à deux hybrides de levure, etc.), l'approche à haut débit, les programmes de prédiction computationnelle et les capacités de traitement des données offrent d'énormes possibilités pour étudier l'expression, l'interaction et la modification des ligands de *P. vivax*. Cependant, les données protéomiques seules semblent être insuffisantes pour établir une analyse fine du processus d'invasion et proposer des modèles testables. Elles doivent être intégrées dans une connaissance plus globale et fondamentale de la biologie de *P. vivax* [91].

5. Conclusion

Pendant longtemps, le paludisme à *P. vivax* a été considéré comme une infection bénigne. Par conséquent, la recherche sur *P. vivax* a reçu une priorité moindre de la part des chercheurs, des décideurs politiques et des agences de financement. En outre, l'accès limité au matériel biologique et le manque de systèmes robustes pour la culture *in vitro* ont entravé le développement d'études fonctionnelles.

Pour résoudre ce problème, les scientifiques ont pris en compte les données provenant de familles de protéines homologues chez d'autres espèces de *Plasmodium* et ont produit des protéines recombinantes pour étudier les ligands de *P. vivax* potentiellement impliqués dans le processus d'invasion. Mais ces méthodes n'ont pas permis d'obtenir des informations complètes sur les fonctions réelles des ligands natifs. En fait, la question cruciale pour la recherche sur le paludisme à *P. vivax* recoupe plusieurs disciplines. En effet, les technologies génomiques apportent un certain éclairage sur la diversité génétique des souches *P. vivax*. Mais elles ne sont peut-être pas suffisantes pour comprendre les conséquences fonctionnelles de ces variations. Alors que les technologies transcriptomiques sont des outils puissants pour caractériser les transcriptions hautement régulées et spécifiques à chacun des stades de développement de *P. vivax*, l'analyse des données reste limitée par l'insuffisance d'échantillons et la difficulté d'étudier les gènes de ménage à expression constante.

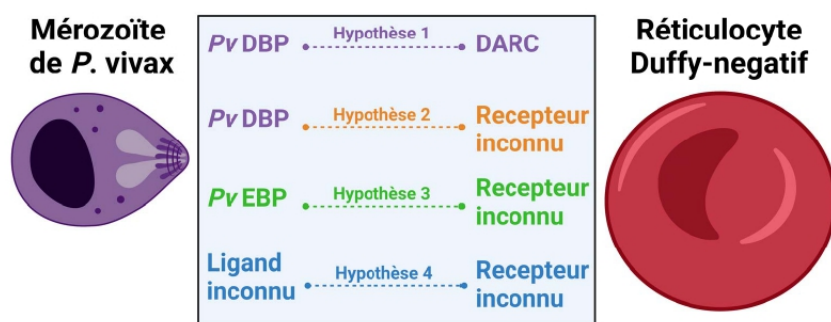


FIGURE 3. Mécanismes hypothétiques d'invasion de *P. vivax* dans les réticulocytes Duffy-négatifs. Hypothèse 1 : Les mérozoïtes de *P. vivax* envahissent les réticulocytes Duffy-négatifs par l'interaction entre le ligand *PvDBP* et le récepteur DARC, ce qui implique que les réticulocytes Duffy-négatifs peuvent exprimer DARC à un niveau faible et/ou transitoire. Hypothèse 2 : Les mérozoïtes de *P. vivax* envahissent les réticulocytes Duffy-négatifs par l'interaction entre le ligand *PvDBP* et un récepteur inconnu exprimé à la surface des réticulocytes Duffy-négatifs. Hypothèse 3 : Les mérozoïtes de *P. vivax* envahissent les réticulocytes Duffy-négatifs par l'interaction entre le ligand *PvEBP* et un récepteur inconnu exprimé à la surface des réticulocytes Duffy-négatifs. Hypothèse 4 : Les mérozoïtes de *P. vivax* envahissent les réticulocytes Duffy-négatifs par l'interaction entre un ligand inconnu exprimé à la surface des mérozoïtes de *P. vivax* et un récepteur inconnu exprimé à la surface des réticulocytes Duffy-négatifs. Figure créée avec BioRender.com.

Cependant, de récentes applications des technologies dites « single-cell » ont permis de produire des profils transcriptomiques à la fois de parasites au stade hépatique et des cellules hôtes pour explorer les interactions hôte-parasite [92]. Il est donc possible que cette technologie soit appliquée à l'étude des facteurs de l'hôte dans la voie d'invasion des réticulocytes humains. En plus des études omiques, divers modèles *in vivo* ont été utilisés pour étudier les voies d'invasion de *P. vivax*. Les modèles de primates non humains (PNH) ont été largement utilisés dans le passé, malgré leurs limites inhérentes telles que les problèmes éthiques et les variations de la sensibilité de l'hôte à l'infection par des isolats de terrain de *P. vivax* nécessitant une adaptation avant la réplique, ce qui modifie probablement les caractéristiques biologiques des souches adaptées [55]. Plus récemment, des efforts ont été entrepris pour développer des systèmes de souris humanisées comme modèles pour reproduire *in vivo* les infections à *P. vivax* [93]. Bien qu'encourageants, ces systèmes très fragiles et coûteux n'en sont qu'à leurs débuts et doivent être perfectionnés pour permettre le maintien continu d'une infection à *P. vivax* asexuée au

stade sanguin et le développement de parasites *P. vivax* génétiquement modifiés [94].

Dans ce contexte, les technologies protéomiques se concentrent sur le décryptage des diverses interactions entre les ligands impliqués dans le processus d'invasion de *P. vivax* et leurs récepteurs spécifiques. Néanmoins, elles ne parviennent pas à fournir une vision générale des mécanismes d'invasion de *P. vivax*, notamment en expliquant comment le parasite est capable d'envahir les réticulocytes Duffy-négatifs. Le développement d'études multi-omiques intégrées et complémentaires ainsi que les études d'invasion *in vitro* devraient nous permettre de mieux comprendre la biologie de ce parasite et soutenir le développement de stratégies de prévention, de contrôle et d'éradication dans le monde entier.

Conflit d'intérêt

Les auteurs déclarent ne pas avoir de conflit d'intérêt.

Remerciements

Cette revue a été soutenue par une subvention de l'Agence Nationale de la Recherche (VIPeRS,

ANR-18-CE15-0026), par l'Institut Pasteur, Paris, le Laboratoire d'Excellence (LabEx) « Alliance Française de Parasitologie pour la Santé » (ANR-11-15 LABX-0024-PARAFRAP) et l'Université de Strasbourg à travers le Programme IdEX 2022 (Attractivité — Dotation d'accueil des professeurs d'université recrutés en 2021).

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II. Molecular epidemiological study on *P. vivax* invasion pathways

The second objective of my thesis project consisted in investigating the natural variations of the genome of *P. vivax* strains circulating in Africa. For this purpose, we collected almost 300 *P. vivax* field samples within 2 years thanks to our collaborations with the French National Reference Center for Malaria, University of Addis-Ababa, University of Nouakchott Al Aasriya, Oswaldo Cruz Foundation, and Institut Pasteur Madagascar. Then, we processed and sequenced them with the help of the Biomics platform in Institut Pasteur Paris. Our first genomic analysis compared all African *P. vivax* isolates to South American and Asian ones. Our second and third molecular epidemiological studies focused on Ethiopian *P. vivax* isolates.

1. Genomic analysis of *P. vivax* strains circulating in Africa

Despite many advances in genomic technologies, only a few *P. vivax* strains circulating in Africa have been sequenced, let alone annotated. This is due to the fact that the collection of field samples is difficult, time demanding and expensive⁸⁸. Besides, the characteristic low parasitemia found in clinical and field samples hampers the acquisition of high-quality sequence data⁸⁸. Yet, describing the genetic structure and diversity of *P. vivax* field isolates is an important step towards understanding dynamics of *P. vivax* infection in Duffy-negative individuals and patterns of transmission in Sub-Saharan Africa. We sequenced the genome of 133 *P. vivax* field isolates from 10 African countries (Angola, Burundi, Comoros, Djibouti, Egypt, Eritrea, Ethiopia, Madagascar, Mauritania, and Sudan) using selective whole genome amplification (sWGA) and whole genome sequencing (WGS). Among them, 102 *P. vivax* field isolates were obtained from individuals living in endemic zones and 26 *P. vivax* field isolates were obtained from travelers coming back from Africa. Then, we enriched our data set with published sequences of 204 *P. vivax* field isolates from 3 African countries (Ethiopia, Madagascar Mauritania), 3 Asian countries (Cambodia, Thailand, Vietnam), 3 Pacific coral triangle countries (Indonesia, Malaysia, Papua New Guinea), and 2 South American countries (Brazil, Columbia) for a total data set comprising 337 samples from 18 different countries. Finally, we investigated the genetic structure and diversity of *P. vivax* populations, with respect to the global distribution of Duffy-negativity. Preliminary results identified four distinct geographic clusters (Eastern Africa, Indian Ocean, Latin America, and Asia/Pacific coral triangle) characterized with low levels of admixture. Notably, they revealed the existence of contrasts between Eastern Africa and Indian Ocean clusters. However, they did not show any

association between genetic diversity and adaptation to Duffy-negative human hosts in the genes encoding proteins involved in *P. vivax* invasion pathways. Overall, this study suggests that *P. vivax* did not evolve towards an alternative invasion pathway. It also highlights the breadth of approaches that may be required to decipher *P. vivax* invasion mechanisms. The draft of a scientific article is being written and is presented below.

Genomic analysis of *Plasmodium vivax* field isolates circulating in Africa

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Abstract

Vivax malaria is a major public health issue in south America, southeast Asia, Middle East, Pacific coral triangle, eastern Africa, and southern Africa but it is considered rare in sub-Saharan Africa. However, recent studies have reported a growing number of *P. vivax* infection cases in Duffy-negative patients across Africa, raising the concern that the parasite has evolved an alternative invasion pathway to overcome Duffy-negativity.

Here, we investigated the global genetic structure and diversity of *P. vivax* populations with respect to the global distribution of Duffy-negativity at broad a geographical scale and we attempted to identify molecular signatures of adaptation to Duffy-negative hosts. We sequenced the genome of 133 *P. vivax* field isolates from 10 African countries (Angola, Burundi, Comoros, Djibouti, Egypt, Eritrea, Ethiopia, Madagascar, Mauritania, and Sudan) using selective whole genome amplification and whole genome sequencing. We enriched our data set with published sequences of 204 *P. vivax* field isolates from 3 African countries (Ethiopia, Madagascar Mauritania), 3 southeast Asian countries (Cambodia, Thailand, Vietnam), 3 countries in the Pacific coral triangle (Indonesia, Malaysia, Papua New Guinea) and 2 south American countries (Brazil, Columbia) for a total of 337 samples from 18 different countries. Four distinct geographical clusters (eastern Africa, Indian Ocean, south America, and southeast Asia/Pacific coral triangle) as well as clear contrasts between Eastern African and Indian Ocean clusters were identified. The analysis of genotypic data did not reveal any associations between genetic diversity and adaptation to Duffy-negative hosts in invasion-related genes.

Background

Vivax malaria is a major public health issue in south America, southeast Asia, Middle East, western Pacific, eastern Africa and southern Africa¹. Historically, it was thought that *vivax* malaria was rare or absent in Sub-Saharan Africa due to the predominance of Duffy-negative human populations¹ and it was established that *P. vivax* merozoites exclusively invade Duffy-positive erythrocytes through the interaction between the receptor Duffy Antigen Receptor for Chemokines (DARC) and the ligand *P. vivax* Duffy Binding Protein (*PvDBP*)². Through the years, this paradigm has been supported by experimental infections demonstrating the natural resistance of Duffy-negative individuals to *vivax* infections and cases of microscopically confirmed *vivax* malaria in regions where Duffy-negativity was either rare, less frequent or present but admixed with Duffy-positive populations³. However, recent studies have reported a growing number of *P. vivax* infection cases in Duffy-negative patients across Africa⁴⁻¹⁴. Although such observations are still scarce, they indicate that *P. vivax* transmission may occur in sub-Saharan Africa and raise the concern that the parasite has evolved an alternative invasion pathway to overcome Duffy-negativity¹⁵.

Despite recent advances in genomic technologies, analysis of *P. vivax* field isolates remains technically challenging due to low parasitemia in clinical samples hampering the acquisition of high-quality sequence data¹⁶. Consequently, molecular mechanisms of *P. vivax* invasion into reticulocytes remain poorly understood. Previous whole-genome sequencing studies of monkey-adapted *P. vivax* strains and field isolates revealed duplications of the *pvdbp* gene in several *P. vivax* isolates from Madagascar¹⁸, a setting where Duffy-positive and Duffy-negative individuals coexist, but also from Southeast Asia, where Duffy-negativity is virtually absent¹⁷. Additional genomic study, using long reads without relying on the reference genome, identified a homologue of *PvDBP*: the *P. vivax* erythrocyte binding protein (*PvEBP*). But to date, its role in a DARC-independent invasion pathway is not clearly defined. Indeed, *PvEBP* was shown to preferentially bind to immature (CD71^{high}) and Duffy-positive reticulocytes¹⁷ while minimal binding was observed with Duffy-negative reticulocytes. No binding was observed with mature red blood cells or normocytes. Lastly, recent *in vitro* studies demonstrated that the ligands *P. vivax* Reticulocyte Binding Protein 2b (*PvRBP2b*) and *P. vivax* Reticulocyte Binding Protein 2a (*PvRBP2a*) are crucial for reticulocyte recognition prior to invasion¹⁸⁻²⁰. However, attempts to identify a new invasion-related ligand or to determine whether genetic polymorphisms allow interaction with other erythrocyte receptors have failed.

Here, we sequenced the genome of 133 *P. vivax* field isolates from 10 African countries (Angola, Burundi, Comoros, Djibouti, Egypt, Eritrea, Ethiopia, Madagascar, Mauritania, and

Sudan) using selective whole genome amplification and whole genome sequencing. Then, we enriched our dataset with published sequences of 204 *P. vivax* field isolates from 3 African countries (Ethiopia, Madagascar Mauritania), 3 southeast Asian countries (Cambodia, Thailand, Vietnam), 3 countries in the Pacific coral triangle (Indonesia, Malaysia, Papua New Guinea) and 2 south American countries (Brazil, Columbia) for a total data set comprising 337 samples from 18 countries. Our aim was to investigate the global genetic structure and diversity of African *P. vivax* populations in relation to the global distribution of Duffy-negativity, at a broad geographical scale, and to identify potential molecular signatures of adaptation to the Duffy-negative human hosts.

Materials and Methods

Biological samples. All biological samples used in this study were obtained from *P. vivax* infected blood samples after informed consent was obtained. At each location, sample collection was approved by the appropriate local ethics committees. Dried blood spots and / or veinous blood samples were collected from symptomatic patients at health centers in Ethiopia (University of Addis Ababa), Madagascar (Institut Pasteur Madagascar), and Mauritania (University of Nouakchott Al Aasriya) and from symptomatic travelers coming back from Comoros, Djibouti, Madagascar, Mauritania, and Ethiopia (French National Reference Center for malaria). Blood samples from Madagascar were leuko-depleted using CF11-packed columns to minimize the amount of human DNA²¹. We enriched our dataset with 204 published genomic sequences of *P. vivax* strains circulating in southeast Asia (Cambodia, Thailand, Vietnam), Pacific Coral Triangle (Indonesia, Malaysia, Papua New Guinea), south America (Brazil, Columbia), and Africa (Ethiopia, Madagascar, Mauritania) (**Table 1 and Supplementary information**).

DNA extraction. Genomic DNA was extracted from 100 µl of red blood cell pellets or from 6-mm dried blood spot punch using the QIAamp mini-blood DNA kit (Qiagen), according to the supplier's instructions. Total DNA concentration was quantified using a dsDNA HS assay kit and a Qubit fluorometer (Invitrogen).

Plasmodium species screening. The *Plasmodium* species screening of *P. vivax* isolates was performed by real time PCR as previously described²² (**Table 2**).

Duffy genotyping. The Duffy genotype of patients was determined by PCR and Sanger sequencing as previously described⁵ (**Table 2**).

Selective whole genome amplification. Selective whole genome amplification (sWGA) was performed to enrich *P. vivax* DNA as previously described²³ (**Table 2**). PCR products obtained from sWGA were diluted (1:1) with DNase-free, RNase-free water (ThermoFisher Scientific) and purified with AMPure XP beads (Beckman-Coulter) according to the supplier's recommendations.

Whole genome sequencing. Whole genome sequencing was performed as previously described⁵. Sample libraries were prepared using a Truseq Nano kit (Illumina). Briefly, 100-150 ng of DNA was subject to shearing, end-repair, A-tailing, and adapter ligation. When necessary, they were enriched with 10-15 cycles of PCR. Then, sample libraries were multiplexed and loaded. Paired-end sequencing was performed with 2x151-base reads and an 8-base index read using a Nextseq kit (Illumina). Illumina sequence reads were submitted to an archive system.

Variant Calling. The Genome Analysis Toolkit (GATK) Haplotype Caller (version 4.1.7.0)²⁴ was used to identify single nucleotide polymorphisms (SNPs) in isolates following GATK best practices (**Supplementary information**).

Analysis of genotypic data. Unless specified otherwise, all data analyses were done with R 4.3.0 (R Core Team 2023). The genetic structure was studied using both multivariate analysis and clustering methods (*ade4* package), to assess whether individuals from distant origins were genetically differentiated. The genomic islands of genetic differentiation were estimated using Nei's coefficient of genetic differentiation at each SNP. Genetic imprints at invasion-related loci were then investigated on specific loci known or supposed to be involved in reticulocyte invasion pathways using the Nei's coefficient of genetic differentiation at invasion-related genes and the Tajima's *D*. Lastly, we used the following generalized linear model (GLM, with a Binomial family) to estimate the association between the Duffy-status of the host and the genotype: $\text{Duffy_status} = \text{parasite_genotype} + \text{country_of_origin}$. *P*-values were estimated for the effect bound to genotypes and were further adjusted to account for multiple testing, using a Bonferroni procedure. All details are provided in the Supplementary information (**Supplementary methods**).

Results & Discussions

Samples. We collected dried blood spots or whole blood samples from symptomatic patients at health centers in Ethiopia, Madagascar, and Mauritania. We used DNA extracts from samples obtained from symptomatic travelers coming back from Comoros, Djibouti, Madagascar, Mauritania, and Ethiopia. We amplified *P. vivax* DNA by selective whole genome amplification and successfully sequenced 72/133 *P. vivax* genomes with high throughput whole sequencing technologies. The successful sequences were obtained from 18/72 homozygous and 52/72 heterozygous Duffy-positive patients and 2/72 undetermined Duffy status. Unfortunately, *P. vivax* genome sequences from the 18 homozygous Duffy-negative patients could not be properly exploited due to too low parasitemia³. This data set was further enriched with 204 published genomic sequences of *P. vivax* strains circulating in southeast Asia (Cambodia, Cambodia, Thailand, Vietnam), Pacific coral triangle (Indonesia, Malaysia, Papua New Guinea), south America (Brazil, Columbia), and Africa (Ethiopia, Madagascar, Mauritania). All details are provided in the **Table 1** and in the Supplementary information (**Table S2**).

Quality control. We aligned sequencing reads against the PvP01 (v. 48) reference genome and used the GATK 4.0 tools to identify SNPs. We then applied a series of quality control filters to exclude variants of poor quality, samples with low coverage, and SNPs with a high risk of genotype errors. The final data set contained > 300,000 SNPs called in 276 samples. While *P. vivax* infections are often characterized by the presence of multiple *P. vivax* clones in Asia²⁵, we found only a few samples presenting polyclonal infections in Africa. Several factors could explain this observation, including low endemic transmission across the African continent, low exposure of African populations, or potential competition with *P. falciparum*²⁸.

Global genetic diversity. The global genetic diversity and structure of *P. vivax* populations is poorly understood. While genomic studies have already provided evidence of geographic genetic structure in southeast Asia²⁶ and south America²⁷, there is little information on *P. vivax* populations circulating in Africa with the exception of southern Ethiopia²⁸. A study based on a limited number of highly polymorphic markers²⁹ suggested moderate levels of genetic differentiation between Africa and Asia, and ambiguous admixture profiles. In line with such findings, we expected *P. vivax* populations circulating in Africa to be distinct from those circulating in Asia or south America. However, the structure analysis revealed clear genetic clusters (**Figure 1A**), characterized by low levels of admixture overall. Consistent with this, a

recent study suggested some degree of relatedness only between *P. vivax* originating from east African countries and those from Middle Eastern countries, while no admixture could be detected with parasites from southeast Asia. Isolates from south America appeared to be admixed, although the sample size was considered too small to reliably interpret their associated clustering results. The results obtained with a PCO analysis were highly congruent and four geographical groups were defined including an East African cluster (Ethiopia, Djibouti, and Mauritania), an Indian Ocean cluster (Madagascar, Comoros), an Asian/Pacific coral triangle cluster (Cambodia, Indonesia, Malaysia, Papua New Guinea, Thailand, and Vietnam), and a south American cluster (Brazil, Columbia) (**Figure 1B**). Globally, the distinction between African, Asian, and south American clusters was consistent with previous observations of independent East African, South Asian, Southeast Asian, Southern Southeast Asian and South American *P. vivax* populations in SNP-based neighbour-joining tree and admixture ancestral analysis³⁰. In addition, we observed a clear distinction between two African subpopulations with the East African cluster (Ethiopia, Djibouti, and Mauritania) and the Indian Ocean cluster (Madagascar, Comoros) (**Figure 1B**). The Indian Ocean cluster was closer to the East African cluster than to the Asian cluster. This observation does not seem to be consistent with the assumption of a unique Indonesian origin of the Malagasy *P. vivax* population³¹ (**Figure 1A, Figure 1B**). It is likely that the population genetic structure of *P. vivax* primarily reflects past human migrations^{5,32}. An important hypothesis that motivated this study is that natural selection acting on genes involved in adaptation to human hosts, and notably invasion-related genes should result in clear genetic signatures in the corresponding genomic regions³³.

Genomic islands of genetic differentiation Patterns of genetic diversity are rarely homogeneous across the whole genome as some genomic regions may show increased levels of genetic differentiation. Such heterogeneity is common and is likely to result from a combination of microevolutionary processes, particularly those associated with local adaptation, such as linkage and background selection³⁴. The absence of Duffy antigens in the human host may exert strong selective pressure and favor parasites that can invade reticulocytes by alternative invasion pathways. In this study, we investigated whether genomic intervals containing genes involved in the invasion process held genetic imprints of selection and could be associated with any genomic islands of genetic differentiation. We performed a genome-wide analysis of genetic differentiation in *P. vivax*. Clear peaks of differentiation were observed in eight genomic regions on chromosomes 4, 5, 7, 10, 11, 12, 13 and 14 (**Figure 1C**). However, these regions did not appear to be linked with genes encoding parasite ligands involved in

reticulocyte invasion pathways (**Supplementary information**), with the exception of the thrombospondin-related anonymous protein gene (*PVP01_1218700*). However, this protein is expressed by *P. vivax* sporozoites and has been shown to be critical for invasion of human hepatocytes^{35,36}. Taken together, these results indicate that the global structure of *P. vivax* populations is characterized by consistent patterns across the genome (**Supplementary Information**), while some smaller genomic regions show a stronger signal. Among the genes falling into the genomic islands we identified, there were loci associated with genes involved in metabolic pathways, cellular component, or unknown function. It is noteworthy that we detected 3 genes previously associated with antimalarial drug resistance (*mdr-1*, *PVP01_1010900*; *dhfr*, *PVP01_0526600* and *dhps*, *PVP01_1429500*) which probably reflects the existence of different drug pressures worldwide³⁰.

Genetic diversity at invasion-related genes Even though we could not find invasion genes among the genomic regions with the higher levels of genetic structure genome-wide, we restricted our analysis to regions containing these genes of interest³⁰, in particular *pvdbp*, *pvebp*, *pvrpb2a*, and *pvrpb2b* genes (**Table S3**). We therefore performed an analysis of genetic differentiation and estimated Tajima's *D* ratio at invasion-related loci and compared them with values obtained with each individual gene in the genome. The analysis of genetic differentiation shows that invasion-related genes from all *P. vivax* populations fall well within the boundaries defined by all the other genes (**Figure 2A**). Similarly, the analysis of Tajima's *D* within 13 countries shows that invasion-related genes does not appear to differ from any other gene in the genome. Again, we found no evidence of selective pressure acting on invasion-related genes in *P. vivax* (**Figure 2B, Figure 2C**).

Genetic diversity associated with the Duffy genotype of human hosts. The common human Duffy alleles show striking patterns of geographic differentiation³⁷. Assuming the existence of genetic loci controlling the ability of *P. vivax* parasites to invade a Duffy-negative host, one might expect a statistical association between the human Duffy status and the parasite genotype at the relevant locus. Here, we hypothesized that the Duffy allele of patients might be associated with highly diversified invasion-related genes in *P. vivax* African genomes. To test this hypothesis, we performed a GWAS analysis on sequences obtained from 18 *P. vivax* African isolates infecting homozygous Duffy-positive patients and 52 *P. vivax* African isolates infecting heterozygous Duffy-positive patients. Note that, it was not possible to perform this analysis on sequences obtained from African *P. vivax* isolates infecting homozygous Duffy-negative

patients as the parasitemia was too low to achieve good quality of parasite genome sequencing. We were unable to identify an association between patient Duffy allele and parasite genotype at invasion-related genes (**Figure 3**). There was no evidence for a likely molecular signature of adaptation to the Duffy-negative human hosts. Although the existence of an alternative invasion-related ligand binding to DARC or to another unknown reticulocyte receptor may be a plausible hypothesis to explain genetic diversity between Asian and African *P. vivax* populations, it is unlikely to explain *P. vivax* invasion pathways in Duffy-negative patients^{5,12,38,39}. Furthermore, *P. vivax* infections in Duffy-negative patients always show low parasitemia, suggesting that whatever mechanism is involved in invading Duffy-negative reticulocytes, it is less efficient than the PvDBP-DARC pathway used to invade Duffy-positive reticulocytes⁴⁰ (**Figure 3**). Overall, this suggests that the evolution of African *P. vivax* populations may not depend on invasion-related genes or may depend on them in ways too complex to be detected by this analysis.

Conclusion

This comparative genomic analysis provides insights into the genetic structure and diversity of African *P. vivax* populations. The comparison of 337 samples from 18 countries around the world revealed four distinct geographical clusters (East Africa, Indian Ocean, Asia/Pacific coral triangle, and South America) and contrasts between the East African and Indian Ocean clusters. However, we did not find any association between genetic diversity and adaptation to Duffy-negative human hosts in invasion-related genes.

Figures

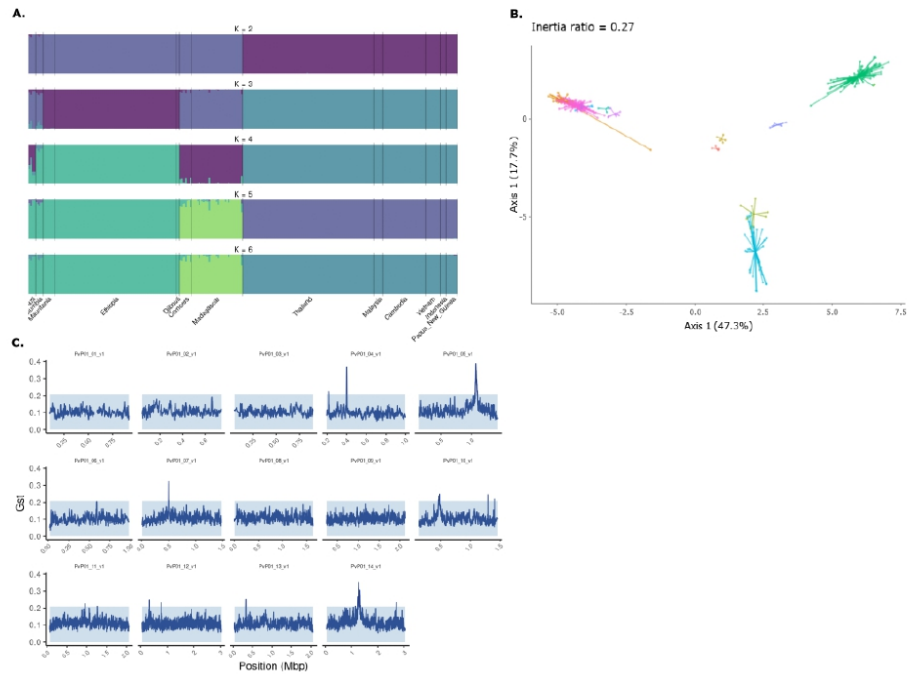


Figure 1: Global genetic diversity of *P. vivax* populations. A. Structure analysis on all chromosomes. **B.** Principal Coordinate analysis (PCO). **C.** Genome-wide genetic differentiation with genomic islands

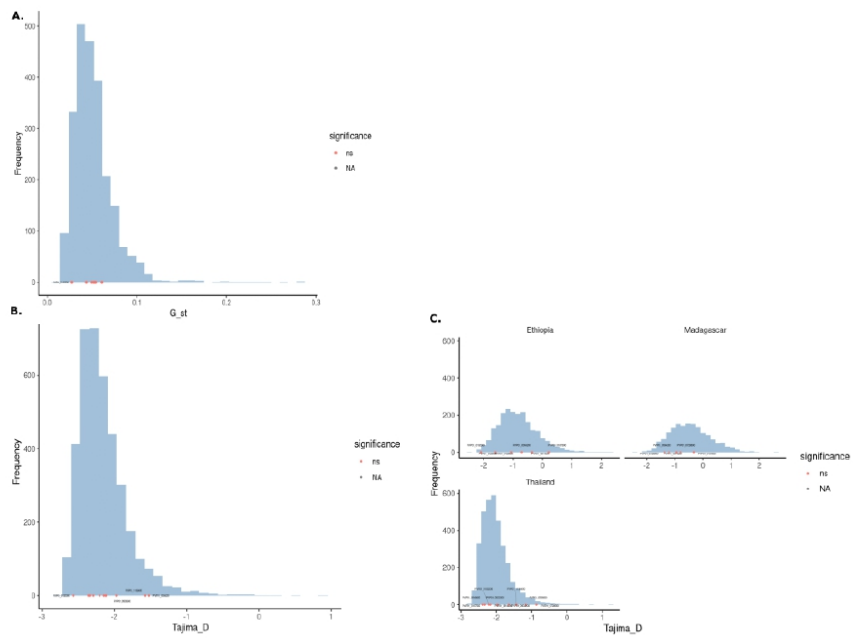


Figure 2: Genetic diversity at invasion-related genes. A. Genetic differentiation of invasion-related genes of all *P. vivax* populations. B. Analysis of Tajima's D within all countries. C. Analysis of Tajima's D within Ethiopia, Madagascar, and Thailand.

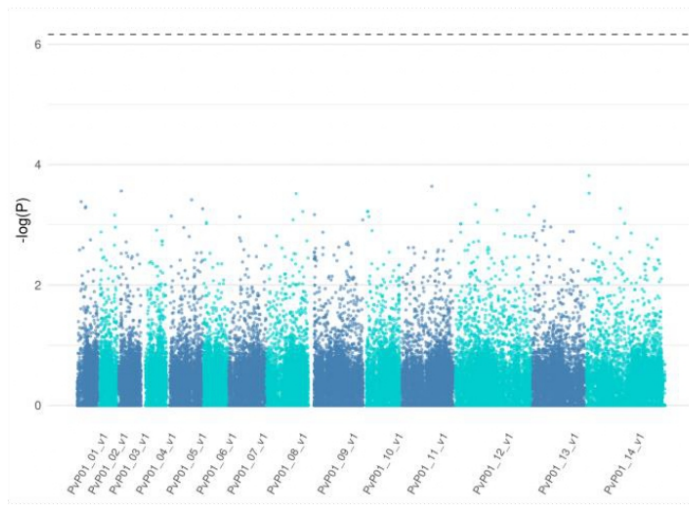


Figure 3: GWAS analysis. GWAS analysis on sequences obtained from 18 *P. vivax* African isolates infecting homozygous Duffy-positive patients and 52 *P. vivax* African isolates infecting heterozygous Duffy-positive patients of invasion-related genes.

Tables

Table 1: List of *P. vivax* field isolates sequenced for this study

Country	Sample	Duffy genotype of patient	Sequence
Angola	ANG71	Duffy-negative (homozygous)	Not interpretable
Angola	ANG83	Duffy-negative (homozygous)	Not interpretable
Angola	ANG168	Duffy-negative (homozygous)	Not interpretable
Angola	ANG172	Duffy-negative (homozygous)	Not interpretable
Angola	ANG189	Duffy-negative (homozygous)	Not interpretable
Angola	ANG192	Duffy-negative (homozygous)	Not interpretable
Angola	ANG193	Duffy-negative (homozygous)	Not interpretable
Angola	ANG201	Duffy-negative (homozygous)	Not interpretable
Angola	ANG207	Duffy-negative (homozygous)	Not interpretable
Angola	ANG209	Duffy-negative (homozygous)	Not interpretable
Angola	ANG210	Duffy-negative (homozygous)	Not interpretable
Angola	ANG212	Duffy-negative (homozygous)	Not interpretable
Burundi	1803016292	Duffy-positive (homozygous)	Not interpretable
Comoros	711035003	Duffy-positive (homozygous)	Interpretable
Comoros	10157	Duffy-positive (homozygous)	Interpretable
Comoros	11315	Duffy-positive (heterozygous)	Interpretable
Comoros	11729	Duffy-positive (heterozygous)	Interpretable
Comoros	8330	Duffy-positive (heterozygous)	Interpretable
Comoros	10555	Duffy-positive (heterozygous)	Not interpretable
Comoros	11812	Duffy-positive (heterozygous)	Not interpretable
Comoros	11994	Duffy-positive (heterozygous)	Not interpretable
Djibouti	1609055605	Duffy-positive (homozygous)	Not interpretable
Djibouti	1812066011	Duffy-positive (homozygous)	Not interpretable
Djibouti	1807003070	Duffy-positive (homozygous)	Interpretable
Egypt	1408007924	Duffy-positive (heterozygous)	Not interpretable
Ethiopia	711390488	Duffy-positive (homozygous)	Interpretable
Ethiopia	A1001	Duffy-negative (homozygous)	Not interpretable
Ethiopia	A1002	Duffy-positive (heterozygous)	Not interpretable
Ethiopia	A1004	Duffy-positive (heterozygous)	Interpretable
Ethiopia	A1005	Duffy-positive (heterozygous)	Interpretable
Ethiopia	A1006	Duffy-positive (heterozygous)	Not interpretable
Ethiopia	Ad8001	Duffy-positive (heterozygous)	Interpretable
Ethiopia	Am008	Duffy-positive (homozygous)	Not interpretable
Ethiopia	Am010	Duffy-positive (heterozygous)	Interpretable
Ethiopia	Am012	Duffy-positive (heterozygous)	Not interpretable
Ethiopia	Aw004	Duffy-positive (heterozygous)	Not interpretable

Ethiopia	Aw0005	Duffy-positive (heterozygous)	Interpretable
Ethiopia	Aw0007	Duffy-positive (heterozygous)	Interpretable
Ethiopia	G6001	Duffy-positive (heterozygous)	Not interpretable
Ethiopia	G6003	Duffy-positive (heterozygous)	Interpretable
Ethiopia	G6005	Duffy-positive (heterozygous)	Interpretable
Ethiopia	G6006	Duffy-positive (heterozygous)	Not interpretable
Ethiopia	G6007	Duffy-positive (heterozygous)	Not interpretable
Ethiopia	H9002	Duffy-positive (heterozygous)	Interpretable
Ethiopia	H9003	Duffy-positive (heterozygous)	Not interpretable
Ethiopia	K101	Duffy-positive (heterozygous)	Interpretable
Ethiopia	K102	Duffy-positive (heterozygous)	Not interpretable
Ethiopia	K107	Duffy-negative (homozygous)	Not interpretable
Ethiopia	MC4005	Duffy-positive (heterozygous)	Interpretable
Ethiopia	MC4008	Duffy-positive (heterozygous)	Not interpretable
Ethiopia	MC4010	Duffy-positive (heterozygous)	Not interpretable
Ethiopia	MC4011	Duffy-positive (heterozygous)	Interpretable
Ethiopia	MC4012	Duffy-positive (heterozygous)	Interpretable
Ethiopia	MC4013	Duffy-positive (heterozygous)	Not interpretable
Ethiopia	MC4014	Duffy-positive (heterozygous)	Not interpretable
Ethiopia	MC4016	Duffy-positive (heterozygous)	Interpretable
Ethiopia	MC4018	Duffy-positive (heterozygous)	Not interpretable
Ethiopia	MC4019	Duffy-positive (heterozygous)	Interpretable
Ethiopia	MC4020	Duffy-positive (homozygous)	Interpretable
Ethiopia	MC4021	Duffy-positive (heterozygous)	Interpretable
Ethiopia	MC4022	Duffy-positive (heterozygous)	Interpretable
Ethiopia	MC4023	Duffy-positive (homozygous)	Interpretable
Ethiopia	MC4024	Duffy-positive (heterozygous)	Interpretable
Ethiopia	MC4027	Duffy-positive (heterozygous)	Interpretable
Ethiopia	MC4030	Duffy-positive (heterozygous)	Not interpretable
Ethiopia	MC4031	Duffy-positive (homozygous)	Interpretable
Ethiopia	MC4032	Duffy-positive (homozygous)	Interpretable
Ethiopia	MC4033	Duffy-positive (heterozygous)	Interpretable
Ethiopia	MC4034	Duffy-positive (heterozygous)	Not interpretable
Ethiopia	MC4035	Duffy-positive (homozygous)	Not interpretable
Ethiopia	MC4036	Duffy-positive (homozygous)	Not interpretable
Ethiopia	MC4037	Duffy-positive (heterozygous)	Interpretable
Ethiopia	MC4038	Duffy-positive (heterozygous)	Interpretable
Ethiopia	MC4040	Not Identified	Interpretable
Ethiopia	MC4043	Duffy-positive (homozygous)	Interpretable
Ethiopia	MC4044	Duffy-positive (heterozygous)	Not interpretable

Ethiopia	MC4046	Duffy-positive (homozygous)	Not interpretable
Ethiopia	MC4047	Duffy-positive (homozygous)	Interpretable
Ethiopia	MC4048	Duffy-positive (heterozygous)	Not interpretable
Ethiopia	MC4049	Duffy-positive (homozygous)	Interpretable
Ethiopia	MC4050	Duffy-positive (heterozygous)	Not interpretable
Ethiopia	MC4052	Duffy-positive (heterozygous)	Interpretable
Ethiopia	MC4053	Duffy-positive (homozygous)	Not interpretable
Ethiopia	MJ0016	Duffy-positive (heterozygous)	Interpretable
Ethiopia	MJ0017	Duffy-positive (heterozygous)	Interpretable
Ethiopia	MJ0018	Duffy-positive (heterozygous)	Interpretable
Ethiopia	MJ7001	Duffy-positive (heterozygous)	Interpretable
Ethiopia	MJ7003	Duffy-positive (heterozygous)	Interpretable
Ethiopia	MJ7004	Duffy-positive (heterozygous)	Interpretable
Ethiopia	MJ7006	Duffy-positive (heterozygous)	Not interpretable
Ethiopia	MT0023	Duffy-negative (homozygous)	Not interpretable
Ethiopia	W5011	Duffy-negative (homozygous)	Not interpretable
Eritrea	11302	Duffy-positive (heterozygous)	Not interpretable
Eritrea	1801078268	Duffy-positive (heterozygous)	Not interpretable
Mauritania	711M21044701	Duffy-positive (homozygous)	Interpretable
Mauritania	713285008	Duffy-positive (homozygous)	Interpretable
Mauritania	714035002	Duffy-positive (homozygous)	Interpretable
Mauritania	714145012	Duffy-positive (heterozygous)	Not interpretable
Mauritania	11034	Duffy-positive (heterozygous)	Not interpretable
Mauritania	MAU24	Duffy-negative (homozygous)	Not interpretable
Mauritania	MAUAT68	Duffy-negative (homozygous)	Not interpretable
Madagascar	MAE-CSB01-001	Duffy-positive (homozygous)	Interpretable
Madagascar	MAE-CSB01-002	Duffy-positive (heterozygous)	Interpretable
Madagascar	MAE-CSB02-001	Duffy-positive (heterozygous)	Interpretable
Madagascar	MAE-CSB02-002	Duffy-positive (heterozygous)	Interpretable
Madagascar	MAE-CSB02-003	Duffy-positive (heterozygous)	Interpretable
Madagascar	MAE-CSB03-002	Duffy-positive (heterozygous)	Interpretable
Madagascar	MAE-CSB03-003	Duffy-positive (heterozygous)	Interpretable
Madagascar	MAE-V01-001	Duffy-positive (heterozygous)	Interpretable
Madagascar	MAE-V01-002	Not identified	Interpretable
Madagascar	MDZ-V02-001	Duffy-positive (homozygous)	Not interpretable
Madagascar	MDZ-V02-006	Duffy-positive (homozygous)	Not interpretable
Madagascar	MDZ-V03-001	Duffy-positive (homozygous)	Not interpretable
Madagascar	MAE-V04-001	Duffy-positive (heterozygous)	Interpretable
Madagascar	MAE-V06-001	Duffy-positive (heterozygous)	Interpretable
Madagascar	MDZ-CSB01-001	Duffy-positive (heterozygous)	Interpretable

Madagascar	MDZ-CSB01-002	Duffy-positive (heterozygous)	Interpretable
Madagascar	MDZ-CSB01-003	Duffy-positive (heterozygous)	Interpretable
Madagascar	MDZ-CSB01-005	Duffy-positive (heterozygous)	Not interpretable
Madagascar	MDZ-V01-001	Duffy-positive (heterozygous)	Interpretable
Madagascar	MDZ-V02-001	Duffy-positive (homozygous)	Interpretable
Madagascar	MDZ-V02-002	Duffy-positive (heterozygous)	Interpretable
Madagascar	MDZ-V02-004	Duffy-positive (heterozygous)	Interpretable
Madagascar	MDZ-V02-005	Duffy-positive (heterozygous)	Interpretable
Madagascar	MDZ-V02-006	Duffy-positive (homozygous)	Interpretable
Madagascar	MDZ-V04-001	Duffy-positive (heterozygous)	Interpretable
Madagascar	MDZ-V04-002	Duffy-positive (heterozygous)	Interpretable
Madagascar	MDZ-V04-003	Duffy-positive (heterozygous)	Interpretable
Madagascar	711185003	Duffy-positive (heterozygous)	Interpretable
Madagascar	713455019	Duffy-positive (homozygous)	Interpretable
Sudan	713405019	Duffy-positive (homozygous)	Not interpretable
Sudan	713485020	Duffy-positive (homozygous)	Not interpretable
Sudan	510m15000201	Duffy-positive (heterozygous)	Not interpretable

Chromosome	Pos. start	Pos. end	ID	Description	Function
4	219558	220885	PVP01_0404900	Plasmodium exported protein, unknown function	Unknown
4	399586	401784	PVP01_0409900	acyl-CoA synthetase, putative	Metabolic pathways
5	1061019	1067947	PVP01_0526300	conserved Plasmodium protein, unknown function	Unknown
5	1070413	1071498	PVP01_0526400	conserved Plasmodium protein, unknown function	Unknown
5	1072310	1074121	PVP01_0526500	mRNA-binding protein PUF2, putative	Metabolic pathways
5	1077362	1079236	PVP01_0526600	bifunctional dihydrofolate reductase-thymidylate synthase, putative	Metabolic pathways
5	1080118	1082726	PVP01_0526700	LETM1-like protein, putative	Metabolic pathways
5	1085714	1102054	PVP01_0526800	conserved Plasmodium protein, unknown function	Unknown
7	495347	505297	PVP01_0709800	cysteine repeat modular protein 1, putative	Cellular component
10	470947	474113	PVP01_1010700	heptatricopeptide repeat-containing protein, putative	Unknown
10	475543	477329	PVP01_1010800	cytochrome b-c1 complex subunit 2, putative	Metabolic pathways
10	478739	483133	PVP01_1010900	ABC transporter B family member 1, putative	Metabolic pathways
10	1302679	1303648	PVP01_1030200	60S ribosomal protein L31, putative	Metabolic pathways
11	961821	964757	PVP01_1121700	acetyl-CoA synthetase, putative	Metabolic pathways
12	323534	324835	PVP01_1208000	6-cysteine protein P47	Cellular component
12	768961	770631	PVP01_1218700	thrombospondin-related anonymous protein, putative	Metabolic pathways
12	771355	773349	PVP01_1218800	conserved Plasmodium protein, unknown function	Unknown
13	332331	342481	PVP01_1307300	cysteine repeat modular protein 3, putative	Unknown
14	1225902	1230319	PVP01_1428700	conserved protein, unknown function	Unknown
14	1231284	1233767	PVP01_1428800	histone-arginine methyltransferase CARM1, putative	Metabolic pathways
14	1235239	1237877	PVP01_1428900	conserved protein, unknown function	Unknown
14	1242635	1248727	PVP01_1429000	CCR4-associated factor 1, putative	Metabolic pathways
14	1254473	1258020	PVP01_1429100	ER membrane protein complex subunit 1, putative	Metabolic pathways

14	1259368	1261188	PVP01_1429200	mitochondrial carrier protein, putative	Unknown	
14	1262207	1264847	PVP01_1429300	cullin-1, putative	Metabolic pathways	ubiquitin-dependent protein catabolic process
14	1267481	1268779	PVP01_1429400	conserved Plasmodium protein, unknown function	Unknown	
14	1269756	1272304	PVP01_1429500	hydroxymethylidihydropterin pyrophosphokinase-dihydropterotate synthase, putative	Metabolic pathways	Folate biosynthesis/Drug resistance
14	1273133	1274237	PVP01_1429600	conserved Plasmodium protein, unknown function	Unknown	
14	1276317	1279088	PVP01_1429700	ATP-dependent RNA helicase DBP1, putative	Metabolic pathways	nucleic acid binding
14	1284812	1287310	PVP01_1429800	protein phosphatase PPM7, putative	Metabolic pathways	protein dephosphorylation
14	1287683	1289728	PVP01_1429900	aquaporin, putative	Metabolic pathways	transmembrane transport
14	1294483	1297856	PVP01_1430000	protein phosphatase PPM5, putative	Metabolic pathways	protein dephosphorylation
14	1299004	1301880	PVP01_1430100	ABC1 family, putative	Metabolic pathways	Purine metabolism
14	1302963	1303546	PVP01_1430200	ribosomal protein L33, apicoplast, putative	Metabolic pathways	translation
14	1310045	1316657	PVP01_1430400	JmjC domain-containing protein, putative	Metabolic pathways	Biosynthesis of secondary metabolites
14	1318305	1322540	PVP01_1430500	conserved Plasmodium protein, unknown function	Unknown	
14	1323658	1326144	PVP01_1430600	RuvB-like helicase 1, putative	Metabolic pathways	Biosynthesis of various antibiotics and secondary metabolites
14	1328328	1344272	PVP01_1430700	peptidase family C50, putative	Metabolic pathways	proteolysis
14	2694842	2697905	PVP01_1462600	conserved Plasmodium protein, unknown function	Unknown	

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Genomic analysis of *Plasmodium vivax* field isolates circulating in Africa

Supplementary information

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Supplementary methods

1.1. Variant Calling

The Genome Analysis Toolkit (GATK) Haplotype Caller (version 4.1.7.0)¹ was used to identify single nucleotide polymorphisms (SNPs) in isolates following GATK best practices. Paired-end sequencing were assigned to proper read group while processing read alignment. Reads were aligned against the reference genome of *P. vivax* (PvP01 v.48, PlasmoDB) using bwa mem². Mapped reads associated with low quality scores were excluded if mapping quality mapQuality < 30, insert size > 1000 bp, or whenever they were not in proper pairs. Duplicated reads were marked and removed. Variants were screened on a per-isolate basis using a haplotype-based algorithm (HaplotypeCaller). Variants were jointly called, and genotypes were determined with GenotypeGVCFs. Sites and genotypes were further hard filtering based on classical criteria. A total of > 300,000 SNPs were used in this study.

1.2. Global genetic structure

We investigated the extent to which population genetic structure in *P. vivax* could match the global distribution patterns of Duffy-negativity in human hosts. Genetic structure was studied using both multivariate analysis and clustering methods (*ade4* package), to assess whether individuals from distant origins were genetically differentiated. Euclidean genetic distances were calculated among isolates based on genotypes and the resulting genetic distance matrix was used in a Principal COordinate Analysis (PCO). An additional multivariate rotation was made with a between-group multivariate analysis to minimize the effects of unbalanced sample sizes depending on the geographical origin. The clustering software fastSTRUCTURE³ was used both to analyze distribution of genetic clusters as well as to estimate the amount of genetic admixture among clusters.

1.3. Genomic islands of genetic differentiation

We then examined whether invasion-related genes could be related to genomic islands exhibiting higher levels of genetic differentiation genome-wide. We calculated Nei's coefficient of genetic differentiation at each SNP. Smoothed estimates of genetic differentiation were obtained locally along the genome using a rolling mean procedure (windows size of 100 SNPs). Significance of genomic islands (i.e., observed peaks of genetic differentiation) was assessed based on a permutation procedure. An iteration of the permutation scheme consisted in (i) permuting coefficient values obtained at SNP level within chromosomes; (ii) calculating

smoothed estimates with rolling means; (iii) recording the maximum value of any peaks obtained genome wide. Repeating this scheme, a large number of iterations simulated the distribution of estimates under the null hypothesis of homogeneous genetic differentiation genome wide.

1.4. Genetic imprints at invasion-related loci

We further focused on specific loci that are either known or supposed to be involved in invasions mechanisms. Our hypothesis was that, if particular subtypes adapted to Duffy-negative hosts were circulating, genomic regions involved in this adaptation would harbor genetic hallmarks of host-related selection. In particular, we expected to detect: (i) higher levels of genetic differentiation at invasion-related genes as compared to other genes as well as (ii) signs of positive selection at such loci. We calculated Nei's coefficient of genetic differentiation at invasion-related genes and compared them to coefficients obtained with other genes, genome wide. In addition, we calculated Tajima's *D* to assess whether a significant excess of nucleotide diversity could be found at such loci and whether it differed from genetic diversity observed at other genes.

1.5. Genome-wide association study

Last, we explicitly tested any associations between parasite genotypes and the Duffy-status of the human host. Our expectation was that parasites that were adapted to Duffy-negative hosts should be positively selected in such hosts, and their genotype at loci controlling invasion mechanisms should relate to the Duffy-status of their host. We used the following generalized linear model (GLM, with a Binomial family) to estimate the association between the Duffy-status of the host and the genotype: $\text{Duffy_status} = \text{Parasite_genotype} + \text{Country_of_origin}$. P-values were estimated for the effect bound to genotypes and were further adjusted to account for multiple testing, using a Bonferroni procedure.

Supplementary Tables

Table S1: Primer sequences and PCR conditions used in the study

Assay	Primer	Sequences (5'-3')	Master mix	Parameters	T°C melt peak
Duffy Genotyping	Duffy	F: AATCCAACCTCAAAACAGGA R: CCCAAATTCACAGTGA	5µl DNA template, 200nM primers (Sigma Aldrich), 1X Hot FirePol PCR mix (Solis BioDyne) for total volume 50µl 5µl PCR product diluted (1:10), 200nM primers (Sigma Aldrich), 1X Hot FirePol PCR mix (Solis BioDyne) for total volume 50µl	94°C for 15min	NA
	GATA-1	F: GAGGCTTGTGCAGGCAGT R: CAAACAGCAGGGAAATGAG		94°C for 30s x 40 cycles 60°C for 40s x 40 cycles 72°C for 90s x 40 cycles	
	FY	F: CCCTCAATCCAGGAGACT R: GCTGAGCCATACCAGACACA		72°C for 10min	
Screening	screening	F: TGGAGTGGATGGTGTITTAGA R: TTGCACCCCAATARCTCATT	5µl DNA template, 150nM primers (Sigma Aldrich), 1X Hot FirePol EvaGreen qPCR mix (Solis BioDyne) for total volume 20µl	95°C for 15min 95°C for 15s x 45 cycles 60°C for 20s x 45 cycles 72°C for 20s x 45 cycles 95°C for 2min 68°C for 2min Increment 0.2°C/0.05s from 68°C to 90°C	76.4 - 78.4°C
Species	All species	F: TGGAGTGGATGGTGTITTAGA R: ACCCTAAAGGATTGTGCTACC	5µl DNA template, 250nM primers (Sigma Aldrich), 1X Hot FirePol PCR mix (Solis BioDyne) for total volume 20µl	94°C for 15min 94°C for 30s x 20 cycles 58°C for 60s x 20 cycles 72°C for 60s x 20 cycles 72°C for 10min	NA
	Pf	F: ATGGATATCTGGATTGATTTTATTATGA R: TCCTCCACATATCCAAATTA CTGC	5µl PCR products diluted (1:10), 250nM primers, 1X Hot FirePol EvaGreen HRM mix (Solis BioDyne) for total volume 20µl	95°C for 15min	78.6 - 79.6°C
	Pv/Pk	F: TGCTACAGGTGCATCTCTGTATTTC R: ATTTGTCCCCAAGGTAAAACG		95°C for 10s x 40 cycles 62°C for 20s x 40 cycles	74.8-75.8°C
	Pm	F: ACAGGTGCATCACTTGATTTTTTC R: TGCTGGAATTGAAGATAATAAATTAGTAATAACT		72°C for 25s x 40 cycles	75.4 - 76.4°C
	Po	F: GTTATATGGTTATGTGGAGGATATACTGTT R: CGAATGGAAGAATAAAATGTAGTACG		95°C for 1min 40°C for 1min	73.2 -74.2°C

Set 1	pvS1: CGTTG*C*G pvS2: TTTTTC*G*C pvS3: TCGTG*C*G pvS4: CGTTTTT*T*T pvS5: TTTTTC*G*T pvS6: CCGTT*C*G pvS7: CGTTC*G*T pvS8: CGTTC*G*C pvS9: CGTTTT*C*G pvS10: TCGTTC*G*T	12.5µl DNA template, 3.5µM primers (Sigma Aldrich), 4mM dNTPs (Biotech biobasic), 30U phi29 DNA polymerase (New England Biolabs), 1X phi29 buffer (New England Biolabs), 1% BSA (Invitrogen) for total volume 50µl The * indicate phosphorothioate bonds that are necessary to prevent degradation by phi29 DNA polymerase	35°C for 10min 34°C for 10min 33°C for 10min 32°C for 10min 31°C for 10min 30°C for 16h 65°C for 10min	NA
sWGA	pvS1: AACGAAGC*G*A pvS2: ACGAAGCG*A*A pvS3: ACGACGA*A*G pvS4: ACGCGCA*A*C pvS5: CAACGCG*G*T pvS6: GACGAAA*G*G pvS7: GCGAAAA*G*G pvS8: GCGAAGC*G*A pvS9: GCGGAAC*G*A pvS10: GCGTCGA*A*G pvS11: GGTTAGCG*G*C pvS12: AACGAAT*C*G			
Set 1920				

Table S2: List of *P. vivax* genomic sequences already published and used in this study

Country	Sample	Sequence
Brazil	ERR018932	public data
Brazil	ERR019040	public data
Brazil	SRR332567	public data
Brazil	SRR332569	public data
Brazil	SRR340133	public data
Columbia	SRR1562975	public data
Columbia	SRR1564650	public data
Columbia	SRR1564664	public data
Columbia	SRR1564665	public data
Columbia	SRR1567977	public data
Cambodia	ERR020103	public data
Cambodia	ERR023039	public data
Cambodia	ERR023040	public data
Cambodia	ERR023041	public data
Cambodia	ERR023042	public data
Cambodia	ERR027119	public data
Cambodia	ERR039234	public data
Cambodia	ERR054080	public data
Cambodia	ERR054082	public data
Cambodia	ERR111729	public data
Cambodia	ERR123849	public data
Cambodia	ERR152408	public data
Cambodia	ERR152410	public data
Cambodia	ERR152413	public data
Cambodia	ERR337538	public data
Cambodia	ERR337542	public data
Cambodia	ERR386533	public data
Cambodia	ERR386534	public data
Cambodia	ERR386535	public data
Cambodia	ERR386536	public data
Cambodia	ERR386537	public data
Cambodia	ERR386538	public data
Cambodia	ERR386539	public data
Cambodia	ERR386541	public data
Cambodia	ERR386542	public data
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Cambodia	ERR388742	public data

Cambodia	SRR572648	public data
Indonesia	ERR054085	public data
Indonesia	ERR337624	public data
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Ethiopia	ERR2678989	public data

Ethiopia	ERR2678991	public data
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Ethiopia	ERR925440	public data

Ethiopia	ERR925441	public data
Mauritania	SRR332410	public data
Mauritania	SRR332413	public data
Mauritania	SRR340129	public data
Madagascar	SRR570031	public data
Madagascar	ERR490350	public data

Table S3: List of validated and suspected *P. vivax* invasion-related genes used in this study

Ligands	Accession number (PvP01)	Role in invasion	Location	Size
MSP1	PVP01_0728900	confirmed	PvP01_07_v1:1.215,432..1.220,636(+)	5205 bp
GAMA	PVP01_0505600	potential	PvP01_05_v1:253,743..255,818(+)	3415 bp utr
MSP1P	PVP01_0728800	potential	PvP01_07_v1:1.206,976..1.212,549(+)	5574 bp
DBP	PVP01_0623800	confirmed	PvP01_06_v1:982,025..985,813(+)	5979 bp intron utr
EBP	PVP01_0102300	potential	PvP01_01_v1:104,127..107,068(-)	3417 bp intron utr
RBP2b	PVP01_0800700	confirmed	PvP01_08_v1:34,100..42,108(+)	10393 bp intron utr
RBP2a	PVP01_1402400	confirmed	PvP01_14_v1:113,324..120,966(+)	8696 bp intron utr
RBSA	PVP01_0004240	potential	Transfer:PvP01_00_11.final:23,781..25,244(+)	1464 bp intron
P12	PVP01_1136400	potential	PvP01_11_v1:1.556,940..1.558,028(+)	1697 bp utr
AMA1	PVP01_0934200	potential	PvP01_09_v1:1.459,296..1.460,984(+)	2784 bp utr
RON	PVP01_0916600	potential	PvP01_09_v1:744,059..746,709(-)	3640 bp intron utr
ETRAMP	PVP01_0532300	potential	PvP01_05_v1:1.361,360..1.362,476(-)	6030 bp intron utr
MSA180	PVP01_0814200	potential	PvP01_08_v1:613,890..618,680(+)	6438 bp utr
RAMA	PVP01_0107500	potential	PvP01_01_v1:375,340..378,546(+)	295 bp intron utr

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2. Epidemiological analysis of *P. vivax* strains circulating in East Shewa, Ethiopia

Although the largest burden of *vivax* malaria is found in South and Southeast Asia, the Horn of Africa harbors about 10% to 20% of all cases thereby representing an important reservoir of infection in Africa⁸⁹. In the Horn of Africa, Ethiopia suffers from the largest number of clinical cases in the region. This partly reflects the co-existence of Duffy-positive and Duffy-negative populations and the potential existence of hidden transmission¹. However, the recent widespread detection of *vivax* malaria in Duffy-negative patients raised questions on *P. vivax* epidemiology in Ethiopia. A few studies already examined the correlation between Duffy genotype and parasitaemia. While it has been suggested that the expression of Fya and Fyb antigens influences the parasitaemia and thereby modulates susceptibility to *vivax* malaria, results seem to be contradictor⁹⁰⁻⁹⁴. Besides, these studies emphasized on the detection of *P. vivax* DNA by polymerase chain reaction (PCR) without estimating the peripheral parasitaemia by microscopic examination. Our study aimed at determining the distribution and effect of Duffy genotype on peripheral parasitaemia in *P. vivax* infected patients in East Shewa, Ethiopia. Between 2019 and 2021, we collected dried blood samples from a cohort of 138 *P. vivax* infected patients in different health centers. On one hand, the Duffy genotype of the patients was determined by PCR and Sanger sequencing. On the other hand, the parasitaemia of *P. vivax* asexual stages was estimated by microscopic examination and quantitative real-time polymerase chain reaction (qPCR). Results showed that all infections were characterized by low parasitaemia (< 110 parasites/ μ L). But interestingly, the median parasitaemia of Duffy-negative patients was significantly lower than the median parasitaemia of Duffy-positive patients (homozygous and heterozygous). This supports the hypothesis that there may be a correlation between Duffy genotype and peripheral parasitaemia. Moreover, this suggests that *P. vivax* invasion pathway may be less efficient in Duffy-negative individuals. Overall, this indicates that Duffy-negative populations may constitute a hidden reservoir of infection and may hamper the control and eradication of *vivax* malaria in Ethiopia¹. The scientific article named « *Vivax* malaria in Duffy-negative patients shows invariably low asexual parasitaemia: implication towards malaria control in Ethiopia » has been published in Malaria Journal, in August 2022 and is presented below.

RESEARCH

Open Access

Vivax malaria in Duffy-negative patients shows invariably low asexual parasitaemia: implication towards malaria control in Ethiopia



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Abstract

Background: The increase in detections of *Plasmodium vivax* infection in Duffy-negative individuals in Africa has challenged the dogma establishing the unique *P. vivax* Duffy Binding Protein–Duffy antigen receptor for chemokines (PvDBP–DARC) pathway used by *P. vivax* merozoites to invade reticulocytes. Information on the impact of Duffy antigen polymorphisms on the epidemiology of *P. vivax* malaria remains elusive. The objective of this study was to determine the distribution of asexual parasitaemia of *P. vivax* according to the Duffy antigen polymorphisms in Ethiopia.

Methods: DNA was extracted from dried blood spots (DBS) collected from prospectively recruited 138 *P. vivax*-infected patients from health centres. The identification and estimation of *P. vivax* asexual parasitaemia were performed by microscopic examination and quantitative real-time polymerase chain reaction (PCR). Duffy genotyping was conducted by DNA sequencing in a total of 138 *P. vivax* infected samples.

Results: The proportion of Duffy-negatives (FY*B^{ES}/FY*B^{ES}) in *P. vivax* infected patients was 2.9% (4/138). Duffy genotype FY*B/FY*B^{ES} (48.6%) was the most common, followed by FY*A/FY*B^{ES} genotype (25.4%). In one patient, the FY*02 W.01/FY*02 N.01 genotype conferring a weak expression of the Fy^D antigen was observed. All *P. vivax* infected Duffy-negative patients showed low asexual parasitaemia (≤ 110 parasites/ μ L). The median *P. vivax* parasitaemia in Duffy-negative patients (53 parasites/ μ L) was significantly lower than those found in homozygous and heterozygous individuals ($P < 0.0001$).

Conclusion: *Plasmodium vivax* in Duffy-negative patients shows invariably low asexual parasitaemia. This finding suggests that the pathway used by *P. vivax* to invade Duffy-negative reticulocytes is much less efficient than that used in Duffy-positives. Moreover, the low asexual parasitaemia observed in Duffy-negative individuals could constitute an ‘undetected silent reservoir’, thus likely delaying the elimination of vivax malaria in Ethiopia.

Keywords: Duffy antigen, Polymorphism, Asexual parasitaemia, Ethiopia

Background

In 2020, the World Health Organization (WHO) estimated that malaria contributed to cause 241 million cases and 627,000 deaths [1]. Most malaria cases were caused by *Plasmodium falciparum*, while recently the prevalence and severity of *Plasmodium vivax* malaria cases has shown a sharp increment [2]. *Plasmodium vivax* is a *Plasmodium* species capable to survive even in temperate

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climates, which explains its wider range of geographical distribution than other species [3]. In East Shewa, Ethiopia, *P. vivax* has been responsible for 54–71.4% of malaria cases [4, 5].

Unlike *P. falciparum*, the invasion of reticulocytes by *P. vivax* requires the dual interactions between two parasite ligands (*P. vivax* Duffy Binding Protein, PvDBP and *P. vivax* reticulocyte Binding Protein 2b, PvRBP2b) and two human receptors (Duffy antigen/receptor for chemokines, DARC/CD234 and transferrin receptor 1, TfR1/CD71) [6–8].

The Duffy antigen has Fy^a and Fy^b variants differing from each other by a single nucleotide polymorphism of replacing Glycine to Aspartic acid at codon 42. Encoded by the FY gene (*ACKR1* located on chromosome 1q23.2), the Duffy glycoprotein has FY*01 (FY^a) and FY*02 (FY^b) codominant alleles. In other words, if the FY^a is inherited from one parent, the FY^b allele will be inherited from the other. Both gene products, Fy^a and Fy^b antigens are expressed on the red blood cells (RBCs). There are four main Duffy phenotypes: Fy(a + b +), Fy(a + b -), Fy(a - b +), and Fy(a - b -). The last one gives rise to Duffy-negative phenotypes [9, 10] and was thought to confer innate resistance to blood-stage infection with *P. vivax* and clinical malaria [11]. Recently, however, the widespread detection of *P. vivax* across individuals lacking Duffy antigen in many African countries [9, 12–17] including Ethiopia [13, 18, 19] further complicates the current understanding of vivax malaria epidemiology in the region.

It has been postulated that hidden transmission could be occurring in Duffy-negative populations in sub-Saharan Africa. A plausible explanation that could support this notion is the observation of Duffy-independent *P. vivax* blood-stage infection across the Duffy-negative population of Africa [20]. Most of the previous reports have given more emphasis on *P. vivax* DNA detection in Duffy-negative individuals by polymerase chain reaction (PCR) without parasite density estimation. It has been suggested that the expression of Fy^a and Fy^b antigens on the reticulocytes influenced asexual *P. vivax* parasitaemia, thereby modulating susceptibility to clinical vivax malaria [21]. For instance, in Papua New Guinea, it has been shown that Duffy heterozygous (FY^a/FY^b) individuals had lower *P. vivax* parasitaemia than those individuals with homozygous (FY^a/FY^a) [22]. To date, the association of clinical *P. vivax* with Duffy antigen is contradicting. For instance, genotypes FY^a/FY^a, as compared to FY^b/FY^b, were reported in the increasing susceptibility of individuals and the severity of *P. vivax* infection [23]. In contrary, some studies have shown that FY^b/FY^b genotypes were highly susceptible to *P. vivax* malaria [21, 24–26].

In Ethiopia, data regarding the epidemiological features of vivax malaria in relation with duffy blood group are scarce [13, 18, 19], while an admixture of people with varying Duffy antigen polymorphism co-exists and *P. vivax* transmission is frequent. This provides the opportunity to study distribution and effect of Duffy antigen polymorphisms on the asexual *P. vivax* parasitaemia. This study was, therefore, initiated to explore this question in the admixed populations of Duffy-positive and Duffy-negative individuals in East Shewa, Ethiopia.

Methods

Study area and population

The study was conducted in Adama City Administration, and its surroundings located 100 km southeast of Addis Ababa, the capital city of Ethiopia from October 2019 up to January 2021 (Fig. 1). Adama City Administration is presented on 1623 m above sea level, 20.5 °C average annual temperature and 808 mm annual rainfalls. *Plasmodium vivax* is reported as the predominant malaria-causing species [4, 5] in East Shewa. A total of 151 individuals were prospectively recruited from all health centres. The study populations were those patients with fever seeking health care in public health institutions at Adama City Administration (malaria diagnostic centre, and 7 health centres), and surroundings (Awash Melkasa, Koka, Metehara, Mojo and Welenchity health centres) in East Shewa Zone. All patients known to have uncomplicated *P. vivax* malaria by microscopy were included when possible. Those with *P. falciparum* and a recent history of antimalarial drugs were excluded from the current study.

Blood sample collection

Clinical *P. vivax* blood samples were collected from patients seeking malaria diagnosis and treatment at health centres. Finger-prick blood samples were collected to determine *Plasmodium* infection. Both thin and thick blood films were prepared in a single slide and labeled with the identification number of participants and date of preparation. The thin smear was fixed with methanol, and both thick and thin blood smears were stained with Giemsa according to the protocol [27]. Each slide was examined independently by two experienced microscopists who were blinded to each other's result under a microscope with 100 × oil immersion. Slides with discordant results were read by a third reader blindly, and the majority results were taken as final. If a *P. vivax* infection was confirmed by microscopy examination, two to four spots of blood, near to ~60 µl each spot, were collected on Whatman 3 MM filter paper for dried blood spots (DBS) for each participant, dried, and stored at room temperature until use.

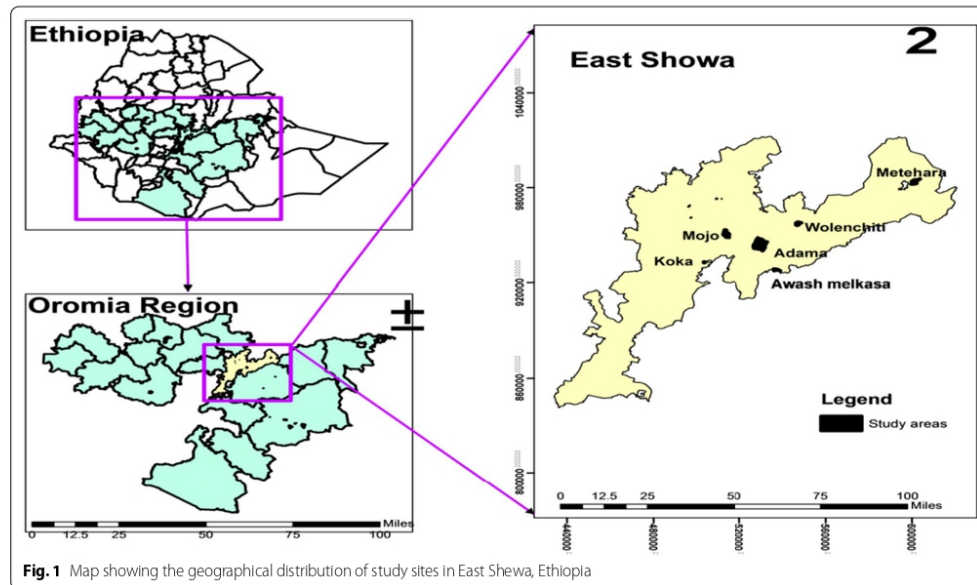


Fig. 1 Map showing the geographical distribution of study sites in East Shewa, Ethiopia

DNA extraction

DBS were cut into small pieces with scissors and transferred into 2 mL microtubes. Genomic DNA was extracted using QIAamp DNA Mini blood kit according to the manufacturer's protocol with some modifications. Briefly, 1 mL of PBS (1X)/saponin (0.5%), 180 μ L of buffer ATL, 20 μ L proteinase K, 200 μ L of Buffer AL, 200 μ L of ethanol (96–100%), 500 μ L of Buffer AW1, 500 μ L of Buffer AW2, and 200 μ L of elution buffer (AE buffer) were added to each tube. The extraction was performed using the recommended incubation temperature and time with manufacturers' centrifugation rate. Finally, the 1.5 mL Eppendorf tubes containing the extracted DNA were stored at -20°C until use.

Identification of *Plasmodium* species by real-time PCR

Plasmodium detection was performed by real-time PCR amplification of species-specific segments of the *cytochrome b* using the protocol described previously [28]. The assay was conducted to confirm and quantify *P. vivax* and mixed infection with *P. falciparum* since only these species were detected in the microscopic examination. The following nucleotide primers were used: forward-5'-TGCTACAGGTGCATCTCTGTATTC-3', and reverse-5'-ATTTGTCCCAAGGTAACG-3' specific for *P. vivax*, and forward-5'-ATGGATATCTGG

ATTGATTTTATTTATGA-3', reverse-5'-TCCTCCACA TATCCAAATTACTGC-3' specific for *P. falciparum*. The PCR amplifications using CFX96™ Real-Time PCR detection system (Bio-Rad Laboratories Ltd) were carried out following 10 μ L of molecular water, 4 μ L of Evagreen HRM Mix (5x) (Sotis biodyne), 0.5 μ L of each primer, and 5 μ L of DNA template with a final volume of 20 μ L. The thermal cycling programs were 94°C for 15 min (minutes) followed by 20 cycles of 30 s (seconds) at 94°C , 58°C for 1 min, 72°C for one and a half min and 72°C for 10 min followed by a cooling step of 20°C . Negative (water) and positive (*P. vivax* DNA from blood sample with parasite density estimated ~ 8000 parasites/ μL and *P. falciparum* DNA at 5 ng/ μL) control samples were used in each PCR run. For each sample positive for *P. vivax*, the qPCR Ct value was estimated based on a threshold defined at 200.

Estimating parasite density from qPCR Ct value

The parasite densities per μL were estimated using qPCR for all 138 samples. However, to observe the correlation between qPCR ct values with microscopy results, the parasite densities per μL of fourteen randomly selected samples were determined on Giemsa-stained blood films. Blood films were read for 30 min under oil-immersion microscope by three experienced technicians (approximately 20,000 red blood cells). The correlation between

qPCR Ct values (triplicate) with microscopy results (triplicate) was estimated using scatter diagrams and correlation coefficients (automatic weighted regression).

Duffy genotyping

Duffy genotypes were determined by PCR amplification from *P. vivax* human isolates since the DBS samples also contained human DNA. The primers for genotyping were forward-5'-GAGGCTTGTGCAGGCAGT-3' and reverse-5'-CAAACAGCAGGGGAAATGAG-3' specific for GATA-1 transcription factor binding site of the FY gene (-33 T → C), and forward-5'-CCCTCAATCCCAGGAGACT-3' and reverse-5'-GCTGAGCCATAC CAGAC ACA-3' specific for exon 2 of the DARC gene (125 G → A) (Sigma Aldrich, France). The PCR was conducted in a 50 µL total PCR volume using 33 µL molecular water, 1 µL of each primer, 10 µL of 5X HOT blend Taq polymerase (Sotis biodyne), and 5 µL of DNA template, as previously described with minor modifications [29]. The PCR conditions were first denaturation at 94 °C for 15 min followed by 40 cycles at 94 °C for 20 s, 60 °C for 20 s, 72 °C for 1 min, and final elongation at 72 °C for 10 min. The PCR products were purified using Agencourt AMPure XP bead-based purification prior to sequencing. The purified PCR products were sent to Biofidal (France) for Sanger sequencing. The sequenced nucleotides were analyzed, using CLC Genomics Workbench 22.0 (Qiagen, Germany) software, on both strands. Sequences produced from the present study were aligned with their respective reference sequences.

Statistical analysis

All statistical analyses were performed with MedCalc software (version 20.014) and Statistical Package for Social Sciences (SPSS) version 25. Descriptive statistics were used to describe the characteristics of study participants, frequencies of Duffy genotypes, phenotypes, and other related variables using figures and tables. Outlier detection was used to detect anomalous observations in sample data by the Tukey test [30]. The outliers were categorized as 'outside' or 'far out' values. An outside value was defined as a value that is smaller than the lower quartile minus 1.5 times the interquartile range, or larger than the upper quartile plus 1.5 times the interquartile range. A far-out value was defined as a value that is smaller than the lower quartile minus 3 times the interquartile range, or larger than the upper quartile plus 3 times the interquartile range.

The regression analysis was used to define the relationship between asexual *P. vivax* parasitaemia estimated by microscopy and qPCR Ct value (defined by the best-fitting regression model), and then to deduce the parasitaemia from qPCR Ct values. The relationship was

then confirmed with the paired t-test, intraclass correlation coefficient (ICC), and Passing-Bablok regression (bootstrap method). The independent non-parametric Mann-Whitney test was used to determine the differences between the median *P. vivax* parasitaemia related to Duffy antigen polymorphism with 95% confidence interval (CI).

The violin plot [31] was used to present the distribution of asexual *P. vivax* parasitaemia by Duffy antigen polymorphism. A P-value < 0.05 was considered statistically significant.

Results

Characteristics of the study population

Out of the total 151 recruited patients, 11 were *P. falciparum* positive (confirmed by two microscopists), and 2 were negative (confirmed by PCR). Thus, 138 symptomatic *P. vivax* infected patients were enrolled in this study; of which 135 were *P. vivax* single infections and the remaining three were mixed infections (*P. vivax* and *P. falciparum*) as determined by microscopy and real-time PCR. Thirty seven percent of the study participants were in the age group of 15–24 years old. The highest number (37%) of study participants were from Adama City Administration. The majority (66.7%) of study participants were males and were recruited during the data collection year of 2020 (67.4%) (Table 1).

Plasmodium vivax parasitaemia deduced by qPCR

Plasmodium vivax DNA was detected by qPCR amplification in all tested samples (N = 138). The median (range) Ct value was 19.07 (12.92–36.62). The outlier

Table 1 Characteristics of study participants in East Shewa, Ethiopia

Variables	Category	Number (%)
Sex	Male	92 (66.7)
	Female	46 (33.3)
Age	≤ 14	16 (11.6)
	15–24	51 (37.0)
	25–34	38 (27.5)
	≥ 35	33 (23.9)
Data collection period	2019	31 (22.5)
	2020	93 (67.4)
	2021	14 (10.1)
Data collection centres/ health institutions	Adama City Administration	51 (37.0)
	Awash Melkasa	7 (5.1)
	Koka	3 (2.2)
	Metehara	29 (21.0)
	Mojo	18 (13.0)
	Welenchity	30 (21.7)

detection revealed 12 (8.7%) outside values in the sample set (Additional file 1: Fig. S1). The correlation between qPCR Ct values with microscopy results from the 14 randomly selected blood samples showed by the best-fitting regression model was estimated with the exponential curve as regression equation (Additional file 1: Fig. S2, Table S1). The ICC value indicated excellent consistency between microscopy and qPCR Ct values (ICC = 0.927; 95% CI 0.865–0.961). The paired *t*-test indicated that the estimated (from microscopy) and deduced (from qPCR Ct values) parasitaemia were not significantly different (mean difference 188.5 parasites/ μ L, 95% CI – 593.2 to 970.2, *P* = 0.629). The Passing–Bablok regression did not present evidence of a systematic or proportional bias. The estimations for the Intercept (95% CI) were – 9.92 (– 163.02 to 37.33), for the Slope (95% CI) 0.93 (0.84–1.14) and for the Spearman rank correlation coefficient (95% CI) 0.87 (0.78–0.93, *P* < 0.00001) (Additional file 1: Fig. S3).

The deduced median (range) *P. vivax* asexual parasitaemia of the 138 infected study participants was 4804(38–26,278) parasites/ μ L. We detected 12 (8.7%) samples with far-out values (\leq 110 parasites/ μ L) (Additional file 1: Fig. S4).

Duffy blood group genotyping

Duffy genotyping was successfully performed on 138 patients as shown in Table 2. The proportion of Duffy-negatives (FY*B^{ES}/FY*B^{ES}) in *P. vivax* infected patients was 2.9% (4/138). The duffy negatives were detected from Adama, Welenchity, Koka and Metehara (one from each site). Duffy genotype FY*B/FY*B^{ES} (48.6%) was the most common followed by FY*A/FY*B^{ES} genotype (25.4%). The FY*02 W.01/FY*02 N.01 genotype conferring a weak expression of the Fy^b antigen was observed in one patient. The FY*02 was the most frequently observed allele (65.9%) followed by FY*01 (42.8%). The most common (54.3%) phenotype detected in the study was Fy(a+b+) (Table 2).

Association between Duffy phenotypes and deduced *P. vivax* asexual parasitaemia

To determine whether and how Duffy phenotypes influence the asexual parasitaemia in *P. vivax* patients, this study analysed the relationship between the deduced parasite densities and the Duffy antigens distribution (deduced from Duffy genotypes). The median (range) *P. vivax* asexual parasitaemia of the 138 infected study participants by Duffy phenotypes are presented in the Table 3.

Table 2 Genotypes and phenotypes frequencies of Duffy blood group among *P. vivax* infected patients in East Shewa, Ethiopia

DbSNP	rs2814778	rs12075	rs34599082	rs13962	Predicted genotype	Predicted antigen	Phenotype	No. (%)
Nucleotide	c.1-67 ^a	c.125 ^b	c.265 ^c	c.298 ^d				
Region	5'UTR	Exon 2	Exon 2	Exon 2				
SNP (amino acid)	C	A(Asp42)	C(Arg89)	G(Ala100)	FY*B ^{ES} /FY*B ^{ES} or FY*02 N.01/ FY*02 N.01	Fy ^a /Fy ^{es}	Fy(a–b–)	4 (2.9)
	T	G(Gly42)	C(Arg89)	G(Ala100)	FY*A/FY*A or FY*01/FY*01	Fy ^a	Fy(a+b–)	7 (5.1)
		A(Asp42)	C(Arg89)	G(Ala100)	FY*B/FY*B or FY*02/FY*02	Fy ^b	Fy(a–b+)	6 (4.3)
		G(Gly42)/ A(Asp42)	C(Arg89)	G(Ala100)	FY*A/FY*B or FY*01/FY*02	Fy ^a /Fy ^b	Fy(a+b+)	17 (12.3)
	C/T	A(Asp42)	C(Arg89)	G(Ala100)	FY*B/FY*B ^{ES} or FY*02/ FY*02 N.01	Fy ^b /Fy ^{es}	Fy(a–b+)	67 (48.6)
		A(Asp42)	C(Arg89)	A(Thr100)	FY*02/ FY*02 N.01	Fy ^b /Fy ^{es}	Fy(a–b+)	1 (0.7)
		A(Asp42)	C(Arg89)/ T(Cys89)	G(Ala100)/ A(Thr100)	FY*B ^{ES} /FY*X or FY*02 W.01/ FY*02 N.01	Fy ^a /Fy ^{es}	Fy(a–b+)	1 (0.7)
		G(Gly42)/ A(Asp42)	C(Arg89)	G(Ala100)	FY*A/FY*B ^{ES} or FY*01/ FY*02 N.01	Fy ^a /Fy ^{es}	Fy(a+b+)	35 (25.4)

NB: the nucleotide position (dbSNP) is based on NCBI data, UTR: untranslated region

^a This polymorphism prevents expression of Fy^b antigen in red blood cells

^b This polymorphism predicts the expression of the Fy^a and Fy^b antigens

^{c,d} These polymorphisms determine weak expression of the Fy^b antigen

Table 3 Association between Duffy antigen polymorphism and *P. vivax* asexual parasitaemia in patients from East Shewa, Ethiopia

Phenotype	Predicted antigen	Deduced parasite density (parasites/ μ L)			
		No. of sample	Range	Median (95% CI)	Interquartile range
Fy(a + b +)	Fy ^a /Fy ^b	17	39–26,278	4584 (2824–6122)	2543–6637
Fy(a + b –)	Fy ^a	7	3905–8750	4725 (4198–8714)	4530–7899
	Fy ^a /Fy ^{es}	35	75–18,247	6042 (4126–7425)	3479–7937
Fy(a – b +)	Fy ^b	6	2811–7053	3893 (2811–6785)	2811–5781
	Fy ^b /Fy ^{es}	68	39–20,100	4987 (3903–6178)	2693–7880
	Fy ^x /Fy ^{es}	1	5943	5943	–
Fy(a – b –)	Fy ^{es} /Fy ^{es}	4	38–76	53	39–72
All		138	38–26,278	4804 (4242–5781)	2811–7663

Plasmodium vivax infected Duffy-negative patients (FY^aB^{ES}/FY^aB^{ES}) showed a regularly low asexual parasitaemia (median, 53 parasites/ μ L) significantly lower (100-fold) than homozygous (FY^aA/FY^aB, FY^aA/FY^aA or FY^aB/FY^aB) and heterozygous (FY^aA/FY^aB^{ES}, FY^aB/FY^aB^{ES} or FY^aB^{ES}/FY^x) individuals ($P < 0.0001$). No significant difference in asexual *P. vivax* parasitaemia was observed between homozygous and heterozygous Duffy-positive individuals. Interestingly, four of the Duffy-negative patients were classified in the ≤ 110 parasites/ μ L group, while the proportions of patients carrying other

Duffy phenotypes who had ≤ 110 parasites/ μ L were lower (5.7% [for FY^aA/FY^aB^{ES}]) and (3.0% [for FY^aB/FY^aB^{ES}]) (Fig. 2).

Discussion

Since the first discovery of Duffy blood groups in the 1950s [32], there have been many investigations focusing on its global distribution [33], its role in blood transfusion [34], and for *P. vivax* invasion pathway into red blood cells [35]. Later, the innate resistance of humans to *P. vivax* infections has been attributed to Duffy blood

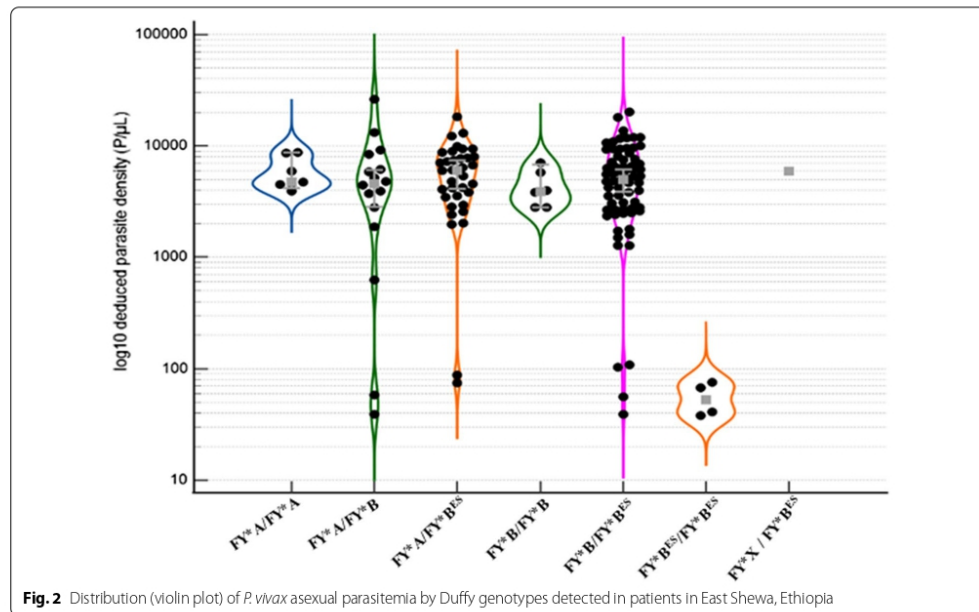


Fig. 2 Distribution (violin plot) of *P. vivax* asexual parasitemia by Duffy genotypes detected in patients in East Shewa, Ethiopia

group polymorphisms [26]; however it is unclear whether Duffy blood group polymorphism confers either only partial or complete resistance against *P. vivax*. This is because of the detection of *P. vivax* infections [36] among the previously refractory Duffy antigen-negative red blood cells [11] as frequently observed in Africa over the last decade [13, 14, 19].

In the current study, *P. vivax* infection in Duffy-negative individuals was identified. However, the proportion (2.9%) of *P. vivax*-infected patients with Duffy-negatives was lower than previous studies conducted at different parts of Ethiopia (10.5–11.9%) [13]. Here, a larger number of FY*B/FY*B^{ES} (Fy^b/Fy^{es}) and FY*A/FY*B^{ES} (Fy^a/Fy^{es}) carriers were observed among *P. vivax* infected patients in agreement with the previous study [19], indicating frequent heterozygous Duffy negativity of the study participants. This was in contrast with the predominant record of homogenous FY*B^{ES} in most African ethnic groups [10, 21], but confirmed the variation in the frequency of the Duffy genotypes across different populations. For instance, FY*B/FY*B was frequently observed in Eastern, while FY*B/FY*B^{ES} was in Southwestern Ethiopia [19]. A higher frequency of FY*B allele carriers was observed compared with the FY*A carriers. A consistent finding is that the probability of having the FY*B allele has increased in Africa [9, 19], in contrast to Asian countries where FY*A was the most frequent variant [25].

Phenotypically, the current results showed that the most common Duffy phenotype was Fy(a – b+) followed by Fy(a + b –) with a frequency of 54.3% and 30.5%, respectively, consistent with previous observations in neighbouring countries, such as Sudan [9]. Although all patients in the current study were clinical, the finding may suggest that individuals with Fy(a + b –) have a relatively low susceptibility to clinical *P. vivax* malaria compared to Fy(a – b+) due to diminishing effect of Fy(a + b –) on the binding of Duffy binding protein (PvDBP) at the erythrocyte surface, as it has been shown in Brazil [21]. The relatively more frequent observation of Fy(a – b+) phenotypes among *P. vivax* infected patients compared to healthy individuals in Sudan [9] also provided potential evidence for an association between Fy(a – b+) and its susceptibility to clinical malaria.

The FY*X (FY*02 W.01) allele encoded the weakly expressed Fy^b antigen due to the reduced amount of Duffy protein caused by the mutation at position 89 (Arg > Cys) [37, 38]. Surprisingly, we observed for the first time that FY*X/FY*B^{ES}(FY*02 W.01/FY*02 N.01) genotype, which is not common in Africa [10], was detected from one *P. vivax* infected patient. The asexual *P. vivax* parasitaemia (5943 parasites/μL) detected in this patient was, however, similar to those found in homozygous and heterozygous Duffy-positives.

To date, it is well known that the asexual parasitaemia in patients with *P. vivax* malaria is influenced by several factors such as immunity, age, delay in seeking treatment, self-treatment behaviour before presentation, and other variety of host and parasite factors even though no significant differences were observed in the current study. Here, this study aimed at exploring an additional factor which is the effect of Duffy antigen polymorphisms that can also modulate asexual parasitaemia in *P. vivax* patients. The current study observed that heterozygous Duffy-positive individuals had similar range of parasite density with homozygous, as supported by previous findings in Ethiopia [13] and Thailand [39], but contrary to reports from Papua New Guinea suggesting that *P. vivax* parasitaemia was significantly lower in heterozygous than homozygous [22]. Particularly, Michon et al. showed the advantages of being heterozygous to reduce Duffy binding protein adherence to erythrocytes connecting with resistance against clinical *P. vivax* malaria [40].

However, the current study confirmed that the parasite density of *P. vivax* was significantly lower in Duffy-negative individuals than the Duffy-positives in line with previous studies elsewhere [9, 13, 22]. From this study, all Duffy-negative patients had parasitaemia ≤ 110 parasites/μL (median 53 parasites/μL), while Duffy-positive patients (homozygous or heterozygous) had an average parasitaemia between 1000 and 5000 parasites/μL. These data suggest two important features regarding vivax malaria epidemiology in Africa.

First, the very low parasite density observed in Duffy-negative patients might provide better estimates of the silent *P. vivax* parasites reservoir that may remain undetected by widely used microscopic examination although all *P. vivax* in Duffy negatives in the current study were detected by microscopy. It is important to note that the current frequent observation of vivax malaria among Duffy negative Africans might be due to the use of a more sensitive molecular tools. This suggests that *P. vivax* malaria was likely missed in Duffy-negative patients in endemic settings such as Africa where malaria diagnosis is routinely made by microscopy. This was supported by the previous findings which indicated that only four of the twenty *P. vivax* infections were microscopic-positive in Duffy-negative patients in other parts of Ethiopia [13]. This, therefore, further pointed out that *P. vivax* might be maintained in the Duffy-negative populations without clinical symptoms [41], and with the difficulty of microscopic examination [13], but could be transmitted between hosts [42].

This hypothesis is further substantiated by the fact that several studies noticed the high probability of transmission of *P. vivax* between hosts from submicroscopic individuals [43–45]. However, further study is

needed to determine the relative contribution of Duffy antigen polymorphism in the transmissibility of *P. vivax* gametocytes to mosquitoes. The observation of *P. vivax* among Duffy-negative individuals, thus, might further complicate the poor understanding of the exact frequency of vivax malaria in Ethiopia and thereby across Africa where both Duffy-negatives and -positives co-exist.

Second, the very low parasite density found in Duffy-negative patients strongly suggests that the invasion pathway(s) used by *P. vivax* merozoites to invade Duffy-negative reticulocytes is (are) far less efficient compared to the classical pathway involving the interaction between the PvDBP and DARC in Duffy positive individuals. One hypothesis that is warranted to be envisioned is to consider that reticulocytes (or less mature erythroid cells from the bone marrow) of Duffy-negative subjects, which were thought to be devoid of the Duffy protein, express transiently and weakly this protein at the course of erythroid cell differentiation. This hypothesis had already been explored by Dechavanne et al. [46], but needs to be confirmed and validated. However, this cannot reject the alternative hypothesis that *P. vivax* pathway used to invade Duffy negative reticulocytes is based on not very effective alternative interactions between unknown parasite ligand and reticulocytes receptors.

The limitation of this study includes failure to well describe the association of factors other than Duffy antigen polymorphism which could affect parasitaemia of *P. vivax* because of small samples. In addition, the number of Duffy negatives was too small for making firm conclusion regarding predictors of the parasite density.

In conclusion, the current study described the emerging perspectives on the relationship of Duffy antigen polymorphisms with parasite density of *P. vivax*. The complex relationships between hosts and parasites have clinical implications including the risk of being a silent reservoir, and thus needs introducing specific and sensitive diagnostic methods in areas where *P. vivax* malaria is endemic. Further investigations using longitudinal study and large samples (both clinical and community) from different parts of Ethiopia are needed to know the exact distribution of Duffy antigen and how Duffy antigen polymorphisms impact and shape the *P. vivax* malaria epidemiology in Ethiopia.

Abbreviations

DARC: Duffy antigen/receptor for chemokines; RBCs: Red blood cells; SNP: Single nucleotide polymorphism; PCR: Polymerase chain reaction; PvDBP: *Plasmodium vivax* Duffy binding protein; PvRBP2b: *Plasmodium vivax* Reticulocyte Binding Protein 2b; TfR1: Transferrin receptor 1; WHO: World Health Organization.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12936-022-04250-2>.

Additional file 1: Table S1. Summarized statistics of the regression equations (weighted least-squares regression) tested as the best-fitting regression model between *P. vivax* parasitemia estimated by microscopy and qPCR Ct values. **Figure S1.** Distribution of the qPCR Ct values of the 138 *P. vivax* blood samples collected from *P. vivax* infected patients, 2019–2021. The median (range) Ct value was 19.07 (12.92–36.62). The Outlier detection using the Tukey test revealed 12 (8.7%) outside values in the sample set (red dots). **Figure S2.** Scatter diagram of the best-fitting regression model (exponential curve as regression equation) between qPCR Ct values and microscopy results from the 14 blood samples. **Figure S3.** Passing–Bablok regression: scatter diagram and regression line of *P. vivax* parasitemia defined by microscopy and parasitemia deduced from qPCR Ct values. The Intercept (95% CI) were -9.92 (-163.02 to 37.33); the Slope (95% CI) 0.93 (0.84 – 1.14) and the Spearman rank correlation coefficient (95% CI) 0.87 (0.78 – 0.93 , $P < 10^{-5}$). **Figure S4.** Distribution of the *P. vivax* parasitemia deduced from qPCR Ct values. The median (range) *P. vivax* asexual parasitemia of the 138 infected study participants was 4804 (38–26,278) parasites/ μ L. The Tukey test (Outlier detection) detected 12 (8.7%) samples with far-out values (below 110 parasites/ μ L).

Acknowledgements

The authors are grateful to Aklilu Lemma Institute of Pathobiology, Addis Ababa University, Bahir Dar University, Bill and Melinda Gates Foundation, and European and Developing Countries Clinical Trials Partnership (EDCTP) for financial support of the study. Our special thanks also go to Malaria Genetics and Resistance Unit, Department of Parasites and Insect Vectors of Pasteur Institute, Paris, France for financial support for all molecular laboratory works. We would like to thank Tewabech Lemma and Tsehay Orlando (WHO certified microscopists at Adama Malaria Diagnostic Centre) for their laboratory support. We are indebted to all the study participants, and data collectors for their cooperation during the fieldwork.

Author contributions

AA, DM and LG conceived and designed the study. AA collected the sample. AA and IB conducted the laboratory procedures. DM, LD and LG conducted the supervision. AA, IB, SM, CDL, DM and LG verified, analysed, and interpreted the data. AA and IB drafted the original version of the manuscript. All authors read and approved the final manuscript.

Funding

This study was supported by the Bill and Melinda Gates Foundation (ID: INV-015996), European and Developing Countries Clinical Trials Partnership (EDCTP), and Malaria Genetics and Resistance Unit, Department of Parasites and Insect Vectors of Pasteur Institute, Paris, France.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author on reasonable request. All relevant data are within the manuscript.

Declarations

Ethics approval and consent to participate

Ethical clearance was obtained from the Institutional Review Board of Addis Ababa University, Aklilu Lemma Institute of Pathobiology (Ref. no. ALIPB IRB/18/2012/20), and National Research Ethics Review Committee (Ref. no. MoSHE/02/152/778/21). Study participants, their parents, or guardians had gotten detailed explanations about their rights, purpose, procedure, and benefit of the study and side effects of the procedure and written informed consent was also obtained. In addition, assent was obtained from children. The study participants were treated according to the national treatment guideline. All participants were assured of confidentiality throughout the study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Received: 2 June 2022 Accepted: 26 July 2022

Published online: 01 August 2022

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3. Epidemiological analysis of *P. vivax* strains circulating in Ethiopia

As already explained for the previous study, *vivax* malaria is prevalent in Ethiopia due to the co-existence of Duffy-positive and Duffy-negative populations and the potential existence of low, hidden transmission⁸⁹. The recent widespread detection of *vivax* malaria in Duffy-negative patients raised questions on *P. vivax* epidemiology in Ethiopia. However, little is known about the correlation between *P. vivax* infections and distribution of Duffy blood groups within the different Ethiopian ethnic groups and geographic endemic areas. Our cross-sectional study aimed at determining the distribution and effect of Duffy genotype on peripheral parasitemia in *P. vivax* infected patients in Ethiopia. Between February 2021 and September 2022, dried blood samples were collected from a cohort of 361 *P. vivax* infected patients in 5 different eco-epidemiological endemic sites in Ethiopia (Arbaminch, Dubti, Gambella, Metehara, and Shewarobit). On one hand, *P. vivax* infections were diagnosed by RDT and microscopy. The parasitemia of *P. vivax* asexual stages was estimated by microscopic examination and qPCR. On the other hand, the molecular identification of the Duffy genotype of patients was based on a novel sequence-specific PCR primers named Amplification Refractory Mutation System (ARMS) that allows amplifications of the GATA-1 transcription factor-binding site of the *ackr1* gene promoter only when the target allele (TCT for Duffy-positive and CCT for Duffy-negative genotypes) is detected in the sample. Results showed that 95.6% of the patients were Duffy-positive with mean asexual parasite density of 12.165p/μl while 4.4% of the patients were Duffy-negative with a mean asexual parasite density of 1.227p/μl. These results confirm that Duffy-negativity does not prevent from *P. vivax* infections and that Duffy-negative Ethiopian populations may constitute a hidden reservoir of infection and transmission. However, no significant association was found between Duffy genotype and geographic site (p=0.12), ethnic group (p=0.50) or gender (p=0.16). Nevertheless, this study suggests that conducting field studies will help in better understanding the epidemiology of *vivax* malaria and development of specific elimination strategies in Africa. The scientific article named « Potential hidden *Plasmodium vivax* malaria reservoirs from low parasitemia Duffy-negative Ethiopians: molecular evidence » has been published in July 2023 in the journal Public Library Of Science Neglected Tropical Diseases and is presented below.

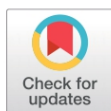
RESEARCH ARTICLE

Potential hidden *Plasmodium vivax* malaria reservoirs from low parasitemia Duffy-negative Ethiopians: Molecular evidence

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OPEN ACCESS

Citation: Abebe A, Bouyssou I, Mabilotte S, Dugassa S, Assefa A, Juliano JJ, et al. (2023) Potential hidden *Plasmodium vivax* malaria reservoirs from low parasitemia Duffy-negative Ethiopians: Molecular evidence. *PLoS Negl Trop Dis* 17(7): e0011326. <https://doi.org/10.1371/journal.pntd.0011326>

Editor: Kamala Thriemer, Menzies School of Health Research, AUSTRALIA

Received: April 24, 2023

Accepted: June 14, 2023

Published: July 3, 2023

Peer Review History: PLOS recognizes the benefits of transparency in the peer review process; therefore, we enable the publication of all of the content of peer review and author responses alongside final, published articles. The editorial history of this article is available here: <https://doi.org/10.1371/journal.pntd.0011326>

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Data Availability Statement: All relevant data are within the manuscript.

Abstract

Background

The interaction between the *Plasmodium vivax* Duffy-binding protein and the corresponding Duffy Antigen Receptor for Chemokines (DARC) is primarily responsible for the invasion of reticulocytes by *P. vivax*. The Duffy-negative host phenotype, highly prevalent in sub-Saharan Africa, is caused by a single point mutation in the GATA-1 transcription factor binding site of the DARC gene promoter. The aim of this study was to assess the Duffy status of patients with *P. vivax* infection from different study sites in Ethiopia.

Methods

A cross-sectional study was conducted from February 2021 to September 2022 at five varying eco-epidemiological malaria endemic sites in Ethiopia. Outpatients who were diagnosed with *P. vivax* infection (pure and mixed *P. vivax/P. falciparum*) by microscopy and Rapid Diagnostic Test (RDT) were subjected to PCR genotyping at the DARC promoter. The associations between *P. vivax* infection, host genotypes and other factors were evaluated.

Result

In total, 361 patients with *P. vivax* infection were included in the study. Patients with pure *P. vivax* infections accounted for 89.8% (324/361), while the remaining 10.2% (37/361) had mixed *P. vivax/P. falciparum* infections. About 95.6% (345/361) of the participants were Duffy-positives (21.2% homozygous and 78.8%, heterozygous) and 4.4% (16/361) were Duffy-negatives. The mean asexual parasite density in homozygous and heterozygous

Funding: The author(s) received no specific funding for this work.

Competing interests: The authors have declared that no competing interests exist.

Duffy-positives was 12,165 p/μl (IQR25-75: 1,640–24,234 p/μl) and 11,655 p/μl (IQR25-75: 1,676–14,065 p/μl), respectively, significantly higher than that in Duffy-negatives (1,227 p/μl; IQR25-75: 539–1,732 p/μl).

Conclusion

This study confirms that Duffy-negativity does not provide complete protection against *P. vivax* infection. The development of *P. vivax*-specific elimination strategies, including alternative antimalarial vaccines should be facilitated by a better understanding of the epidemiological landscape of *vivax* malaria in Africa. More importantly, low parasitemia associated with *P. vivax* infections in Duffy-negative patients may represent hidden reservoirs of transmission in Ethiopia.

Author summary

Plasmodium vivax generally receives less attention than *P. falciparum* and was neglected in sub-Saharan Africa. However, the characteristics of *P. vivax* infection in Duffy-negative individuals, and the distribution of Duffy blood group in different eco-epidemiological zones and ethnic groups of Ethiopia are not well documented. Here, we determined the Duffy genotypes of *P. vivax* infected patients across broad regions of Ethiopia. It is clear that Duffy negative individuals in Ethiopia are not fully protected against *P. vivax* infection, and that these infections in Duffy negatives are often associated with low parasitemia. Our findings lend support to the notion that *P. vivax* may have developed a Duffy-independent erythrocyte invasion pathway and/or evolution in host immune responses.

Introduction

In 2021, there were an estimated 247 million cases and 619,000 deaths from malaria worldwide. Of these, 2% of the cases were due to *P. vivax*, mainly in the Horn of Africa, South America, India and Asia [1]. *P. vivax* malaria is recognized as a cause of severe morbidity and mortality, with a significant negative impact on health in endemic countries [2–4]. The unique biological features of *P. vivax*, such as hypnozoite formation and early gametocytogenesis, pose a challenge to global malaria elimination efforts [5].

The *P. vivax* invasion pathway into reticulocytes was previously thought to be primarily dependent on the interaction between the Duffy binding protein (PvDBP) and its cognate receptor, the Duffy Antigen Receptor for Chemokines (*DARC*) [6–8]. Polymorphisms in the promoter of the *DARC* gene alter the expression of the Duffy antigen protein and determine an individual's Duffy blood group [9]. A point mutation in the GATA-1 transcription factor binding site of the promoter of the gene at position–67 encodes the change of a T nucleotide to a C nucleotide and underlies the Duffy-negative (erythrocyte silent) phenotype [10–12]. Individuals with this mutation do not express the Duffy antigen on the surface of their erythrocytes [13].

Duffy-negative phenotype in the population occurs at low frequency in Caucasians (~3.5%) [14], and has a prevalence of almost 100% in West Africa, > 80% in African Americans [15], 20–36% in East Africa and 84% in southern Africa [16]. In Ethiopia, a study reported 94/416

(~23%) of the febrile patients from the six health centres/hospitals were Duffy-negatives, though only two of them were confirmed with *P. vivax* [17].

The scientific paradigm that *P. vivax* exclusively invades Duffy-positive reticulocytes through the interactions of PvDBP and DARC was established, and it was assumed that *vivax* malaria was rarely transmitted or even absent in regions where Duffy-negativity predominates, such as sub-Saharan Africa [18,19]. However, recent studies have shown that *P. vivax* can infect Duffy-negative reticulocytes [20], and is no longer a barrier to such infections [21]. In East Africa, the prevalence of *P. vivax* in Duffy-negative populations ranges from 3% [22] to ~12% in Ethiopia [16] and 3% [16] to ~18% in Sudan [9]. Although several studies have demonstrated the presence of *P. vivax* infection in Duffy-negative individuals, the distribution of these infections in different eco-epidemiological zones and ethnic groups are not well documented, especially in Ethiopia. This study compares the prevalence of Duffy blood group genotypes of *P. vivax* infected patients among different eco-epidemiological zones of Ethiopia.

Materials and methods

Ethical statement

Ethical approval was obtained from Aklilu Lema Institute of Pathobiology Institutional Review Board (IRB) (Ref No: ALIPB IRB/34/2013/20), Addis Ababa University, and ministry of education (MoE) via its national research ethical review committee (Ref No:7/2-514/m259/35), Addis Ababa, Ethiopia. An official letter was sent to the study sites and the study was initiated after obtaining permission from the respective health facilities. Written informed consent and/or assent was obtained from all study participants, using the local language where appropriate.

Study design, period and areas

A health-facility based cross-sectional study was conducted from February 2021 to September 2022. Based on the annual parasite index (API), malaria risk stratification in Ethiopia is divided into five classes: i) malaria free, ii) very low, iii) low, iv) moderate and v) high malaria risk.

The study sites were selected from different malaria risk stratification areas. The sites include: Arbaminch, Dubti, Gambella, Metehara, and Shewarobit (Fig 1). Arbaminch hospital is located in the Southern Nation and Nationalities People Regional State in the southern part of the country (altitude 1200 m; Lat, Long: 6.02043, 37.56788); Dubti hospital is located in the Afar Regional State in the northern-east part of the country (altitude 379 m; Lat, Long: 11.72654, 41.09440); Gambella hospital is located in Gambella Regional State in the western part of the country (altitude 447 m; Lat, Long: 8.24810, 34.59071); Metehara hospital is located in Oromia Regional State in the eastern part of the country (altitude 959 m, Lat, Long: 8.89932, 39.91726); and Shewarobit hospital is located in Amhara Regional State in the northern part of the country (altitude 1268 m; Lat, Long: 10.01734, 39.91253).

Sample collection

Blood samples diagnosed positive for *P. vivax* (pure or mixed *P. vivax/P. falciparum*) by qPCR from patients seen at health facilities were included to determine their Duffy status. The sample size was calculated based on the proportion of Duffy-negative (20%) cases previously reported in Ethiopia [23].

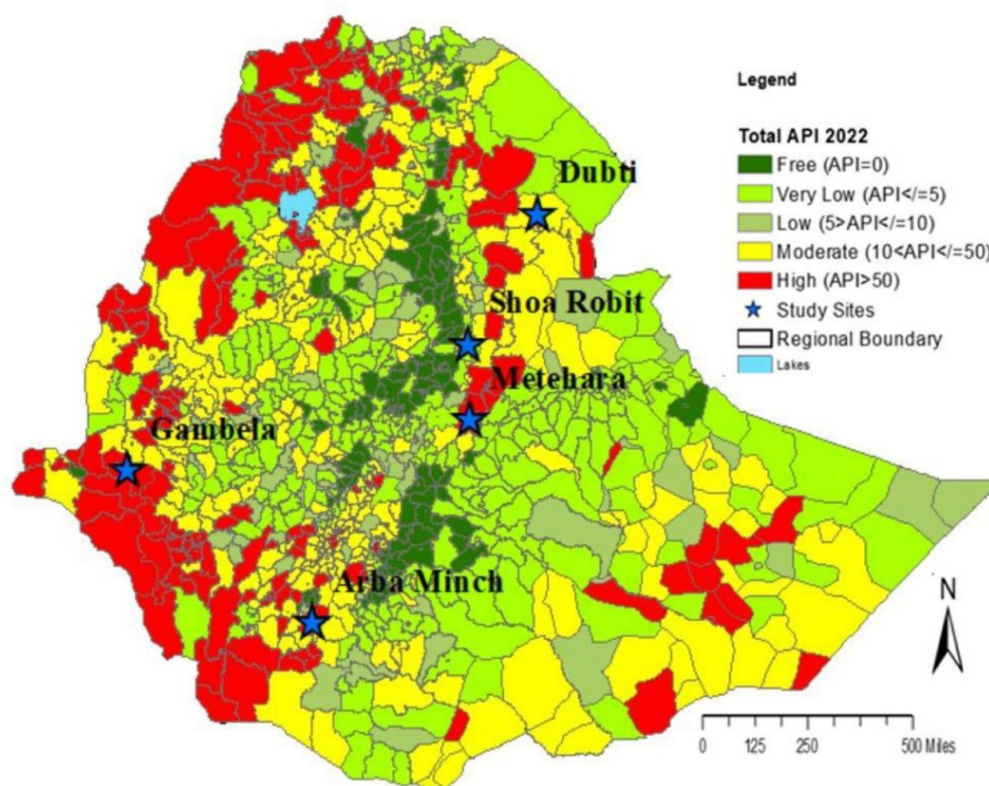


Fig 1. A map of the study sites with malaria risk stratification background, Ethiopia.

<https://doi.org/10.1371/journal.pntd.0011326.g001>

Malaria diagnosis by Rapid Diagnostic Tests (RDT) and microscopy

Finger prick blood samples were collected from self-reported febrile patients seeking malaria diagnosis at a participating health centre. The malaria laboratory diagnosis was carried out for each suspected malaria patient using RDT and microscopy. SD BIOLINE Malaria Ag *Pf*/*Pv* (05FK80) RDT was used for the detection of HRP-II (Histidine-rich protein II) specific for *P. falciparum* and *Plasmodium* lactate dehydrogenase (pLDH) specific for *P. vivax*. Based on WHO public report, the panel detection score of the kit was 96.0% at 200 parasites/ μ l, and 95.0% at 200 parasites/ μ l for *P. falciparum*, and *P. vivax*, respectively [24]. Five microliters (5 μ l) of sample were obtained for the test and the result was interpreted after 15 minutes (up to 30 minutes) according to the manufacturer's guidelines.

Thick and thin blood films (stained with 10% Giemsa staining solution—pH 7.2—for 10 minutes) were prepared from finger-prick blood samples. Parasite densities were determined on thick films by expert malaria microscopists for both sexual and asexual stages of the parasites, considering a minimum of 200 WBCs, the reported number of parasites per microliter of

blood, assuming a total white blood cell count of 8000/ μ L. *P. vivax* sexual stages/gametocytes quantification was performed on the blood film with *P. vivax* (pure) and a mixed species (*P. vivax/P. falciparum*). Parasitemia was then classified as low (<1000 parasites per microliter of blood), intermediate (1000–4999 parasites per microliter of blood) and high (\geq 5000 parasites per microliter of blood) [25]. Slides were declared negative after examination of at least 100 high-power microscope fields [26]. Blood film slides were re-examined and quantified by WHO certified malaria microscopists. Patients who were positive for *P. vivax* (pure) and mixed *P. vivax/P. falciparum* infection by both RDT and microscopy were included in the study. Venous blood samples were collected and spotted onto Whatman 903 Protein Saver Card, US (Dried Blood Spot, DBS), for molecular analysis of the Duffy genotypes.

DNA extraction and molecular diagnosis of Plasmodium species

Genomic DNA (human and parasite) was extracted from a 6 mm diameter spot of a DBS sample and eluted in 100 μ l of TE Buffer (Tris-EDTA) using the QIAamp DNA Extraction Kit (Cat.No: 79216, Lot: 172018338, Germany) according to the manufacturer's instructions for the molecular identification of malaria parasites and Duffy genotypes. The extracted DNA was stored at -20° C for further use.

Primers previously designed to target the mitochondrial species-specific *cytochrome b* (*cytb*) gene were used for the detection and identification of *P. vivax* and *P. falciparum* (Table 1). Given the multiple copies of the mitochondria, this assay allows the detection of low density *Plasmodium* infections in malaria pre-elimination settings [27].

The PCR amplifications were carried out using 10 μ L of Advanced Mix Sybgreen (2x) (Catalog # 1725271, Bio-Rad laboratories Inc. United State), 4 μ L of water, 0.5 μ L of each primer, and 5 μ L of DNA template with the final volume of 20 μ L in a Bio-Rad CFX96 Real-Time PCR detection system.

Molecular identification of the Duffy genotypes

Molecular identification of the Duffy genotype was based on a novel sequence-specific PCR primers (Amplification Refractory Mutation System, ARMS) that allows amplifications of the GATA-1 transcription factor-binding site of the *DARC* gene promoter only when the target allele (TCT for Duffy-positive and CCT for Duffy-negative genotypes) is detected in the sample. Different sets of primers were designed and tested using Duffy-positive and Duffy-negative DNA controls which were validated by PCR and Sanger sequencing (see S1 Appendix [28] to detect the Duffy genotype by qPCR. The forward primers contain a nucleotide mismatch at the 3' end (T for Duffy-positive and C for Duffy-negative genotypes) in addition to a mismatch base near to the SNP at the 3' end (C for Duffy-positive and A for Duffy-negative genotypes), to avoid false-amplification and prevent binding to non-complementary sequence and terminate amplification. The reverse primer are the same (Table 2). All Duffy negative samples found by PCR ARMS have been confirmed by Sanger sequencing.

Table 1. Species-specific primers used for the detection and the species identification of *P. vivax* and *P. falciparum*.

Species	Forward or Reverse	Primer
<i>P. vivax</i>	Forward	5'- TGCTACAGGTGCATCTCTTGATTC -3'
	Reverse	5'- ATTTGTCCCAAGGTAAAACG -3'
<i>P. falciparum</i>	Forward	5'- ATGGATATCTGGATTGATTTTATTTATGA -3'
	Reverse	5'- TCCTCCACATATCCAAATTACTGC -3'

<https://doi.org/10.1371/journal.pntd.0011326.t001>

Table 2. AMRS PCR primers used for the detection of Duffy-positive and -negative genotypes.

Duffy genotype	Forward or Reverse	Primer
positive	Forward	5'- CCCTCATTAGTCCTTGGCTCTCAT -3'
	Reverse	5'- CACCTGTGCAGACAGTTCC -3'
negative	Forward	5'- CCCTCATTAGTCCTTGGCTCTAAC -3'
	Reverse	5'- CACCTGTGCAGACAGTTCC -3'

<https://doi.org/10.1371/journal.pntd.0011326.t002>

Briefly, polymorphisms in the GATA-1 were detected in two separate real-time PCR reactions. Real-time PCR reactions were performed with 10 μ L of ssAdvanced mix sybgreen 2x, 10 μ M of primers and 5 μ L of DNA extract, and amplified on the CFX96 PCR instrument (Bio-Rad, Marnes-la-Coquette, France). PCR amplification was carried out under the following conditions: heating at 98°C for 3 min, followed by 35 cycles of heating at 95°C for 20 s, and of annealing/extension at 62°C for 30 s and a final extension step at 72°C for 10 min. After completion of the amplification reaction, a melting curve (65–90°C) was generated to detect non-specific PCR amplification. For each run, homozygous (TCT/TCT), heterozygous (TCT/CCT) Duffy-positive, and homozygous Duffy-negative (CCT/CCT) samples were included as positive controls and a no template (water) as negative control (see [S2 Appendix](#)).

Statistical analyses

Data were analysed using Statistical Package for Social Sciences (SPSS, version 25). Chi-squared, or Fisher's exact tests were used for frequency data (expressed as percentages). Associations between Duffy genotypes and different variables were assessed and two-sided p-values less than 0.05 were considered as statistically significant.

Results

Characteristics of study participants

A total of 361 PCR-confirmed *P. vivax* (mono or mixed) infected samples were included for the molecular analysis of Duffy status. Among them, 126 (34.9%) were female, the mean age was 25 years old (ranging from 1–80 years old), 108 (29.9%) had no occupation, and 152 (42.1%) participants live in rural areas ([Table 3](#)).

Distribution of the Duffy genotypes by study sites, study participants, ethnicity, gender, and age

From the 361 samples tested, 345 (95.6%) of the *P. vivax* patients were Duffy-positives (272 (78.8%) were heterozygous and 73 (21.2%) were homozygous) and 16 (4.4%) were Duffy-negatives.

Based on Duffy genotyping, 73 (20.2%) were TCT/TCT, 272 (75.4%) were TCT/CCT and 16 (4.4%) were CCT/CCT. The Duffy-negative patients (CCT/CCT genotype) from Arba-minch, Gambella, Metehara and Shewa robit was 2.7%, 8.1%, 4.0%, and 7.1%, respectively ([Fig 2](#)). No Duffy-negatives were detected from Dubti. The highest proportion of Duffy-negatives was in Gambella (8.1%, 6/74) and the lowest in Arbaminch (2.7%, 2/74). There was no significant association between Duffy genotype and study site ($p = 0.12$). The highest proportion of Duffy-negatives was observed in the Nuer ethnic group (12.5%, 2/16) followed by the Agnuwak group (7.7%, 1/13). No Duffy-negatives was identified among the Somale, Afar, Tigre, Welayita and Hadiya ethnic groups. No significant association was found between Duffy genotype and ethnic group ($p = 0.50$). About 5.5% (13/235) male and 2.4% (3/126)

Table 3. Sociodemographic characteristics of study participants in Ethiopia (n = 361).

Variables	Category	Number	Percent (%)
Gender	Female	126	34.9
	Male	235	65.1
Age stratification in years	<14	40	11.1
	15–24	135	37.4
	25–34	122	33.8
	≥35	64	17.7
Ethnic	Amhara	106	29.4
	Anuak	13	3.6
	Somale	6	1.7
	Oromo	76	21.1
	Afar	52	14.4
	Tigre	11	3.0
	Welayita	30	8.3
	Gamo	37	10.2
	Sidama	11	3.0
	Hadiya	3	0.8
	Nuer	16	4.4
Study Sites	Arbaminch	74	20.5
	Dubti	68	18.8
	Gambella	74	20.5
	Metehara	75	20.8
	Shewarobit	70	19.4

<https://doi.org/10.1371/journal.pntd.0011326.t003>

female participants were Duffy-negatives, with no significant association between Duffy genotype and gender ($p = 0.16$). From different age stratification [22], the lowest proportion of Duffy-negatives was identified from the age groups ≤ 14 (2.5%, 1/40) and 25–34 (2.5%, 3/122), while the highest proportion was identified from the age group ≥ 35 (7.8%, 5/64), no significant association between Duffy genotype and age groups ($p = 0.337$). (Table 4). Similarly, among the Duffy-positive participants, the relative proportions of heterozygous and homozygous were not associated with either study site ($p = 0.47$), ethnic group ($p = 0.47$) or gender ($p = 0.17$) (Table 5).

Distribution of the Duffy genotype by *P. vivax* infections

For the 16 *P. vivax* infections in Duffy negatives, 12 (75%) were mono-infection and four (25%) were mixed *P. vivax/P. falciparum* infections. For the 345 *P. vivax* infections in Duffy positives, 312 (90%) were mono-infections and 33 (~10%) were mixed *P. vivax/P. falciparum* infections. The proportion of mixed *P. vivax/P. falciparum* infections was two-fold higher in Duffy-negative than in Duffy positive individuals ($p = 0.047$), despite small samples for the Duffy negatives (Table 6). Of the 73 homozygous Duffy-positives, 68 (93.2%) were pure *P. vivax* infections and 5 (6.8%) were mixed *P. vivax/P. falciparum* infections, slightly lower than that observed in heterozygous Duffy-positives of which 244 (78.2%) were pure *P. vivax* infections and 28 (10.3%) were mixed *P. vivax/P. falciparum* infections ($p = 0.37$) (Table 6).

Asexual and sexual parasitemia comparison by Duffy groups

Of the samples with an asexual stage density of $<1,000$ p/ μ L and ranging from 1,000 to 4,999 p/ μ L, 9/60 (15.0%) and 7/131 (5.3%) were obtained from Duffy-negative individuals,

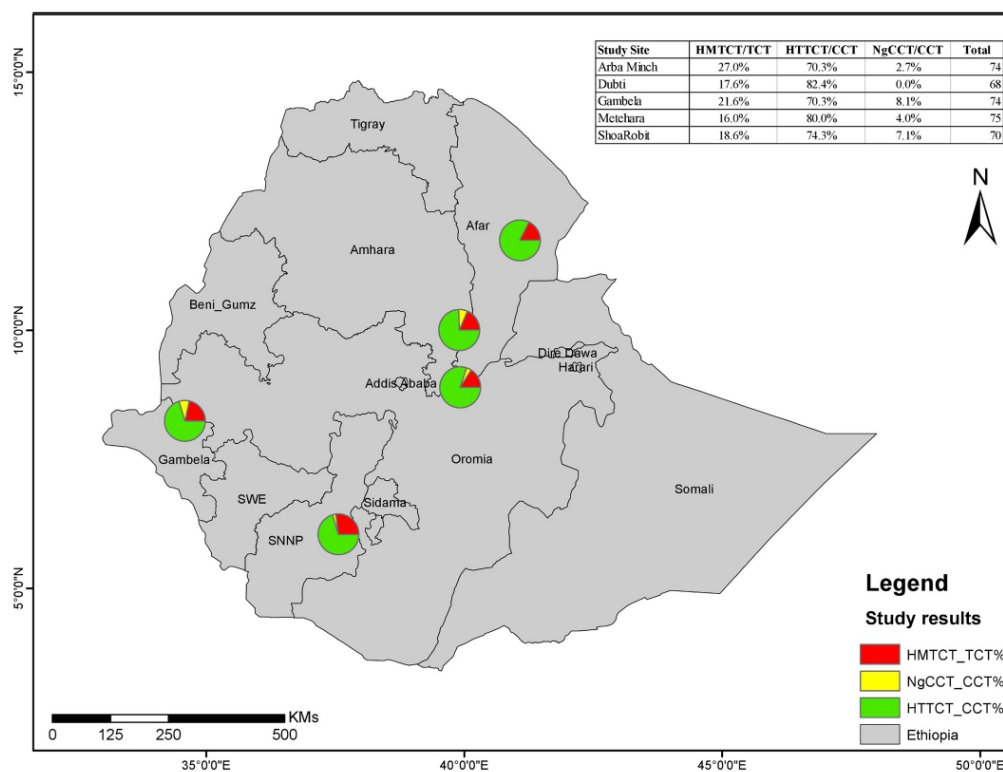


Fig 2. Map showing the distribution of the Duffy genotypes at five different malaria endemic sites in Ethiopia.

<https://doi.org/10.1371/journal.pntd.0011326.g002>

respectively. Interestingly, no Duffy-negatives were detected with asexual parasite density ≥ 5000 p/μL but only Duffy positive patients (n = 170; Table 7). The mean asexual parasite density in homozygous and heterozygous Duffy-positives was 12,165 p/μL (IQR25-75: 1,640–24,234 p/μL) and 11,655 p/μL (IQR25-75: 1,676–14,065 p/μL), respectively, significantly higher than that in Duffy-negatives (1,227 p/μL; IQR25-75: 539–1,732 p/μL) ($p < 0.001$; Fig 3).

Of the samples with a sexual gametocytemia of $< 1,000$ p/μL, 8/180 (4.4%) were detected in Duffy-negative individuals (Table 7). The other eight *P. vivax* infections in Duffy negatives were detected with no gametocytes. By contrast, 172/180 (95.6%) of the Duffy-positives were detected with sexual gametocytemia of $< 1,000$ p/μL, 31 were detected with gametocytemia of 1,000–4999 p/μL, and 4 with gametocytemia of $\geq 5,000$ p/μL.

Discussion

We report here the association between 361 pure *P. vivax* and mixed *P. vivax/P. falciparum* infections collected from five eco-epidemiological study sites in Ethiopia and the Duffy genotype of the patients. We assessed malaria infection by qPCR and polymorphisms in the

Table 4. Analysis of Duffy status by study sites (altitude), study participants ethnicity and gender (n = 361).

Variables		Duffy Status			P-value
		Positive n (%)	Negative n (%)	Total	
Study sites (altitude)	Arbaminch(1200 m)	72 (97.3)	2 (2.7)	74	0.089
	Dubti (379 m)	68 (100)	0	68	
	Gambella (447 m)	68 (91.9)	6 (8.1)	74	
	Metehara (959 m)	72 (96.0)	3 (4.0)	75	
	Shewarobit(1268 m)	65 (92.9)	5 (7.1)	70	
Ethnic of Study participant	Amhara	100 (94.3)	6 (5.7)	106	0.41
	Agnuwak	12 (92.3)	1 (7.7)	13	
	Somale	6 (100)	0	6	
	Oromo	71 (93.4)	5 (6.6)	76	
	Afar	52 (100)	0	52	
	Tigre	11 (100)	0	11	
	welayita	30 (100)	0	30	
	Gamo	35 (94.6)	2 (5.4)	37	
	Sidama	11 (100)	0 (0)	11	
	Hadiya	3 (100%)	0	3	
	Nuer	14 (87.5)	2 (12.5)	16	
	Gender	Female	123 (97.6)	3 (2.4)	
Male		222 (94.5)	13 (5.5)	235	
Age in years	≤14	39 (97.5%)	1 (2.5%)	40	0.34
	15–24	128 (94.8%)	7 (5.2%)	135	
	25–34	119 (97.5%)	3 (2.5%)	122	
	≥35	59 (92.2%)	5 (7.8%)	64	

<https://doi.org/10.1371/journal.pntd.0011326.t004>

GATA-1 promoter affecting Duffy status by a novel ARMS-PCR approach. Here, we observed that the proportion of Duffy-negative (4.4%) among *P. vivax* infected patients was lower than previous studies conducted in different countries; Madagascar (9.3%) [28], different parts of Ethiopia (10.5–11.9%) [16], and Sudan 17.9% [9], but relatively higher than study reports such as Ethiopia 2.9% [22], south-eastern Iran 1.3% [29], Brazilian Amazon 0.6% [18]. Duffy blood groups remain highly polymorphic between different populations [30]. Historically, individuals with the Duffy-negative phenotype were thought to be resistant to *P. vivax* infection [18,19,31–33], but recent reports have shown *P. vivax* infection in Duffy-negative individuals [20,34–38] in many parts of Africa.

This study identified 4.4% (16/361) *P. vivax* infections in Duffy-negative patients. This is different from Central or West Africa, where the Duffy-negative genotype is much more common and almost all *P. vivax* infections occur among Duffy-negative individuals [37,39,40], despite relatively low number of *P. vivax* cases compared to East Africa. Several hypotheses have been put forward to explain the change in the pattern of infection in Duffy-negative individuals. One is a change in the host susceptibility, which in turn may drastically affect the distribution of *P. vivax* [9] and another is that *P. vivax* may have evolved to adapt to a wider range of hosts, environments, and highly competent transmission vectors in Africa [34]. Alternatively, *P. vivax* parasites may have developed alternative mechanisms to bind and invade reticulocytes as other host cell receptors have been shown to be involved in erythrocyte invasion [41].

Polymorphisms in Duffy blood group among different ethnic groups may contribute to the changing epidemiology of *vivax* malaria in Africa [42]. The Ethiopian population has a very

Table 5. Analysis of heterozygous and homozygous Duffy positive patients with study site (altitude), ethnicity, and gender (n = 312).

Variables		Duffy Positive			P-value		
		Homozygous n (%)	Heterozygous n (%)	Total			
Study Sites (altitude)	Arbaminch	20 (27.8)	52 (72.2)	72	0.47		
	Dubti	12 (17.6)	56 (82.4)	68			
	Gambella	16 (23.5)	52 (76.5)	68			
	Metehara	12 (16.7)	60 (83.3)	72			
	Shewarobit	13 (20.0)	52 (80.0)	65			
Ethnicity of study participants	Amhara	20 (20.0)	80 (80.0)	100	0.47		
	Agnuwak	4 (33.3)	8 (66.7)	12			
	Somale	0 (0.0)	6 (100)	6			
	Oromo	13 (18.3)	58 (81.7)	71			
	Afar	10 (19.2)	42 (80.8)	52			
	Tigre	2 (18.2)	9 (81.8)	11			
	welayita	7 (23.3)	23 (76.7)	30			
	Gamo	10 (28.6)	25 (71.4)	35			
	Sidama	5 (45.5)	6 (54.5)	11			
	Hadiya	0 (0)	3 (100)	3			
	Nuer	2 (14.3)	12 (85.7)	14			
	Gender	Female	31 (25.2)	92 (74.8)		123	0.17
		Male	42 (18.9)	180 (81.1)		222	
Age in years	≤14	4 (10.3%)	35 (89.7%)	39	0.284		
	15–24	31 (24.2%)	97 (75.8%)	128			
	25–34	24 (20.2%)	95 (79.8%)	119			
	≥35	14 (23.7%)	45 (76.3%)	59			

<https://doi.org/10.1371/journal.pntd.0011326.t005>

heterogeneous ethnic composition. Eleven different ethnic groups were included in this study and the proportion of Duffy-negative and Duffy-positive varied between ethnic groups and study sites. A high proportion of Duffy-negative samples were observed among the Nuer followed by the Agnuwak ethnic groups, but no Duffy-negative was identified among the Somale, Afar, Tigre, Welayita and Hadiya ethnic groups. Other studies have also showed variation of Duffy genotype between ethnic groups. For example, in Sudan the Duffy-negative phenotype was identified in 83% of Shagia and 77% of Manasir tribes [43]; in Mauritania 54% of Moors were Duffy-positives while only 2% of black ethnic groups were Duffy-positives [42]; in Colombia Duffy genotypes were significantly associated with ethnicity [44]. These findings suggest that ethnic groups may be genetically distinct, resulting in different levels of susceptibility to *P. vivax* infection, in addition to different hypothesis such as *P. vivax* trains with a

Table 6. Analysis of *P. vivax* pure and mixed *P. vivax/P. falciparum* infections with Duffy genotype (n = 361).

Variables		<i>P. vivax</i> infections			Chi-Square	P-value
		Pure n (%)	Mixed n (%)	Total		
Duffy genotype	positive	312 (90.4)	33 (9.6)	345	3.96	0.047
	negative	12 (75)	4 (25)	16		
	Total	324 (89.8)	37 (10.2)	361		
DuffyPositive	homozygous	68 (93.2)	5 (6.8)	73	0.79	0.37
	heterozygous	244 (89.7)	28 (10.3)	272		
	Total	312 (90.4)	33 (9.6)	345		

<https://doi.org/10.1371/journal.pntd.0011326.t006>

Table 7. Association of Duffy status with asexual parasite density (n = 361).

Variables	Duffy Status			Chi-Square	p-value
	Positive n (%)	Negative n (%)	Total		
Asexual stage density	<1000	51 (85.0)	9 (15.0)	23.96	<0.001
	1000–4999	124 (94.7)	7 (5.3)		
	≥5000	170 (100)	0 (0)		

<https://doi.org/10.1371/journal.pntd.0011326.t007>

new capacity for erythrocyte invasion [28], or use several receptor-ligand interactions to tightly bind erythrocytes in the absence of a Duffy receptor [21].

Duffy-negatives with pure *P. vivax* and mixed *P. vivax/P. falciparum* infections was identified in the current study. The proportion of mixed *P. vivax/P. falciparum* infections in Duffy-negative was 25% in the present study, which was similar with the findings from Nigeria (25%)

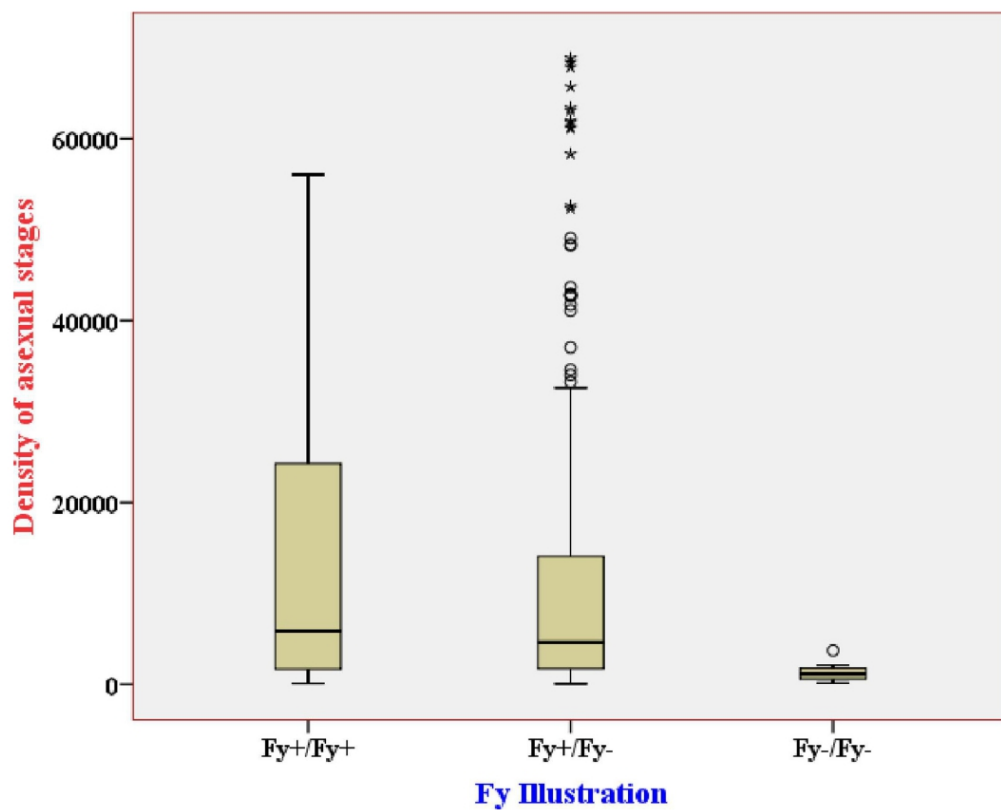


Fig 3. Box plot showing the distribution of asexual parasite density among identified Duffy status. Key: Fy+/+; homozygous Duffy-positive, Fy+/-; Heterozygous Duffy-positive, Fy-/-; Duffy-negative.

<https://doi.org/10.1371/journal.pntd.0011326.g003>

[45], and Cameroon (25%) [46], while it was lower than the study conducted in Madagascar (26.7%) [47], and Democratic Republic of the Congo (64%) [39]. The results provide evidence that Duffy-negative individuals can be infected by pure *P. vivax* alone or together with *P. falciparum*.

The mean asexual parasite density among Duffy-negatives (CCT/CCT) was 1,227 p/μl, around tenfold lower compared to homozygous Duffy-positives (TCT/TCT; 12,165 p/μl) and heterozygous Duffy-positive (TCT/CCT; 11,655 p/μl). Several prior studies have shown that *P. vivax* infections observed in Duffy-negatives have a significantly lower parasitemia than in Duffy-positive infections and are asymptomatic [13,16]. The low parasite densities found in Duffy-negative malaria patients may indicate that *P. vivax* uses an alternative pathway to invade Duffy-negative reticulocytes, which can be much less efficient than the classical pathway involving the interaction between PvDBP and DARC [22]. Alternatively, it has been hypothesized that the reticulocytes of Duffy-negative individuals may express the DARC protein in a transient and weak manner, thereby mediating a low invasion capacity [16]. Among the 16 Duffy negative samples detected in this study, half were detected with low-level gametocytes. The presence of gametocytes among Duffy negatives may further imply that these infections have the potential contributing to onward transmission.

To conclude, this study reports heterogeneous proportions of Duffy-negative individuals among *P. vivax* malaria patients across Ethiopia, confirms that Duffy-negativity does not provide complete protection against *P. vivax* infection, and these infections are often associated with low parasitemia, which may represent hidden reservoirs that can contribute to transmission. Therefore, the development of *P. vivax*-specific elimination strategies, including alternative antimalarial vaccines other than a Duffy-binding protein-based vaccine, should be facilitated by a better understanding of the relationship between Duffy-negativity and the invasion mechanisms of *P. vivax*.

Supporting information

S1 Appendix. Figures summarizing Sanger sequencing data.
(TIF)

S2 Appendix. Figures summarizing AMRS PCR-based allelic discrimination amplification curves in a control samples validated by PCR/Sanger sequencing.
(TIF)

Acknowledgments

We would like to thank i) SIDA project, ii) Human, Heredity and Health in Africa (H3Africa) [H3A-18-002]. H3Africa is managed by the Science for Africa Foundation (SFA Foundation) in partnership with Wellcome, NIH and AfSHG and iii) the Institute Pasteur, Paris, the University of Strasbourg, and the French Government (Agence Nationale de la Recherche; ANR-18-CE15-0026, ANR-21-CE35-0006 and the Laboratoire d'Excellence 'French Parasitology Alliance for Health Care', ANR-11-15 LABX-0024-PARAFRAP), and iv) EPHI for the materials support. We would also like to thank all the individuals who contributed technically to this study: the laboratory staff at the study sites, the staff at ALIPB (parasitology department), the staff at the Institute of Parasitology and Tropical Diseases, University of Strasbourg (Cécile Doderer-Lang), and those who were provided support on Arc Map and statistical analysis (Dereje Dilu, Abriham Keraleme and Chalie). We must express our sincere gratitude to all the regional health offices and the study sites for their cooperation. Last but not least, our sincere thanks go to all the participants who took part in this study.

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III. Functional study on *P. vivax* invasion pathways

The third objective of my thesis project was to further investigate *P. vivax* invasion pathways in Duffy-negative patients. I was very enthusiastic about the opportunity to design and perform *in vitro* functional assays. In close collaboration with the Red Cell Haematology Laboratory of King's College London, we first characterized Duffy-negative erythroid progenitors during terminal erythroid differentiation. Based on this characterization, we designed custom *in vitro* invasion assays to assess whether Duffy-negative erythroblasts are permissible to *P. vivax* merozoites.

1. The DARC side of *P. vivax* in Africa

It is not that I am a big fan of the Star Wars saga, but the title « The DARC side of *P. vivax* in Africa: invasion pathways in Duffy-negative erythroblasts » really made sense when we realized why *P. vivax* infections in Duffy-negative patients were so silent and insidious. It all started when we tried to mature *P. vivax* parasites and isolate enough reticulocytes to perform *in vitro* invasion assays. Noticing a lack of information on the development of Duffy-negative erythroblasts and learning from a few failed attempts, we decided to seek advice from hematologists. First, we hypothesized that Duffy-negative erythroblasts express DARC during terminal erythroid differentiation¹⁸. We isolated Duffy-positive and Duffy-negative CD34+ hematopoietic stem and progenitor cells (HSPCs), placed them in a two-phases culture system and followed their differentiation kinetics, apoptosis rate, enucleation yield as well as their expression of DARC and CD71 during terminal erythroid differentiation. Results revealed that a subset of Duffy-negative erythroblasts express DARC. Second, we hypothesized that this subset of Duffy-negative erythroblasts expresses functional DARC. We designed custom *in vitro* invasion assays and assessed whether Duffy-negative erythroblasts were permissible to *P. vivax* merozoites. Results revealed that *P. vivax* merozoites can invade Duffy-negative erythroblasts that express both CD71 and DARC. Thus, our functional study provides the first biological evidence that Malagasy and Ethiopian *P. vivax* merozoites can invade Duffy-negative erythroblasts. It also supports the assumption that Duffy-negative African populations may be a silent reservoir of infection with all the consequences that this entails for the control and eradication of *vivax* malaria in Sub-Saharan Africa¹. The draft of a scientific article named « the DARC side of *P. vivax* in Africa: invasion pathway in Duffy-negative erythroblasts » is being written and is presented below.

**The DARC side of *vivax* malaria in Africa:
unveiling invasion pathways into Duffy-negative erythroblasts**

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Abstract

Vivax malaria was long thought to be absent from sub-Saharan Africa owing to the high prevalence of people lacking the Duffy antigen receptor for chemokines (DARC) in their erythrocytes. The interaction between *P. vivax* Duffy binding protein (PvDBP) and DARC is assumed to be the main mechanism by which *P. vivax* merozoites invade human erythrocytes. However, increasing numbers of *vivax* malaria cases in Duffy-negative African individuals has raised questions about *P. vivax* invasion pathway(s). Since *P. vivax* has a tropism for CD71+ immature reticulocytes and the hematopoietic niches of the bone marrow may be a hidden reservoir of *P. vivax* parasites, we hypothesized that *P. vivax* merozoites may be able to invade Duffy-positive (DP) and Duffy-negative (DN) erythroblasts.

Here, we recapitulated *in vitro* infection of DP and DN erythroblasts with *P. vivax* merozoites. We showed that a subset of DN erythroblasts express DARC during terminal erythroid differentiation and that *P. vivax* merozoites, irrespective of their origin (Madagascar and Ethiopia), can invade DN erythroblasts expressing DARC. This study provides the first biological evidence of the ability of *P. vivax* to invade genotypically DN erythroblasts and highlights that African human populations may represent a silent reservoir of *P. vivax* infections, with major implications for the control and elimination of *vivax* malaria in sub-Saharan Africa.

Key words: malaria, *Plasmodium vivax*, Duffy-negativity, erythropoiesis, invasion pathways

Introduction

More than one-third of the world's population is affected by *vivax* malaria, an acute debilitating disease caused by *Plasmodium vivax* and transmitted by female *Anopheles* mosquitoes. In 2021, there were an estimated 5 million *vivax* cases worldwide, mainly in Asia, Southeast Asia, South America, Western Pacific, Eastern Mediterranean regions, Eastern Africa, and Southern Africa (*WHO malaria report 2022*).

For a long time, *vivax* malaria was thought to be absent from sub-Saharan Africa. This was based on clinical and field studies conducted in the 1940s showing natural resistance to *vivax* malaria in Africans and Afro-Americans (*Boyd and Stratman-Thomas 1933; Bray 1958; Young et al. 1955; Young, Ellis, and Stubbs 1947*). Later, the identification of Duffy blood groups (*Marie Cutbush, Mollison, and Parkin 1950; M. Cutbush and Mollison 1950*) and the demonstration of the molecular origin of Duffy-negativity, as a single point mutation in the GATA-1 promoter sequence of the *ackr1* gene (*Tournamille et al. 2005; 1995*), linked the natural resistance to *vivax* malaria to the absence of Duffy antigen receptors for chemokines (DARC) on the surface of human erythrocytes (*Miller et al. 1976*). Further pioneering works in the 1980s and 1990s identified *P. vivax* Duffy binding protein (PvDBP) as a specific ligand for DARC and confirmed that the interaction between PvDBP and DARC is critical for reticulocyte invasion (*Barnwell, Nichols and Rubinstein 1989; Chitnis et Miller 1994*). More recently, additional studies revealed CD71 (transferrin receptor, TfR1) and CD98 as the respective cognate receptors for the *P. vivax* reticulocyte binding protein 2b (PvRBP2b) and the *P. vivax* reticulocyte binding protein 2a (PvRBP2a) (*Benoit Malleret et al. 2015; Gruszczyk et al. 2018; Benoît Malleret et al. 2021*). Collectively, these findings led to a scientific paradigm by which *P. vivax* recognize genotypically Duffy-positive (DP) reticulocytes through interactions with PvDRBP2b-CD71 and PvRBP2a-CD98 and then invade them through interaction with PvDBP-DARC (*Bouyssou et al. 2022*).

However, this paradigm has been called into question. With the advent of PCR technologies, an increasing number of *P. vivax* infections has been reported in genotypically Duffy-negative (DN) sub-Saharan African populations (*Albsheer et al. 2019; Ménard et al. 2010; Mendes et al. 2011; Ngassa Mbenda et Das 2014; Niangaly et al. 2017; Poirier et al. 2016; Rubio et al. 1999; Russo et al. 2017; Ryan et al. 2006; Woldearegai et al. 2013; Wurtz et al. 2011*). In many cases, these infections were characterised by submicroscopic or very low levels of parasitaemia (*Baird 2022*). In parallel, it was shown that *P. vivax* preferentially invades CD71⁺ immature reticulocytes, which are mainly found in extravascular erythropoietic tissues, such as the bone marrow and the spleen (*Benoit Malleret et al. 2015*). Finally, a recent study showed that the hematopoietic niches of the bone marrow may be a hidden reservoir of sexual and asexual *P. vivax* parasites (*Silva-Filho et al. 2020*).

To study and identify host receptors used by *P. vivax* for invasion of erythroblasts, here, we recapitulated *in vitro* infection of DP and DN erythroblasts with *P. vivax* merozoites. We performed *in vitro* erythropoiesis assays of genotypically DN and DP erythroid progenitors to critically evaluate erythroid differentiation as well as CD71 and DARC expression profiles over time. Then, we conducted *in vitro* invasion assays to investigate whether DARC expressed on genotypically DN erythroblasts is functional for *P. vivax* invasion. We showed that a subset of genotypically DN erythroblasts express DARC during terminal erythroid differentiation and that this subpopulation is susceptible to *P. vivax* merozoite invasion. This study provides the first biological evidence that *P. vivax* is capable of invading DN erythroblasts.

Results

Genotypically DP and DN erythroid progenitors show similar kinetics of differentiation.

To determine whether DN erythroid progenitors have similar erythroid differentiation kinetics to genotypically DP erythroid progenitors, we performed isolation of CD34⁺ hematopoietic stem and progenitor cells (HSPCs) from the peripheral blood of four DP and four DN healthy

donors, expansion, and differentiation *in vitro* (**Table 1 & Figure 1A**). The kinetics of terminal erythroid differentiation, apoptosis rates, and enucleation yields were monitored every other day, for 12 days, by optical microscopy and flow cytometry. We observed that both DP and DN erythroid progenitors presented the expected pattern with pro-erythroblasts maturing into early basophilic erythroblasts, late basophilic erythroblasts, polychromatic erythroblasts, and orthochromatic erythroblasts (*Hu et al. 2013*) (**Figures 1B-1D**). The mean percentage of DP and DN erythroblasts at orthochromatic stage was respectively 0.08% and 0% at D0, 19.8% and 6.7% at D6 and 85% and 62.1% at D12 (**Figure 1C**). Overall, no significant difference between the kinetics of terminal erythroid differentiation of DP and DN erythroblasts was observed over time (ANCOVA, $p=0.4$ for pro-erythroblasts, $p=0.2$ for early basophilic erythroblasts, $p=0.6$ for late basophilic erythroblasts, $p=0.9$ for polychromatic erythroblasts and $p=0.08$ for orthochromatic erythroblasts). However, we found considerable variation within DP and DN erythroblasts. The relative standard deviation (RSD) of the proportions of erythroblast stages varied from 18.1% to 200% for DN erythroblasts and from 5.3% to 179.6% for DP erythroblasts. No significant difference in variability was detected between DP and DN erythroblasts ($p=0.15$, Mann-Whitney test).

The enucleation rate was also monitored at D8 (mean: 4.6% for DP and 1.3% for DN, $n=3$) and at D10 (mean: 28.2% for DP and 12.0% for DN, $n=4$). Although we found no significant difference in the proportions of enucleated cells between DN and DP erythroblasts (ANCOVA, $p=0.14$), we observed a slight delay in the enucleation of DN erythroblasts (**Figures S1A-B**). Finally, low, and similar proportions of GPA⁺ Annexin V⁺ cells (reflecting apoptosis) were found in both DP and DN erythroblasts, indicating that they survived in similar rates during *in vitro* differentiation (**Figures S1C-D**). These results suggest that both genotypically DP and DN erythroblasts have similar kinetics of terminal erythroid differentiation.

A subset of genotypically DN erythroblasts express DARC during terminal erythroid differentiation. To determine the proportions of genotypically DP and DN erythroblasts expressing DARC and CD71 during terminal erythroid differentiation, we quantified the DARC/CD71 expression profiles every other day, for 12 days, by flow cytometry (*Figure 2A*). We observed that the mean percentage of DP erythroblasts expressing DARC varied from 24.7% at D0 (mostly proerythroblasts) to 78.3% at D8 (mostly polychromatic and orthochromatic erythroblasts) and to 51% at D12 (mostly orthochromatic erythroblasts) (*Figures 2B & 2D*). The proportion of DARC+ DP erythroblasts did not differ significantly over time ($p=0.07$, one-way analysis of variance), but was highly variable within the four donors. The RSD ranged from 57% (D8) to 112% (D0) (*Figure S2A*). We observed that a subset of DN erythroblasts also expressed DARC. The mean percentage of DN erythroblasts expressing DARC was lower than DP erythroblasts, varying from 0.95% at D0 (mostly proerythroblasts) to 1.6% at D8 (mostly polychromatic and orthochromatic erythroblasts) and to 3.2% at D12 (mostly orthochromatic erythroblasts) (*Figures 2C-D*). Again, the proportion of DARC+ DN erythroblasts did not differ significantly over time ($p=0.7$, one-way analysis of variance) but was highly variable within the four donors, with a RSD ranging from 7% (D10) to 141% (D0) (*Figure S2A*).

We further confirmed the expression of DARC on DP and DN erythroblasts and erythrocytes by Western blot analysis (*Figures 2E-F*). We detected DARC expression on both DP and DN erythroblasts (*Figures 2E-F*). However, DN erythrocytes were not DARC+ (*Tournamille et al. 1995*) (*Figure 2F*). Finally, the expression of DARC on a subset of DN erythroblasts and on DP erythroblasts at D8 of terminal differentiation was confirmed by fluorescence microscopy (*Figures 2G-H*).

We found similar CD71 expression profiles in both DP and DN erythroblasts (*Figure S2B*, *Figure S3A*). The mean percentage of DP erythroblasts expressing CD71 was 97.3% at D0 and

95.3% at D12 (**Figures S3B & S3D**), similar to the mean percentage of DN erythroblasts expressing CD71 (100% at D0 and 94.3% at D12) (**Figures S3C-D**). These results show that most of DP erythroblasts express both DARC and CD71, the two main surface receptors required for *P. vivax* invasion, while only a subset of DN erythroblasts does (**Figures S3E-H**).

***P. vivax* merozoites can invade DN erythroblasts during terminal erythroid differentiation.** To determine whether the subset of DN erythroblasts express functional DARC and CD71 receptors for *Pv*DBP and *Pv*RBP2b and allow *P. vivax* merozoites invasion, we performed isolation of CD34+ HSPCs from the peripheral blood of two DP and two DN healthy donors, *in vitro* expansion, and differentiation (**Table 1 & Figure 3A**). Both DP and DN erythroid progenitors differentiated and reached the terminal differentiation stage as above. DARC and CD71 expression profiles were consistent with our findings above for both DP and DN erythroblasts. At D7 of terminal differentiation, we thawed and cultured parasite isolates obtained from two Malagasy and seven Ethiopian patients infected with *P. vivax* (**Table S1 & Figure 3A**). After approximately 24-30 hours of *in vitro* maturation (at D8 of terminal differentiation for DP and DN erythroblasts) (**Figure S4**), each *P. vivax* parasite culture was placed in co-culture with either DP or DN erythroblasts for 24-48 hours. We assessed the infection of DP and DN erythroblasts by *P. vivax* parasites by light microscopy. About 50,000 cells were cytospin onto glass microscope slides and stained with May-Grunwald-Giemsa stain. Of the 22 co-cultures, we were able to detect *P. vivax* infection in both DP and DN co-cultures (**Table S1**). We observed very few parasites, either at ring-stages (**Figures 3B, 3C & 3F, Figures S6A-C**) or at more mature stages with large chromatin dots and hemozoin (**Figures 3B & 3G, Figure S6D**). No Schuffner's dots were seen in the infected erythroblasts. We confirmed the infection of DP and DN erythroblasts with *P. vivax* parasites by fluorescence microscopy. About 3×10^6 erythroblasts per condition were stained with *P. vivax* 18S rRNA FISH probe,

anti-*Plasmodium* HSP70, anti-DARC and anti-CD71 antibodies. Of the 22 co-cultures, we detected *P. vivax* infections in both DP and DN co-cultures (**Figures 3B-G, Figure S5**). Dual staining with the 18S rRNA FISH probe and the anti-*Plasmodium* HSP70 antibody confirmed the presence of *P. vivax* parasites in 1/4 DP and 2/4 DN co-cultures (**Figures 3B-C**). Successful invasion for one given *P. vivax* strain was observed either in both DP and DN erythroblasts or in none of them (**Table S1**). As observed by light microscopy, both *P. vivax* parasites in DP and DN erythroblasts had a similar profile in that, close to the erythroblast nuclei, we could observe the parasite nuclei, sometimes surrounded by signals corresponding to the *P. vivax* 18S rRNA and/or HSP70, together with the expression of CD71 and DARC on the surface of the erythroblasts (**Figures 3B-G, Figure S5, Figure S6**). We found that all the DP and DN erythroblasts that were infected with *P. vivax* were CD71+ and DARC+. These results confirm that *P. vivax* can invade DP and DN erythroblasts expressing both DARC and CD71, irrespective of the origin of the isolates.

Discussion

Our study provides some answers regarding *P. vivax* invasion pathways in DN patients. We explored the terminal erythroid differentiation of DN erythroid progenitors, their potential to express DARC and CD71, and the invasion pathway of *P. vivax* merozoites in DN erythroblasts. The expression profile of DARC in DP erythroblasts was consistent with previous observations, in that most DP erythroblasts expressed DARC (*Southcott, Tanner, et Anstee 1999*). Interestingly, we detected that a subset of DARC + DN erythroblasts. This suggests that a single point mutation in the GATA-1 site of the *ackr1* gene promoter can significantly reduce the number of DARC+ cells (*Tournamille et al. 1995*). This suggests that the transcription factor GATA-1 may be able to bind to the promoter of the *ackr1* gene and activate the transcription of the DARC mRNA in a subset of DN erythroblasts. This observation was further confirmed

by the detection of DARC in cytoplasmic protein extracts from DN erythroblasts, but not in the extracts from DN erythrocytes by Western immunoblotting. *In vivo*, the maturation of orthochromatic erythroblasts into reticulocytes involves various changes including enucleation, haemoglobinization, remodelling of the membrane and the expression or depletion of certain proteins (Moras, Lefevre, et Ostuni 2017). Our data suggest that DN erythroblasts may lose their ability to express DARC at the end of terminal erythroid differentiation when orthochromatic erythroblasts enucleate to produce reticulocytes or when reticulocytes migrate to the peripheral blood and mature into erythrocytes. However, the analysis performed here did not provide robust evidence as to whether or not the subset of DN erythroblasts expressing DARC give rise to DN reticulocytes expressing DARC. We cannot exclude that the sensitivity of the technique, which is based on measuring total cytoplasmic proteins, may not be sufficient to detect DARC⁺ cells in a subset of DN reticulocytes or erythrocytes due to the presence of large amounts of other proteins such as haemoglobin.

We found that the expression profiles of both DP and DN erythroblasts showed high inter-individual variability. This may be due to genetic variation in the *ackr1* locus or upstream regulation in the expression of the transcription factor GATA-1 and / or the transcription of mRNA. However, the expression profiles of CD71 in both DP and DN erythroblasts were consistent with previous observations (Dong, Wilkes, et Yang 2011). Indeed, CD71 is highly expressed in erythroid precursors at all stages of maturation and then progressively reduced as reticulocytes mature (Brun, Gaudernack, et Sandberg 1990; Kono et al. 2009; Benoît Malleret et al. 2013). Given the specific tropism of *P. vivax* merozoites for CD71⁺ immature reticulocytes (Benoit Malleret et al. 2015) and the importance of the PvRBP2b-CD71 interaction for the recognition of DP reticulocytes prior to invasion (Gruszczyk et al. 2018), it is likely that *P. vivax* infections are restricted to CD71⁺ erythroid progenitors, erythroblasts and / or immature reticulocytes.

Based on the characterization of DN erythroblasts and because the development of such functional assays requires a large number of erythroblasts, we established the co-cultures at D8 of terminal differentiation. Both DP and DN erythroblasts were infected by either Malagasy or Ethiopian *P. vivax* merozoites. In co-cultures with *P. vivax* parasites and DN erythroblasts, only DARC+ DN erythroblasts were invaded by the parasites. These surprising observations indicate that DN erythroblasts express functional DARC and that *P. vivax* merozoites can use the PvDBP-DARC invasion pathway, irrespective of the origin and genetic background of *P. vivax* strains. Our findings likely rule out the possibility that *P. vivax* has evolved an alternative invasion pathway to overcome Duffy-negativity (Kanjee et al. 2021; Ntumngia et al. 2016). Taken together, our data support the emerging evidence that the hematopoietic niches of the bone marrow may represent a hidden reservoir of *P. vivax* parasites (Silva-Filho et al. 2020) and that *P. vivax* parasites may have established stable transmission at low levels in sub-Saharan Africa (Baird 2022). The bone marrow hosts the majority of all hematopoietic stem cells, progenitor cells and CD71+ immature reticulocytes (Orkin et Zon 2008; Ivanovs et al. 2017). As only a subset of DN erythroblasts express DARC, the scarcity of permissible cells and their restriction to the bone marrow may explain why *vivax* malaria in DN patients is often characterized by very low parasitaemia. (Baird 2022). These findings have important implications for the development of therapeutic approaches or vaccines against *vivax* malaria. Indeed, the evidence that *P. vivax* merozoites invade DARC+ DN erythroblasts, suggests that the leading vaccine candidate targeting the PvDBPII binding domain of PvDBP in *P. vivax* merozoites (Payne et al. 2017; Singh et al. 2018) could be used to prevent *vivax* malaria in both DP and DN populations.

Although this experimental work represents a breakthrough in our understanding of *P. vivax* invasion pathways, it can be further optimized. For example, the high inter-individual variability observed has prevented us from fully characterizing DN erythroblasts *in vitro*.

Furthermore, *in vitro* invasion assays may not fully recapitulate the *in vivo* invasion mechanisms used by *P. vivax*. They resulted in few infected cells, suggesting that the successful invasion of DN erythroblasts by *P. vivax* merozoites is likely to be rare. In fact, the successful invasion of DN erythroblasts by *P. vivax* merozoites may strongly depend on culture conditions, such as the viability of *P. vivax* parasites from cryopreserved parasite samples, parasitaemia, etc. In our hands, the low number of infected cells made it difficult to quantify and evaluate the inhibition of *P. vivax* invasion into erythroblasts by anti-DARC antibodies or by *in vitro* assays using Duffy-null erythroblasts from immortalized erythroid cell lines (knock-out of the *ackr1* gene). Therefore, *ex vivo* field studies are needed to obtain fresh *P. vivax* isolates, overcome technical problems, and perform further investigations.

In conclusion, this study provides new insights into how genotypically DN erythroblasts can be infected by *P. vivax* merozoites. Our data suggest that *P. vivax* merozoites use the PvDBP-DARC invasion pathway to invade the subset of DARC + DN erythroblasts. However, their scarcity and restriction to the bone marrow may explain the low parasitaemia observed in DN patients (Baird 2022). This study also suggests that the classification of Duffy blood groups may be more complex than expected and highlights the potential existence of a hidden reservoir of *P. vivax* parasites in the bone marrow in sub-Saharan Africa. However, this study does not completely exclude the potential existence of an alternative invasion pathway (Kanjee *et al.* 2021; Ntumngia *et al.* 2016).

Acknowledgments

We would like to thank all the patients and donors who agreed to provide blood samples, Sabine Haas (Etablissement Français du Sang de Strasbourg) for coordinating the blood donations, Olivier Silvie (Centre d'Immunologie et des Maladies Infectieuses de Paris) for providing anti-HSP70 polyclonal antibody, and Pascal Kessler (Centre de Recherche Biomédicales de Strasbourg) for helping with the image acquisition (fluorescent microscopy). This study was supported by a grant overseen by the French National Research Agency (VIPeRs, ANR-18-CE15-0026), by the Institut Pasteur, Paris, the Laboratoire d' Excellence (LabEx) "French Parasitology Alliance for Health Care" (ANR-11-15 LABX-0024-PARAFRAP) and the University of Strasbourg through the Programme IdEX 2022 (Attractivite — Dotation d' accueil des professeurs d' universite recrutes en 2021). IB has received financial supports from the French National Research Agency (VIPeRs, ANR-18-CE15-0026), the FRM (Fondation pour la Recherche Médicale, FRM FDT202204014872) and the Fondation Thérèse Lebrasseur (DM was the winner of the Fondation Thérèse Lebrasseur award in 2021). SEH has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 101024970.

Author contributions

IB, SEH, JS and DM designed the study; MS, IVW and LG provided the biological samples; IB, CDL and SEH, JS and DM conducted and supervised the experiments; IB and CDL acquired the data; IB, CDL, CEC, SEH, JS and DM analyzed data; IB, CEC, SEH, JS and DM wrote the manuscript. All authors read and edited the manuscript.

Disclosure of conflicts of interest

The authors declare no conflict of interest.

Materials & Methods

Biological samples

Whole blood samples

Whole blood samples from Duffy-positive (DP) and Duffy-negative (DN) healthy donors were provided by the Etablissement Français du Sang (EFS) in Strasbourg, according to the Ethics Committees of EFS and the University of Strasbourg. All patients provided informed consent and when participants were children, parents or guardians provided consent.

***P. vivax* isolates**

Clinical *P. vivax* isolates were obtained from symptomatic patients infected with *P. vivax* from health centers in Madagascar and in Ethiopia, according to the declaration of Helsinki with approval from the Ministry of Health, National Ethics Committee. Samples were cryopreserved in liquid nitrogen (*Supplementary Methods*).

Duffy genotyping

Duffy genotype of blood donors was determined by Polymerase Chain Reaction (PCR) and Sanger sequencing as previously described (Ménard *et al.* 2010) (*Supplementary Methods*).

Isolation of human CD34+ cells

Human DP and DN CD34+ cells were isolated by positive magnetic sorting using a CD34+ progenitor cell isolation kit (Miltenyi biotec) and following the supplier's protocol (*Supplementary Methods*).

***In vitro* differentiation of human erythroid progenitors**

Human DP and DN CD34+ cells were differentiated using the two-phase *in vitro* culture system, as previously described (El Hoss et al. 2021) (*Supplementary Methods*).

***In vitro* invasion assays**

Human DP and DN CD34+ cells were expanded and differentiated using a two-phase *in vitro* culture system as previously described (El Hoss et al. 2021). At Day 7 of terminal differentiation, *P. vivax* cryopreserved isolates were thawed and placed in culture for maturation. After approximately 24-30 hours of *in vitro* maturation, *P. vivax* parasites were placed in co-culture with either DP or DN erythroblasts and incubated at 37°C under gas conditions (5% O₂, 5% CO₂, 90% N₂) for 48h (*Supplementary Methods*).

May-Grünwald Giemsa staining and microscopy

Cells were harvested, washed, and spun on glass slides at 3000rpm for 3min using a cytospin centrifuge (Shandon). The dot smears were stained with May-Grünwald Giemsa (MGG) staining (Sigma-Aldrich) following the supplier's instructions and then observed by optical microscopy using 20X, 40X and 50X objectives (Echo).

Flow cytometry

For staining assays, 1x10⁵ cells per condition were harvested, washed, incubated with fluorescently conjugated antibodies (BD Biosciences, BD Pharmingen, IBGRL, Miltenyi biotec) and dyes (Invitrogen, BD Biosciences) and analyzed using the Fortessa or Attune NxT Acoustic Focusing flow cytometers. Data were analyzed using FlowJo software version 10 (BD Biosciences) (*Supplementary Methods*).

Protein extraction and Western immunoblot

Approximately 3×10^6 cells per condition were harvested and treated with NE-PER lysis buffer (Invitrogen) according to the supplier's protocol. Cytoplasmic proteins were electrophoresed through 4-12% SDS-polyacrylamide gel and electroblotted to a nitrocellulose membrane using the Trans-Blot Turbo system (Bio-Rad) and according to the supplier's recommendations. Membranes were blocked and incubated with primary and secondary antibodies. Proteins were revealed using the chemiluminescent detection assay Clarity Max Western ECL (Bio-Rad) and the Chemidoc gel documentation system (Bio-Rad). Quantification of immunodetected proteins was performed using the Image Lab software version 6.1 (Bio-Rad) (*Supplementary Methods*).

Fluorescent microscopy

Approximately 3×10^6 cells per condition were harvested after 24h and 48h of invasion. They were washed, fixed, blocked and / or permeabilized. Cells were incubated with fluorescently conjugated antibodies (Fisher, Invitrogen) and / or a *P. vivax* RNA 18S FISH probe (Stellaris) or anti-*Plasmodium* HSP70 at 37°C in the dark. They were counter stained using ProlongDiamond antifade mountant with diamidino phenylindole (DAPI) (Fisher) and covered using coverslips (EpreDia). *P. vivax* infected erythroblasts were visualized at 20X and 63X magnification lenses using an Apotome microscope (Zeiss). Analysis was performed using Zen software version 3.7 (Zeiss) (*Supplementary Methods*).

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Tables

Table 1: Duffy genotyping of the healthy blood donors.

Blood donor	Mutation GATA-1	Mutation Fy A/B	Mutation Fy			Phenotype	Use in this study
			Codon 49	Codon 89	Codon 100		
DP001	TCT	GAT (b)	GCC	CGC	ACA	Fy (a-b+)	Characterization
DP002	TCT	GAT (b)	GCC	CGC	GCA/ACA	Fy (a-b+)	Characterization
DP003	TCT	GAT (b) / GGT (a)	GCC	CGC	GCA	Fy (a+b+)	Characterization
DP004	TCT	GAT (b) / GGT (a)	GCC	CGC	GCA/ACA	Fy (a+b+)	Characterization
DP007	TCT	GAT (b) / GGT (a)	GCC	CGC	GCA	Fy (a+b+)	Invasion
DP010	TCT	GAT (b) / GGT (a)	GCC	CGC	GCA/ACA	Fy (a+b+)	Invasion
DN001	CCT	GAT (b)	GCC	CGC	GCA	Fy (a-b-)	Characterization
DN002	CCT	GAT (b)	GCC	CGC	GCA	Fy (a-b-)	Characterization
DN003	CCT	GAT (b)	GCC	CGC	GCA	Fy (a-b-)	Characterization
DN005	CCT	GAT (b)	GCC	CGC	GCA	Fy (a-b-)	Characterization & Invasion
DN006	CCT	GAT (b)	GCC	CGC	GCA	Fy (a-b-)	Characterization

Figures

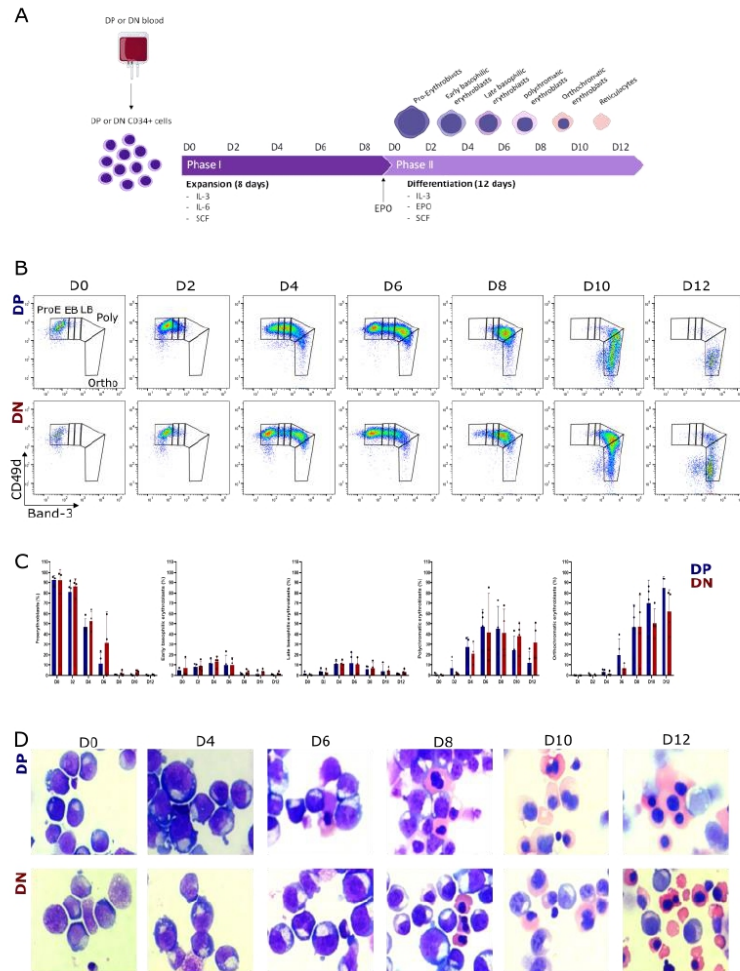


Figure 1. Genotypically DP and DN erythroid progenitors show similar kinetics of differentiation. **A.** Diagram illustrating the two-phase *in vitro* culture system for the isolation of CD34+ cells, the expansion and differentiation of erythroid progenitors. Details are provided in the Supplementary Information. **B.** Representative flow cytometry contour plot of GPA+ cells from one DP and one DN donors, showing the distribution of cell populations with respect to expression of Band-3 (x axis) and CD49d (y axis) at D0, D2, D4, D6, D8, D10 and D12 of terminal differentiation. **C.** Bar graphs representing the percentage of pro-erythroblasts, early basophilic erythroblasts, late basophilic erythroblasts, polychromatic erythroblasts, and orthochromatic erythroblasts in four DP (Duffy-positive, blue) and four DN (Duffy-negative, red) GPA+ cell populations at D0, D2, D4, D6, D8, D10 and D12 of terminal differentiation (mean \pm SD with n=4). **D.** May-Grünwald Giemsa staining showing erythroid cells from one DP and one DN donors at D0, D4, D6, D8, D10 and D12 of terminal differentiation.

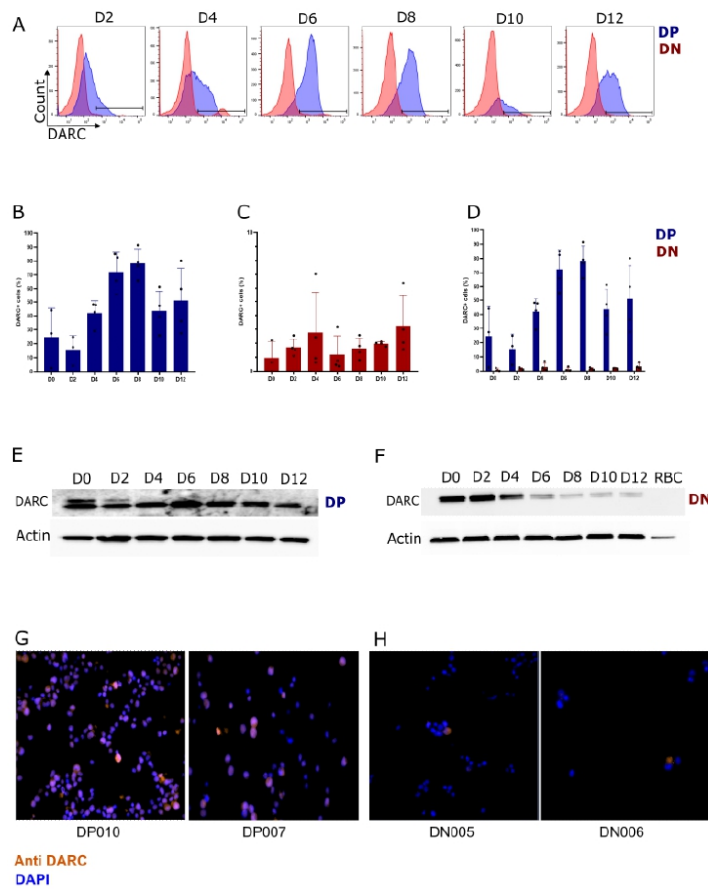
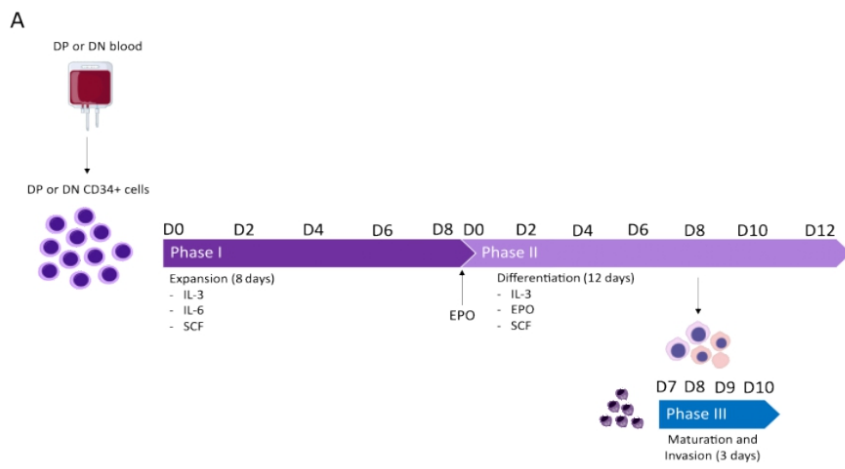
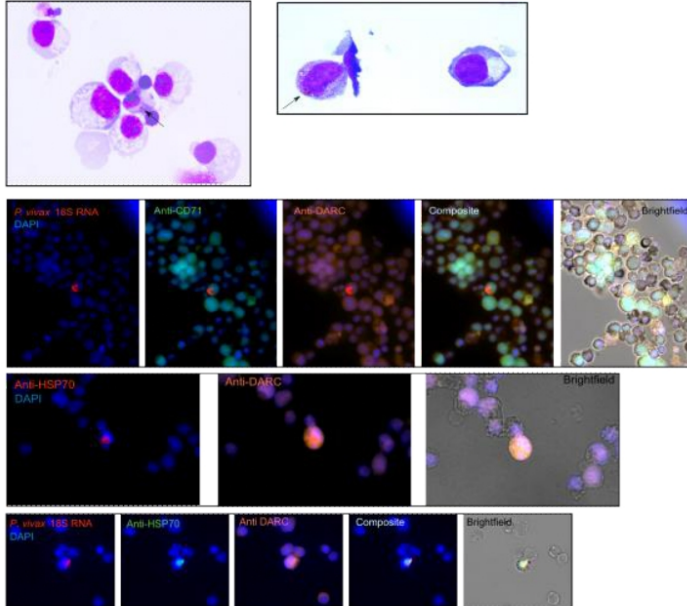


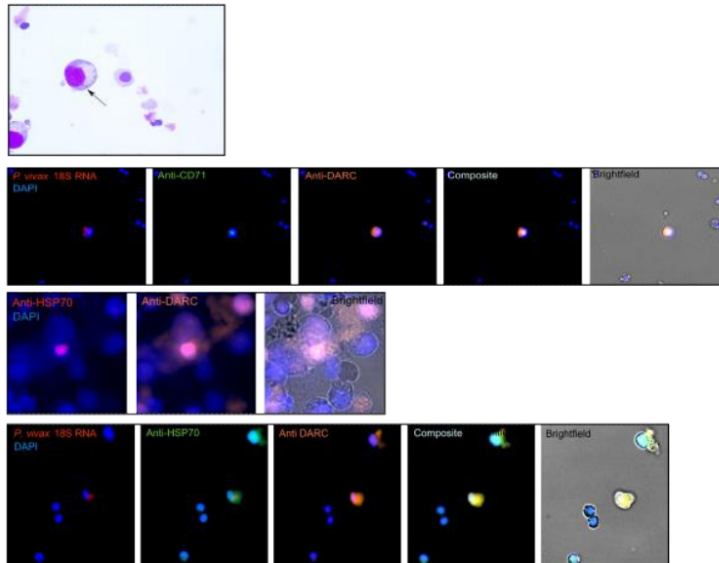
Figure 2. A subset of genotypically DN erythroblasts expresses DARC during terminal erythroid differentiation. **A.** Representative histogram showing the percentage of GPA+ DARC+ cells in erythroid cells from one DP and one DN donors at D2, D4, D6, D8, D10 and D12 of terminal differentiation. **B.** Bar graph representing the percentages of GPA+ DARC+ cells in a total of four DP donors at D0, D2, D4, D6, D8, D10 and D12 of terminal differentiation (mean \pm SD with n=4). **C.** Bar graph representing the percentages of GPA+ DARC+ cells in a total of four DN donors at D0, D2, D4, D6, D8, D10 and D12 of terminal differentiation (mean \pm SD with n=4). **D.** Bar graph representing the percentages of GPA+ DARC+ cells in a total of four DP and four DN donors at D0, D2, D4, D6, D8, D10 and D12 of terminal differentiation (mean \pm SD with n=4). **E.** Representative Western blot analysis of DARC and Actin as a control, performed on cytoplasmic protein extracts of erythroid cells from one DP donor at D0, D2, D4, D6, D8, D10 and D12 of terminal differentiation. **F.** Representative Western blot analysis of DARC and Actin as a control, performed on cytoplasmic protein extracts of erythroid cells from one DN donor at D0, D2, D4, D6, D8, D10 and D12 of terminal differentiation and at erythrocyte stage. **G.** Fluorescence microscopy of paraformaldehyde fixed DP cells at D9 of terminal differentiation. DNA was stained with DAPI (blue) and DARC was stained with anti-DARC antibody (orange). Objective 63X. **H.** Fluorescence microscopy of paraformaldehyde fixed DN cells at D9 of terminal differentiation. DNA was stained with DAPI (blue) and DARC was stained with anti-DARC antibody (orange). Objective 63X.



B Co-culture DP010 - MC4038



C Co-culture DN005 - MC4038



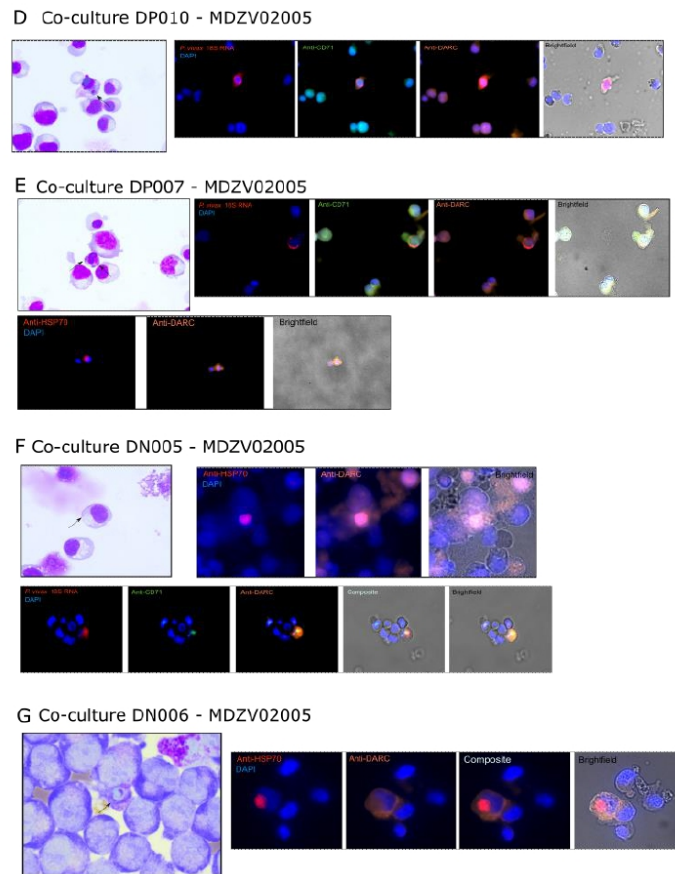


Figure 3. *P. vivax* merozoites can invade DN erythroblasts during terminal erythroid differentiation. **A.** Diagram illustrating the two-phase *in vitro* culture system for the isolation of CD34+ cells, the expansion and differentiation of erythroid progenitors, the maturation of *P. vivax* cryopreserved isolates and *in vitro* invasion assays. Details are provided in the Supplementary Information. **B-G:** *P. vivax* parasites were observed after 24 hours of co-culture on May-Grunwald-Giemsa-stained slides by light microscopy and by fluorescence microscopy [RNA 18S FISH probe (red) + anti-CD71 antibody (green) + anti-DARC antibody (orange) + DAPI (blue) or anti-HSP70 antibody (red), + anti-CD71 antibody (green) + anti-DARC antibody (orange) + DAPI (blue) or dual staining RNA 18S FISH probe (red)/anti-HSP70 antibody (green) + anti-DARC antibody (orange) + DAPI (blue) , objective 63X]. **B.** DP erythroblasts (DP010 at D8 of terminal differentiation) infected with an Ethiopian *P. vivax* isolate (MC4038) by light microscopy (ring stage in the top left panel and mature stage with hemozoin in the top right panel) and by fluorescence microscopy (second, third and fourth lines panel) **C.** DN erythroblasts (DN005 at D8 of terminal differentiation) infected with the same Ethiopian *P. vivax* isolate (MC4038) by light microscopy (ring stage, top panel) and by fluorescence microscopy (second, third and fourth lines panel) **D.** DP erythroblasts (DP010 at D8 of terminal differentiation) infected with a Malagasy *P. vivax* isolate (MDZV02005) by light microscopy (mature ring stage, left panel) and by fluorescence microscopy (right panel) **E.** DP erythroblasts (DP007 at D8 of terminal differentiation) infected with the same Malagasy *P. vivax* isolate (MDZV02005) by light microscopy (two ring stages, first left panel) and by fluorescence microscopy (first right panel and second line panel). **F.** DN erythroblasts (DN005 at D8 of terminal differentiation) infected with the same Malagasy *P. vivax* isolate (MDZV02005) by light microscopy (ring stage, first left panel) and by fluorescence microscopy (first left panel and second line panel) **G.** DN erythroblasts (DN006 at D8 of terminal differentiation) infection with the same Malagasy *P. vivax* isolate (MDZV02005) by light microscopy (mature stage, first left panel) and by fluorescence microscopy (first right panel).

**The DARC side of *vivax* malaria in Africa:
unveiling invasion pathways in Duffy-negative erythroblasts**

Supplementary information

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1. Supplementary Methods

1.1. Whole blood from healthy donors

Whole blood samples from Duffy-positive and Duffy-negative healthy donors were provided by the Etablissement Français du Sang (EFS) in Strasbourg, according to the Ethics Committees of EFS and the University of Strasbourg. All patients provided informed consent and when participants were children, parents or guardians provided consent.

1.2. *P. vivax* clinical isolates

Clinical *P. vivax* isolates were obtained from symptomatic patients infected with *P. vivax* and collected in health centers in Madagascar according to the declaration of Helsinki with approval from the Malagasy Ministry of Health, National Ethics Committee. All patients provided informed consent and when participants were children, parents or guardians provided consent. Whole blood samples were cryopreserved in liquid nitrogen.

Clinical *P. vivax* isolates were obtained from symptomatic patients infected with *P. vivax* and collected in health centers in Ethiopia.

1.3. Duffy genotyping

Duffy genotype of blood donors was determined by Sanger sequencing. Briefly, genomic DNA was extracted from blood samples using a QiaAmp DNA kit (Qiagen) and according to the supplier's recommendation. Duffy gene amplification was performed in a nested polymerase chain reaction (PCR) to search for potential GATA-1 and Fy mutations. The PCR products were sequenced by Sanger sequencing, as previously described (Ménard *et al.* 2010).

1.4. Isolation of CD34+ cells.

Duffy-positive and Duffy-negative peripheral blood mononuclear cells (PBMCs) were isolated from whole blood after a Ficoll fractionation (G&E Healthcare). Then, CD34+ cells were isolated by positive magnetic sorting using a CD34+ progenitor cell isolation kit (Miltenyi biotec) and following the supplier's protocol. CD34+ cells were frozen in freezing media containing 10% dimethyl sulfoxide (DMSO) in liquid nitrogen and used for downstream analysis (El Hoss *et al.* 2021).

1.5. *In vitro* erythropoiesis assays

Duffy-positive and Duffy-negative erythroid progenitors were placed in a two-phases *in vitro* culture system (El Hoss *et al.* 2021). Briefly, CD34+ cells were expanded in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco) supplemented with 15% BIT9500 medium (Stem Cell Technologies), 100 U/ml Penicillin Streptomycin (Gibco), 2mM L-Glutamine (Gibco), 10ng/ml of human recombinant interleukin-3 (IL-3), 100ng/ml of human recombinant interleukin-6 (IL-6) and 50ng/ml human recombinant stem cell factor (SCF) (Miltenyi biotec). This expansion phase lasted for 8 days. Cells were kept at 2×10^5 cells/ml and media change was performed every other day. Differentiation was initiated by using IMDM (Gibco) supplemented with 15% BIT9500 medium (Stem Cell Technologies), 100 U/ml Penicillin Streptomycin (Gibco), 2mM L-Glutamine (Gibco), 10ng/ml of human IL-3, 2U/ml of erythropoietin (EPO) and 50ng/ml human SCF (Miltenyi biotech). This differentiation phase lasted for 12 days. Cells were kept at 2×10^5 cells/ml up until day 8 and then placed at 5 and media change was performed every other day.

1.6. *In vitro* invasion assays

Duffy-positive and Duffy-negative erythroid progenitors were placed in a two-phases (expansion and differentiation) *in vitro* culture system (El Hoss *et al.* 2021). At Day 7 of terminal differentiation, *P. vivax* cryoisolates were thawed, placed in IMDM (Gibco) supplemented with 10% AB+ human serum (Sigma Aldrich), 10mM hypoxanthine (Dutcher) and 50µg/ml gentamycin (Fisher Scientific)

and incubated at 37°C under gas conditions (5% O₂, 5% CO₂, 90% N₂) for ~30h. At Day 8 of terminal differentiation, *P. vivax* parasites were placed in co-culture with either Duffy-positive or Duffy-negative erythroblasts and incubated at 37°C under gas conditions (5% O₂, 5% CO₂, 90% N₂) for 48h.

1.7. May-Grünwald Giemsa staining and microscopy

Erythroblasts were harvested, washed, and spun on glass slides at 3000rpm for 3min using a cytospin centrifuge (Shandon). The dot smears were stained with May-Grünwald Giemsa (MGG) staining (Sigma-Aldrich) following manufacturer instructions then observed by optical microscopy using 20X, 40X and 50X objectives (Echo).

1.8. Antibodies and fluorescent dyes

The BV421-conjugated anti-glycophorin A (GPA) mouse monoclonal antibody, PECy7-conjugated anti-GPA mouse monoclonal antibody, BV421-conjugated anti-CD71 mouse monoclonal antibody, APC-conjugated anti-CD36 mouse monoclonal antibody (BD Biosciences), APC-conjugated anti-CD49d mouse monoclonal antibody, PE-conjugated anti-DARC rabbit monoclonal antibody (BD Pharmingen), FITC-conjugated anti-band 3 mouse monoclonal antibody (IBGRL), APC-conjugated anti-DARC rabbit monoclonal antibody, APC-conjugated anti-GPA mouse monoclonal antibody, FITC-conjugated anti-CD71 mouse monoclonal antibody (Miltenyi biotec), 7-Aminoactinomycin D (7AAD) fluorochrome, FITC-conjugated annexin V dye (BD Biosciences) and Hoechst 33342 dye (Invitrogen) were used for the follow up of the characterization of Duffy-negative erythroblasts and invasion assays by flow cytometry. The anti-DARC rabbit monoclonal antibody (Sigma-Aldrich) and the anti-rabbit IgG goat monoclonal antibody (Cell signaling) were used for the detection of DARC by western blotting. The anti-DARC rabbit monoclonal antibody, anti-CD71 mouse monoclonal antibody (Fisher), Alexa fluor 555-conjugated anti-rabbit IgG goat monoclonal antibody, Alexa fluor 488-conjugated anti-mouse IgG goat monoclonal antibody (Invitrogen) and the set of RNA Stellaris FISH probes specific to *P. vivax* 18S RNA and conjugated to quasar-670 (Stellaris) were used for fluorescence microscopy. The sequences of the probes were designed with the Stellaris® FISH probe designer (Biosearch technologies) available online at www.biosearchtech.com/stellarisdesigner.

1.9. Flow cytometry

Waterfall staining assay. Cells were harvested and stained for surface expression of GPA, Band 3 and CD49d at D0, D2, D4, D6, D8, D10 and D12 of terminal differentiation. Specifically, 1x10⁵ cells were washed with PBS (1X) + 0.2% BSA and incubated with fluorescence-conjugated antibodies at room temperature for 30 min in the dark. They were washed twice with PBS (1X) + 0.2% BSA then incubated with 7-AAD for 5min prior to analysis. Samples were analyzed using a LSR Fortessa flow cytometer and acquired using the Diva software version 8 (BD Biosciences). Data were analyzed using FlowJo software version 10 (BD Biosciences).

Duffy staining assay. Cells were harvested and stained for surface expression of GPA, DARC and CD71 at D0, D2, D4, D6, D8, D10 and D12 of terminal differentiation. Specifically, 1x10⁵ cells were washed with PBS (1X) + 0.2% BSA and incubated with fluorescence-conjugated antibodies at room temperature for 30min in the dark. They were washed twice with PBS (1X) + 0.2% BSA. The cells were analyzed using a LSR Fortessa flow cytometer and a Attune NxT Acoustic Focusing flow cytometer. Acquisition was performed using the Diva software (BD Biosciences) and the Attune NxT software. Data were analyzed using FlowJo software v.10 (BD Biosciences).

Apoptosis staining assay. Cells were harvested and stained to measure the percentage of apoptotic cells at D0, D2, D4, D6, D8, D10 and D12 of terminal differentiation. Specifically, 1x10⁵ cells were

washed once in annexin buffer then incubated with FITC-conjugated annexin V dye (BD Biosciences) at room temperature for 20min in the dark. They were analyzed using a LSR Fortessa flow cytometer and acquired using the Diva software version 8 (BD Biosciences). Data were analyzed using FlowJo software version 10 (BD Biosciences).

Enucleation staining assay. Cells were harvested and stained to measure the percentage of enucleated cells at D8, D10 and D12 of terminal differentiation. Specifically, 1×10^5 cells were washed with PBS (1X) + 0.2% BSA and incubated with PE-Cy7-conjugated anti-GPA mouse monoclonal antibody and APC-conjugated anti-CD36 mouse monoclonal antibody (BD Biosciences) at room temperature for 30min in the dark. They were washed twice with PBS (1X) + 0.2% BSA then incubated with Hoechst 33342 dye (Invitrogen) for 60min at 37°C. They were analyzed using a LSR Fortessa flow cytometer and acquired using the Diva software version 8 (BD Biosciences). Data were analyzed using FlowJo software version 10 (BD Biosciences).

Protein extraction and Western immunoblot. Erythrocytes from both Duffy-positive and Duffy-negative donors were resuspended in lysis buffer and incubated at 4°C for 45min. They were centrifuged at 1500rpm for 5min. The supernatants containing total protein fractions were transferred to new tubes. During differentiation, Duffy-positive and Duffy-negative erythroblasts were harvested, and cytoplasmic extracts were obtained using the NE-PER Plus membrane protein extraction kit (Thermo Fisher) following manufacturer's instructions. Proteins were quantified using the BCA protein quantification kit (Thermo Fisher) according to the supplier's instructions. For each sample, 10µg of proteins were electrophoresed through 4-12% SDS-polyacrylamide gel and electroblotted to a nitrocellulose membrane using the Trans-Blot Turbo system (Bio-Rad). Membranes were blocked in TBS (1X) + 5% milk at room temperature for 60min then probed with rabbit monoclonal anti-DARC antibody (Sigma) in TBS (1X) + 5% milk or mouse monoclonal anti-actin antibody (Cell signaling) in TBS (1X) + 5% BSA at 4°C overnight. Membranes were washed then probed with a goat anti-rabbit antibody or goat anti-mouse antibody (Cell Signaling) at room temperature for 60min. Proteins were revealed using the chemiluminescent detection assay Clarity Max Western ECL (Biorad) and the Chemidoc imaging system (Biorad). The quantification of the proteins was performed using the Image Lab software version 6.1 (Biorad).

1.10. Fluorescence microscopy

RNA FISH staining assay. Cells were harvested and stained to detect *P. vivax* infected erythroblasts by fluorescence microscopy. Briefly, 3×10^6 cells were harvested after 24h and 48h of invasion and washed with PBS (1X) + 0.2% BSA. They were fixed in PBS (1X) supplemented with 4% paraformaldehyde and 0.0075% glutaraldehyde at room temperature for 30min. Then, cells were blocked in PBS (1X) + 3% BSA at room temperature for 60min. They were incubated with anti-DARC rabbit monoclonal antibody and anti-CD71 mouse monoclonal antibody (Fisher) at 37°C for 60min in the dark. Cells were washed twice in PBS (1X) + 1% BSA and incubated with Alexa fluor 555-conjugated anti-rabbit IgG goat monoclonal antibody and Alexa fluor 488-conjugated anti-mouse IgG goat monoclonal antibody (Invitrogen) at room temperature for 60min in the dark. Cells were washed twice in PBS (1X) + 1% BSA and permeabilized at room temperature for 15min in the dark. Cells were then hybridized using a set of RNA Stellaris FISH probes specific to *P. vivax* 18S RNA and conjugated to quasar-670 (Stellaris) at 37°C overnight. They were washed twice in wash buffer A, incubated at 37°C for 30min in the dark, then washed once in wash buffer B. Cells were spun on glass slides at 1000rpm for 3min using a cytospin centrifuge (Shandon) and left to dry. They were counter stained using Prolong Gold antifade mountant with diamidino phenylindole (DAPI) (Fisher) and covered using coverslips (Epredia). *P. vivax* infected erythroblasts were visualized at

63X magnification lenses using an Apotome microscope (Zeiss). Analysis was performed using Zen software v. 3.7 (Zeiss).

Antibody staining assay. Cells were harvested and stained to detect *P. vivax* infected erythroblasts by fluorescence microscopy. Briefly, 3×10^6 cells were harvested after 24h and 48h of invasion and washed with PBS (1X) + 0.2% BSA. They were fixed in PBS (1X) supplemented with 4% paraformaldehyde and 0.0075% glutaraldehyde at room temperature for 30min. Then, cells were blocked in PBS (1X) + 3% BSA at room temperature for 60min. They were incubated with anti-DARC rabbit monoclonal antibody (Fisher) at 37°C for 60min in the dark, then washed twice in PBS (1X). Cells were incubated with Alexa fluor 555-conjugated anti-rabbit IgG goat monoclonal antibody at 37°C for 60min in the dark then washed twice in PBS (1X). They were permeabilized in PBS (1X) supplemented with 0.1% Triton (100X) at room temperature for 15min. Cells were incubated with HSP70 mouse polyclonal antibody (Biology and Immunology of malaria research unit, CIMI) at 37°C for 60min in the dark then washed twice in PBS (1X). Cells were incubated with Alexa fluor 680-conjugated anti-mouse IgG goat monoclonal antibody (Invitrogen) at room temperature for 60min in the dark, then washed twice in PBS (1X) and resuspended in 100 μ l of PBS (1X) 0.2% BSA. They were spun on glass slides at 1000rpm for 3min using a cytospin centrifuge (Shandon) and left to dry. They were counter stained using Prolong Gold antifade mountant with DAPI (Fisher) and covered using coverslips (Epreidia). *P. vivax* infected erythroblasts were observed at 63X magnification lenses using an Apotome microscope (Zeiss). Analysis was performed using Zen software version 3.7 (Zeiss).

RNA FISH and antibody staining assay. Cells were harvested and stained to detect *P. vivax* infected erythroblasts by fluorescence microscopy. Briefly, 3×10^6 cells were harvested after 24h and 48h of invasion and washed with PBS (1X) + 0.2% BSA. They were fixed in PBS (1X) supplemented with 4% paraformaldehyde and 0.0075% glutaraldehyde at room temperature for 30min. Then, cells were blocked in PBS (1X) + 3% BSA at room temperature for 60min. They were incubated with anti-DARC rabbit monoclonal antibody (Fisher) at 37°C for 60min in the dark, then washed twice in PBS (1X). Cells were incubated with Alexa fluor 555-conjugated anti-rabbit IgG goat monoclonal antibody at 37°C for 60min in the dark then washed twice in PBS (1X). They were permeabilized in PBS (1X) supplemented with 0.1% Triton (100X) at room temperature for 15min. Cells were incubated with HSP70 mouse polyclonal antibody (Biology and Immunology of malaria research unit, CIMI) at 37°C for 60min in the dark then washed twice in PBS (1X). Cells were incubated with Alexa fluor 680-conjugated anti-mouse IgG goat monoclonal antibody (Invitrogen) at room temperature for 60min in the dark, then washed twice in PBS (1X). Then, cells were then hybridized using a set of RNA Stellaris FISH probes specific to *P. vivax* 18S RNA and conjugated to quasar-670 (Stellaris) at 37°C overnight. They were washed twice in wash buffer A, incubated at 37°C for 30min in the dark, then washed once in wash buffer B. Cells were spun on glass slides at 1000rpm for 3min using a cytospin centrifuge (Shandon) and left to dry. They were counter stained using Prolong Gold antifade mountant with diamidino phenylindole (DAPI) (Fisher) and covered using coverslips (Epreidia). *P. vivax* infected erythroblasts were visualized at 63X magnification lenses using an Apotome microscope (Zeiss). Analysis was performed using Zen software v. 3.7 (Zeiss).

2. Supplementary Figures

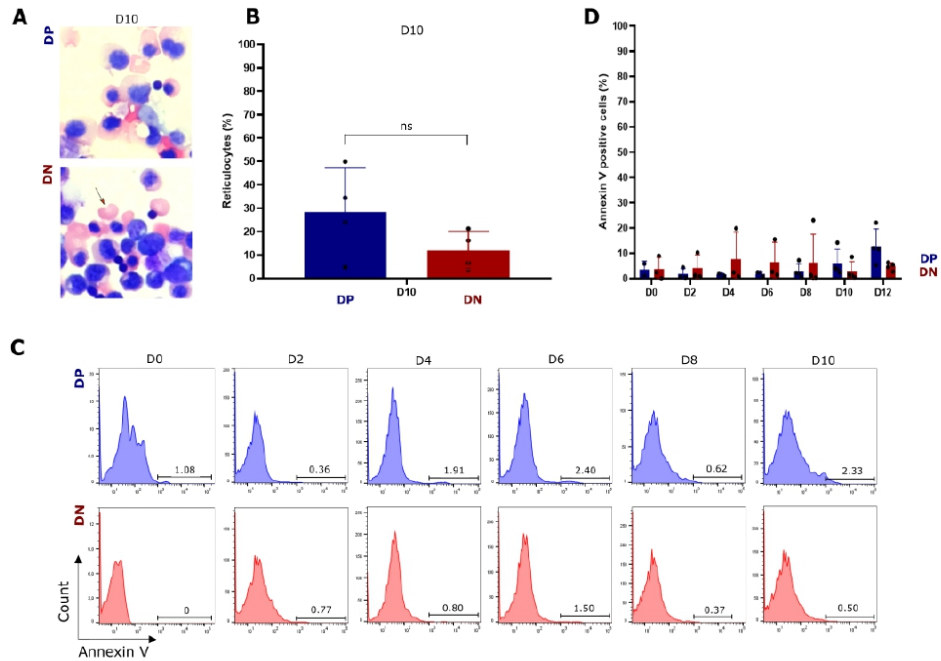


Figure S1. Enucleation and apoptosis of genotypically DP and DN erythroblasts during *in vitro* terminal differentiation

A. May-Grünwald Giemsa staining showing one DP and one DN cell populations at D10 of terminal differentiation. The red arrow shows the presence of reticulocytes in the cell cultures. **B.** Bar chart representing the percentage of reticulocytes in four DP (blue) and four DN (red) cell populations at D10 of terminal differentiation (mean \pm SD with $n=4$). **C.** Representative histograms representing the percentage of GPA⁺ Annexin⁺ cells (apoptotic cells) in one DP (blue) and one DN (red) cell populations at D0, D2, D4, D6, D8, D10 and D12 of terminal differentiation. **D.** Bar graph representing the percentage of GPA⁺ Annexin⁺ cells (apoptotic cells) in four DP (blue) and four DN (red) cell populations at D0, D2, D4, D6, D8, D10 and D12 of terminal differentiation (mean \pm SD with $n=4$).

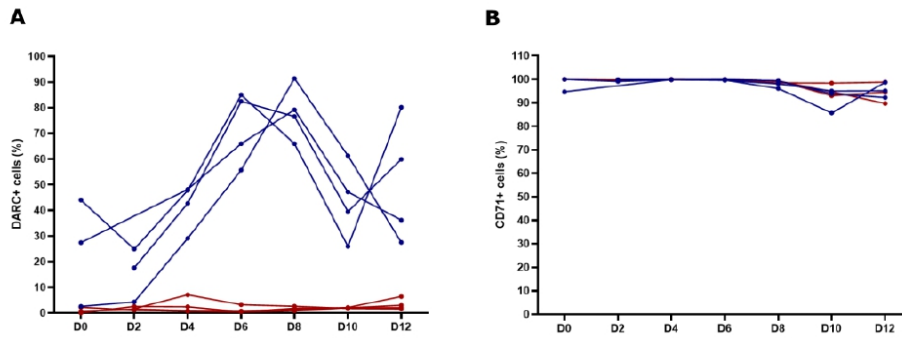


Figure S2. Expression of DARC and CD71 by genotypically DP and DN erythroblasts during terminal differentiation

A. Graphs representing the percentage of GPA+ DARC+ cells in four DP (blue) and four DN (red) cell populations at D0, D2, D4, D6, D8, D10 and D12 of terminal differentiation. **B.** Graphs representing the percentage of GPA+ CD71+ cells in three DP (blue) and three DN (red) cell populations at D0, D2, D4, D6, D8, D10 and D12 of terminal differentiation.

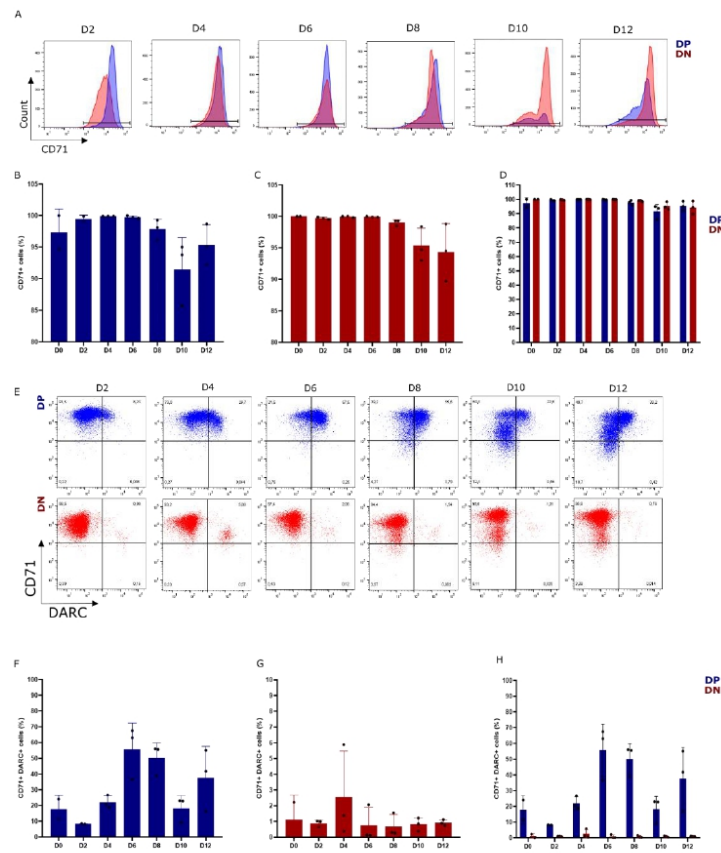


Figure S3. Correlation between the expression of DARC and the expression of CD71 by genotypically DP and DN erythroblasts during terminal differentiation

A. Representative histograms representing the percentage of GPA+ CD71+ cells in one DP (blue) and one DN (red) cell populations at D0, D2, D4, D6, D8, D10 and D12 of terminal differentiation. **B.** Bar graph representing the percentage of GPA+ CD71+ cells in three DP (blue) cell populations at D0, D2, D4, D6, D8, D10 and D12 of terminal differentiation (mean \pm SD with n=3). **C.** Bar graph representing the percentage of GPA+ CD71+ cells in three DN (red) cell populations at D0, D2, D4, D6, D8, D10 and D12 of terminal differentiation (mean \pm SD with n=3). **D.** Bar graph representing the percentage of GPA+ CD71+ cells in three DP (blue) and three DN (red) cell populations at D0, D2, D4, D6, D8, D10 and D12 of terminal differentiation (mean \pm SD with n=3). **E.** Representative graph representing the distribution of GPA+ cells in one DP (blue) and one DN (red) cell populations with respect to the expression of DARC (x axis) and CD71 (y axis) at D2, D4, D6, D8, D10 and D12 of terminal differentiation. **F.** Bar charts representing the percentage of GPA+ CD71+ DARC+ cells in three DP (blue) cell populations at D2, D4, D6, D8, D10 and D12 of terminal differentiation (mean \pm SD with n=3). **G.** Bar charts representing the percentage of GPA+ CD71+ DARC+ cells in three DN (red) cell populations at D2, D4, D6, D8, D10 and D12 of terminal differentiation (mean \pm SD with n=3). **H.** Bar charts representing the percentage of GPA+ CD71+ DARC+ cells in three DP (blue) and three DN (red) cell populations at D2, D4, D6, D8, D10 and D12 of terminal differentiation (mean \pm SD with n=3).

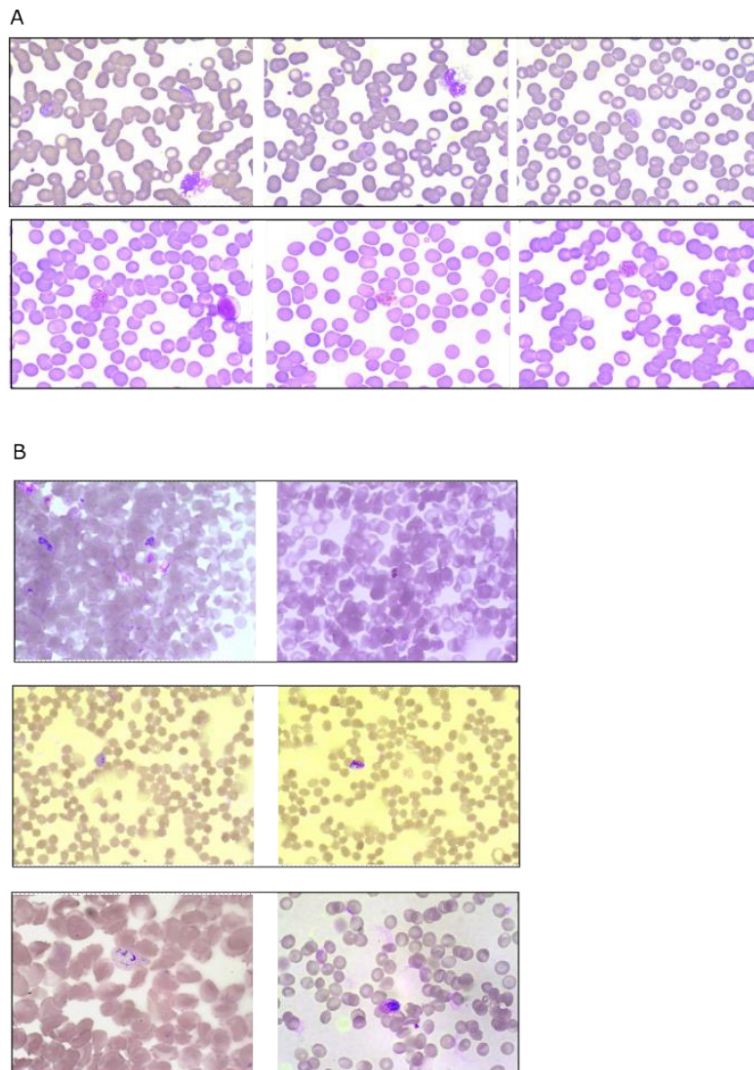
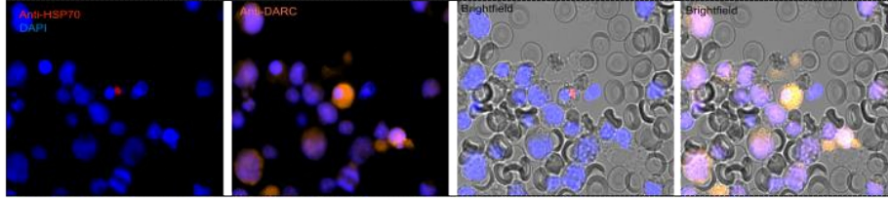


Figure S4. *P. vivax* parasites from Malagasy and Ethiopian cryoisolates after thawing and maturation (light microscopy)

A. May-Grünwald Giemsa staining showing two Malagasy *P. vivax* isolates, respectively MDZV02005 (above) and MDZV02004 (below), after thawing. **B.** May-Grünwald Giemsa staining showing one Ethiopian *P. vivax* isolate (first line) and two Malagasy *P. vivax* isolates (second and third lines), after thawing and *in vitro* maturation for approximately 30 hours.

A Co-culture DP010 - MDZV02004



B Co-culture DN005 - MDZV02004

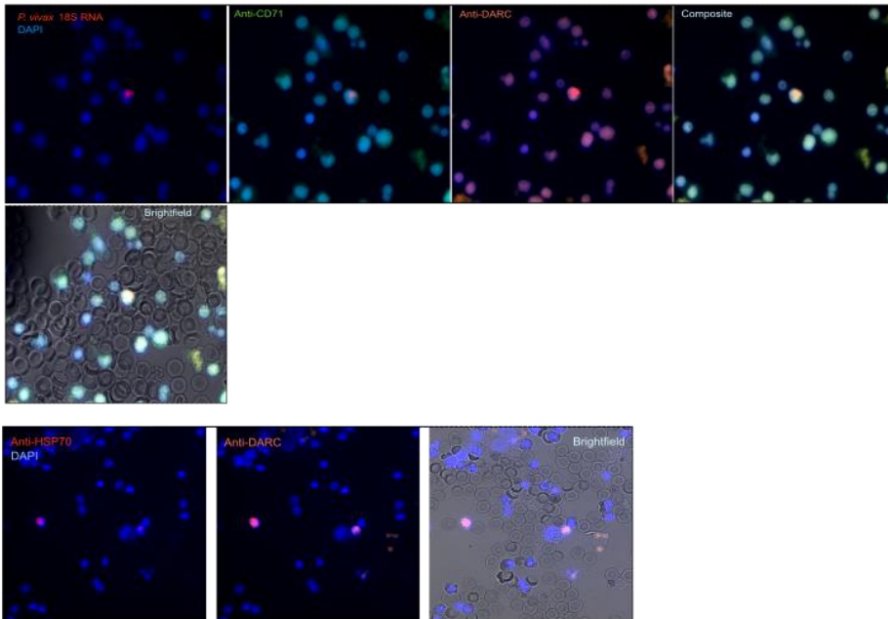


Figure S5. Additional *P. vivax* infections of genotypically DP and DN erythroblasts with Malagasy *P. vivax* isolate (fluorescence microscopy)

A. Infection of DP erythroblasts (DP010 at D8 of terminal differentiation) with a Malagasy *P. vivax* isolate (MDZV02004). Observation of paraformaldehyde fixed DP erythroblasts stained with anti-HSP70 antibody (red), anti-DARC antibody (orange) and DAPI (blue) by fluorescence microscopy (objective 63X). **B.** Infection of DN erythroblasts (DN005 at D8 of terminal differentiation) with the same Malagasy *P. vivax* isolate (MDZV02004). Observation of paraformaldehyde fixed DN erythroblasts stained with *P. vivax* RNA 18S FISH probe (red), anti-CD71 antibody (green), anti-DARC antibody (orange) and DAPI (blue) (first/second line panel, objective 63X) or with anti-HSP70 antibody (red), anti-DARC antibody (orange) and DAPI (blue) (third line panel) by fluorescence microscopy (objective 63X). Details are provided in the Table S1.

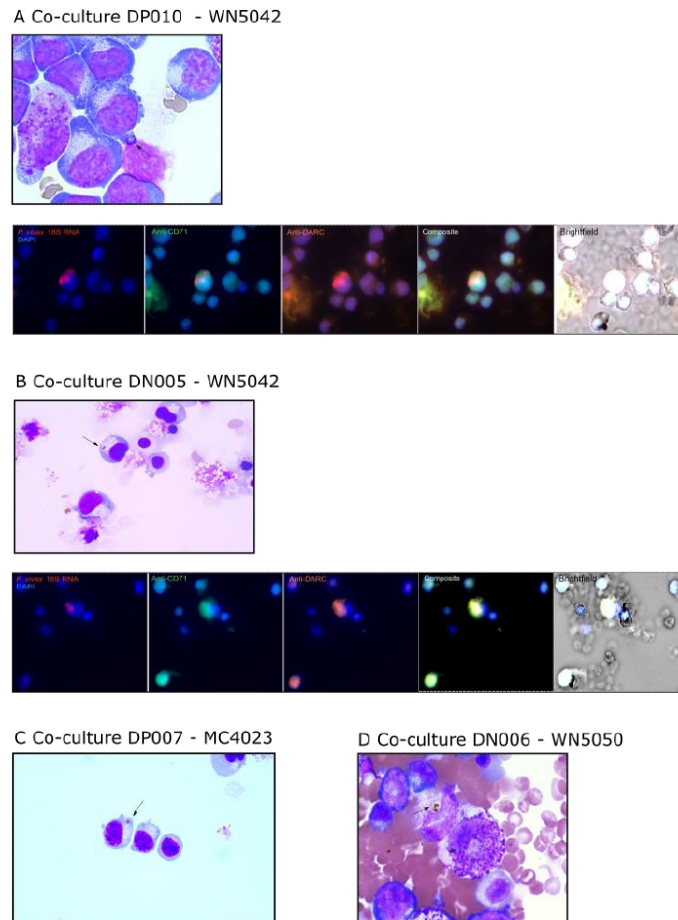


Figure S6. Additional *P. vivax* infections of genotypically DP and DN erythroblasts with Ethiopian *P. vivax* isolate (light and fluorescence microscopy)

A. Infection of DP erythroblast (DP010 at D8 of terminal differentiation) with an Ethiopian *P. vivax* isolate (WN5042) was observed by May-Grunwald-Giemsa staining at 24 hours of co-culture (mature ring stage, first panel) and confirmed by fluorescence microscopy of paraformaldehyde fixed DP erythroblasts stained with *P. vivax* RNA 18S FISH probe (red), anti-CD71 antibody (green), anti-DARC antibody (orange) and DAPI (blue) (second line panel, objective 63X). **B.** Infection of DN erythroblast (DN005 at D8 of terminal differentiation) with the same Ethiopian *P. vivax* isolate (WN5042) was observed by May-Grunwald-Giemsa staining at 24 hours of co-culture (ring stage, first panel) and confirmed by fluorescence microscopy of paraformaldehyde fixed DP erythroblasts stained with *P. vivax* RNA 18S FISH probe (red), anti-CD71 antibody (green), anti-DARC antibody (orange) and DAPI (blue) (second line panel, objective 63X). **C.** Infection of DP erythroblast (DP007 at D8 of terminal differentiation) with an Ethiopian *P. vivax* isolate (MC4023) was observed by May-Grunwald-Giemsa staining at 24 hours of co-culture (ring stage). **D.** Infection of DN erythroblast (DN006 at D8 of terminal differentiation) with an Ethiopian *P. vivax* isolate (WN5050) was observed by May-Grunwald-Giemsa staining at 24 hours of co-culture (mature stage with hemozoin). Details are provided in the Table S1.

3. Supplementary Tables

3.1. Table S1. List of *P. vivax* isolates and erythroblasts used for *in vitro* invasion assays

<i>P. vivax</i> isolate	Origin	Invasion Assay	HSC source	<i>P. vivax</i> infection detection					Successful	Figure	Comment
				MGG staining	18S rRNA FISH probe	anti-HSP70	18S rRNA FISH probe & anti-HSP70				
AW007	Ethiopia	IA 6	DP010	Negative	Positive	Negative	Not done	Yes			
			DN005	Negative	Negative	Negative	Not done	No			
MC4023	Ethiopia	IA 5	DP007	Positive	Negative	Negative	Not done	Yes	Figure S6C	Tiny pellet obtained after thawing of the cryoisolate	
			DN006	Negative	Negative	Negative	Not done	No			
MC4038	Ethiopia	IA 6	DP010	Positive	Positive	Positive	Positive	Yes	Figure 3A		
			DN005	Positive	Positive	Positive	Positive	Yes			
MC4043	Ethiopia	IA 5	DP007	Negative	Negative	Negative	Not done	No		Lots of red blood cell aggregates	
			DN006	Negative	Negative	Negative	Not done	No			
MC4049	Ethiopia	IA 5	DP007	Positive	Negative	Negative	Not done	Yes			
			DN006	Negative	Negative	Negative	Not done	No			
WN5042	Ethiopia	IA 6	DP010	Negative	Positive	Negative	Negative	Yes	Figure S6A		
			DN005	Positive	Positive	Negative	Negative	Yes			
WN5050	Ethiopia	IA 5	DP007	Negative	Negative	Negative	Not done	No		Lots of red blood cell aggregates	
			DN006	Positive	Negative	Negative	Not done	Yes			
MDZV02004	Madagascar	IA 5	DP007	Negative	Not done	Not done	Not done	No		Tiny pellet obtained after thawing of the cryoisolate	
			DN006	Negative	Negative	Negative	Not done	No			
		IA 6	DP010	Negative	Negative	Positive	Not done	Yes	Figure S5A		
			DN005	Negative	Positive	Positive	Not done	Yes			
		IA 5	DP007	Negative	Positive	Positive	Negative	Yes	Figure 3D		
			DN006	Positive	Negative	Positive	Negative	Yes			
MDZV02005	Madagascar	IA 6	DP010	Positive	Positive	Negative	Negative	Yes	Figure 3C		
			DN005	Positive	Positive	Positive	Positive	Yes			
									Figure 3E		

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Discussion

Deciphering *P. vivax* enigmatic invasion pathways in Duffy-negative patients has proved to be quite challenging. At first, our approach consisted in taking advantage of the collection of a large panel of *P. vivax* isolates in Madagascar to identify the molecular mechanisms of invasion in Duffy-negative reticulocytes and define the role of key invasion-related ligands. However, things turned out differently than anticipated. Because we faced many hurdles in the collection of *P. vivax* field samples and the isolation of human reticulocytes during the Covid-19 pandemic, we had to reconsider our approach and open new opportunities. Throughout our investigations, we stepped up our collaborative efforts to collect *P. vivax* isolates in Ethiopia and built up our experimental skills to culture human erythroid cells, resulting in the following outcomes.

1. Key knowledge and tool gaps in *vivax* malaria research

Research on *vivax* malaria has long been neglected for the benefit of the more deadly *falciparum* malaria⁴². It offers now rich opportunities to explore *P. vivax* biology and pathogenicity⁹⁵. In this context, our first objective was to expose existing knowledge and recent progress made to address the global burden of *vivax* malaria. We found that the common perception of *vivax* malaria as infecting relatively few people, causing a benign disease and being treatable with widely available therapies was biased⁴². Certainly, the era of malaria therapy paved the way for important advances in malaria research as observations resulting from thousands of treated patients provided the opportunity to study the early stages of infection, development of immunity, and efficacy of various antimalarial drugs^{96,97}. However, we found that the morbidity and mortality from *vivax* malaria remain under-appreciated⁴². Because *P. vivax* infections are often submicroscopic and asymptomatic, they are difficult to detect by light microscopy or RDTs^{15,67}. Despite the low parasite density, *P. vivax* infections can cause severe illness, complications, and death⁹⁸. In fact, reporting of clinical cases of *vivax* malaria is inconsistent in some endemic regions, making the appreciation of its real burden even more difficult⁹⁹. Finally, the strategies of prevention, control and elimination developed for *P. falciparum* showed limited effect on *P. vivax* suggesting the need for new tools⁶⁷. Nevertheless, we found that omics technologies have recently revolutionized *vivax* malaria research⁸⁸. Indeed, the development of innovative and affordable techniques allowed a more comprehensive characterization of *P. vivax* genes^{100,101}, transcripts¹⁰², and proteins^{103,104} for a better

understanding of its mechanisms. In particular, they have significantly contributed to our understanding of *P. vivax* invasion mechanisms with the acquisition of *P. vivax* genomic sequences, identification of a few potential invasion-related genes, and characterization of their expression. Thus, the constant improvement of omics technologies is promising, but the implementation of integrated multi-omics studies will demand substantial funding and long-term commitment.

2. Contribution of field studies in deciphering *P. vivax* invasion pathways

Describing *P. vivax* genomic traits is an important step towards understanding its invasion mechanisms in Duffy-negative patients and transmission patterns in Sub-Saharan Africa¹⁰⁵. Therefore, our second objective was to investigate the genetic structure and diversity of *P. vivax* African populations and to identify potential molecular signatures in genes validated or suspected to encode proteins involved in *P. vivax* invasion pathways. Globally, the common Duffy alleles present striking patterns of geographic differentiation⁹⁰. In the comparative genomic analysis, we investigated in which extent the genetic structure of *P. vivax* populations could match the global distribution of Duffy-negative human populations. As previously described¹⁰⁶, we found four distinct geographic clusters (Eastern Africa, Indian Ocean, South America, and Asia/Pacific coral triangle). Then, we examined whether genomic intervals that contained genes involved in the invasion mechanisms held genetic imprints of selection and could be related to any genomic islands of genetic differentiation. However, we found no association between genetic diversity and genes encoding proteins involved in *P. vivax* invasion pathways. Finally, we explicitly tested whether parasites could be positively selected in Duffy-negative human hosts. Again, this approach did not allow the identification of any association between parasite genotypes and Duffy status of human hosts. Taken together, these results suggest that even though the existence of an alternative invasion-related ligand binding to DARC or to another unknown reticulocyte receptor may be a plausible hypothesis to explain genetic diversity between Asian and African *P. vivax* populations, it is unlikely to explain *P. vivax* invasion pathways in Duffy-negative patients^{3,5,12,107}. Although several studies have reported cases of *vivax* malaria in Ethiopia, little is known about its epidemiology. The two molecular epidemiological studies that we conducted in Ethiopia showed no association between the Duffy genotype of patients and geographic sites, ethnic groups, or genres. However, we found that *P. vivax* parasitemia was systematically lower in Duffy-negative patients than in Duffy-positive patients, suggesting that Ethiopian Duffy-negative population

may constitute an undetected silent reservoir of infection, if malaria diagnosis is based on microscopy and RDTs¹⁰⁸. We could not conclude on the potential existence of an alternative invasion pathway. But whatever be the mechanism involved in the invasion of Duffy-negative reticulocytes, it appears to be less efficient than the one used in the invasion of Duffy-positive reticulocytes. Overall, molecular epidemiological and genomic studies provide important information in absence of robust *in vitro* culture system for *P. vivax*. But the analysis of *P. vivax* genome remains technically challenging because field and clinical samples present low parasitemia and high human DNA contamination that make the acquisition of sufficient amount of high-quality parasite DNA difficult⁸⁸. For instance, we could not perform genome wide association study (GWAS) on sequences obtained from Duffy-negative patients. In addition, the use of sWGA technology prior to WGS proved to be efficient but not sufficient to enrich *P. vivax* DNA in samples obtained from Duffy-negative patients. It also prevented us from detecting Copy Number Variations (CNVs) in *pvd* and *pve* genes. The uneven distribution of primer-targeted motifs in *P. vivax* genome results in peaks and troughs in mean genome coverage that can confound CNV detection methods¹⁰⁹. Nevertheless, we contributed to advance knowledge in *P. vivax* genomes and enrich the global genetic database with new African *P. vivax* genomic sequences.

3. Contribution of laboratory studies in deciphering *P. vivax* invasion pathways

The design and implementation of *in vitro* assays were by far the most challenging aspect of this thesis project. Indeed, the absence of robust *in vitro* culture systems for *P. vivax* has always restricted investigations to field and clinical sampling and hampered the development of functional assays¹¹⁰. With this in mind, our third objective was to find an experimental approach suited for deciphering *P. vivax* invasion mechanisms in Duffy-negative erythroblasts. We found that a subset of Duffy-negative erythroblasts expresses functional DARC and that *P. vivax* merozoites can invade them. These outcomes represent a major breakthrough in *vivax* malaria research as they contrast with the established scientific paradigm by which *P. vivax* invades exclusively Duffy-positive erythrocytes through the interaction PvDBP-DARC³⁸. They corroborate the conclusions of our molecular epidemiological studies as the parasite may not need to evolve to an alternative invasion pathway to infect Duffy-negative patients. However, they do not completely exclude the existence of such an alternative invasion pathway that would be used as an additional feature helping in the invasion process^{111,112}. Besides, these outcomes

support the emerging evidence that the bone marrow may represent a hidden reservoir for *P. vivax* parasites⁷⁰. Considering the harm caused by liver-stage *P. vivax* hypnozoites^{113,114}, the existence of another parasite reservoir may further complicate the surveillance and elimination of *vivax* malaria in Sub-Saharan Africa. Finally, *P. vivax* may have succeeded in an ultimate adaptation to its human host, its silent and insidious strategy being more efficient to ensure its proliferation, transmission, and survival than the blazing and bursting one used by *P. falciparum*, that sometimes results in the death of its human host. Although we managed to reach our objective, we faced many hurdles in the design, development, and optimization of these *in vitro* assays. As observed for the genomic studies, the quality of *P. vivax* isolates and CD34+ HSPCs appeared to be crucial for the success of *in vitro* invasion assays. Besides, the scarcity of permissible Duffy-negative erythroblasts prevented us from quantifying and scoring *P. vivax* infections through *in vitro* inhibition assays. Overall, this reflects the importance of undertaking field studies to have easily access to fresh *P. vivax* isolates and establish *ex vivo* experiments.

4. Future perspectives

Surely new perspectives will arise from inter-disciplinary collaborations and substantial investments in both fundamental and applied research. First, further efforts in the species-specific description of severe *vivax* malaria would facilitate medical care⁹⁹. Second, the improvement of surveillance strategies and the development of adapted diagnosis tools would allow better estimates of asymptomatic, uncomplicated, or severe *P. vivax* infections and deaths¹⁰⁸. Third, the widespread safe access to radical cure would overcome *P. vivax* resilience to control strategies and substantially impact the parasite reservoirs⁶⁷. Fourth, investments in the development of high-throughput technologies and the establishment of integrated and complementary multi-omics studies would help to overcome remaining technical issues⁸⁸. Fifth, the development of efficient therapeutic treatments or vaccines for *vivax* malaria would definitely achieve the long-term goal of elimination¹¹⁵. In this aspect, the outcomes of this thesis project confirm that the leading vaccine candidate targeting PvDBP is promising to ensure the protection of both Duffy-positive and Duffy-negative populations^{116,117}.

In conclusion, this thesis project provides important insights in *P. vivax* invasion mechanisms for the infection of Duffy-negative patients. Indeed, it unveils the invasion pathway used by *P. vivax* parasites to invade Duffy-negative erythroblasts and to maintain low levels of transmission in Sub-Saharan Africa. This thesis project also emphasizes the importance of recruiting interest of policy makers, funding agencies, and researchers to conduct both field and laboratory studies and thereby advance *vivax* malaria research worldwide.

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