ASYMPTOMATIC P. VIVAX MALARIA BURDEN AND NATURALLY-ACQUIRED HUMORAL RESPONSE TO PvMSP19 IN A LOW ENDEMIC MALARIA AREA IN THE PERUVIAN AMAZON BASIN

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Abstract

**Objective:** *P. vivax* (*Pv*) malaria is the most widely distributed human malaria species in the world, affecting millions of people annually, and the most prevalent species in the Americas. One of the main challenges for the control and elimination of malaria in the region is the presence of *Pv* malaria and its ability to relapse, as well as the presence of asymptomatic individuals who go undiagnosed, facilitating the persistence of disease. This study sought to improve our understanding of the naturally-acquired humoral response and epidemiology of *Pv* malaria in a low-endemicity area in the Peruvian Amazon Basin through the analysis of antibodies (Ab) against *PvMSP1*\(_{19}\) recombinant protein, a leading vaccine candidate antigen.

**Methods:** A large dataset of eight community-wide cross-sectional microscopy surveys conducted from 2004-2011 and 294 repository plasma samples of *Pv*-infected individuals were analyzed using statistical and immunological methods to describe the changing malaria burden in the area, as well as to study naturally-acquired anti-*PvMSP1*\(_{19}\) humoral response in a low-endemic setting. Specifically, we studied the Ab response before, during and after a documented microscopy-positive infection, assessing differences in circulating mean IgG levels and changes in serostatus between symptomatic and asymptomatic subjects both prior and during patent infection, as well as Ab maintenance in symptomatic individuals, assessing the absolute change in IgG levels and serostatus eight to 21 months after infection. Risk factors associated with asymptomatic infection and maintenance of Ab levels were assessed using GLM logistic regression.
**Results:** In the retrospective surveillance study, we observed a significant decline in malaria prevalence in the first years of the survey, followed by sustained low-prevalence, with asymptomatic cases being most common after the sharp decline, and with male individuals, individuals aged 15-44 years, and farmers and laborers at increased risk of infection. Serological analysis revealed that mean pre-infection anti-PvMSP1\textsubscript{19} IgG was indistinguishable between clinical groups while mean IgG elicited during infection was significantly higher in symptomatic individuals, with pre-infection Ab associated with asymptomatic infection while Ab elicited during infection with symptomatic malaria. The Ab maintenance study found that 43% of seropositive individuals maintained their serostatus eight to 15 months after patent infection, with an estimated half-life of 2.3 years. Increasing age was associated with reduced Ab decline and with increased risk of remaining seropositive over time.

**Conclusions:** The cross-sectional microscopy surveillance confirms that asymptomatic malaria is prevalent in low endemic areas in the Peruvian Amazon Basin, posing a challenge to malaria control efforts in the area. Although Ab can be both a marker of exposure and infection, circulating Ab months prior and during patent \textit{Pv} infection reflect different risks associated with clinical presentation. Despite low malaria transmission, seropositive anti-PvMSP1\textsubscript{19} IgG can be maintained eight to 15 months after natural infection, with over 2 years of estimated half-life. The present study supports the hypothesis that malaria infection in areas with reduced transmission can effectively stimulate an Ab response, eliciting naturally-acquired humoral responses capable of controlling clinical disease even under infrequent boosting circumstances. Conducting immuno-epidemiological studies on asymptomatic malaria, with a special focus on \textit{Pv},
will be crucial for better understanding malaria epidemiology both in Peru and the Americas, and contribute to the successful control and possible elimination of malaria in the region.
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<tbody>
<tr>
<td>ACT</td>
<td>Artemisinin-based Combination Therapy</td>
</tr>
<tr>
<td>AIC</td>
<td>Akaike Information Criterion</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
</tr>
<tr>
<td>AMI</td>
<td>Amazon Malaria Initiative</td>
</tr>
<tr>
<td>ANOVA</td>
<td>One-way Analysis of Variance</td>
</tr>
<tr>
<td>API</td>
<td>Annual Parasite Index</td>
</tr>
<tr>
<td>AU</td>
<td>ELISA Arbitrary Units</td>
</tr>
<tr>
<td>CD4</td>
<td>Cluster of Differentiation 4</td>
</tr>
<tr>
<td>CD8</td>
<td>Cluster of Differentiation 8</td>
</tr>
<tr>
<td>CDC</td>
<td>US Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>CSS</td>
<td>Cross-Sectional Survey</td>
</tr>
<tr>
<td>DARC</td>
<td>Duffy Antigen Chemokine Receptor</td>
</tr>
<tr>
<td>DDT</td>
<td>Dichlorodiphenyltrichloroethane</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene-diamine-tetraacetic Acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>EIR</td>
<td>Entomological Inoculation Rate</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ELISPOT</td>
<td>Enzyme-Linked ImmunoSpot</td>
</tr>
<tr>
<td>ENSO</td>
<td>El Niño Southern Oscillation</td>
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<tr>
<td>FCRL4</td>
<td>Fc-Receptor-Like-4</td>
</tr>
<tr>
<td>FOI</td>
<td>Force of Infection</td>
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<tr>
<td>G6PD</td>
<td>Glucose-6-phosphate Dehydrogenase</td>
</tr>
<tr>
<td>GFATM</td>
<td>Global Fund to Fight Aids, Tuberculosis and Malaria</td>
</tr>
<tr>
<td>GLM</td>
<td>Generalized Linear Model</td>
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<tr>
<td>GPI</td>
<td>Glycophosphatidylinositol</td>
</tr>
<tr>
<td>Hb</td>
<td>Hemoglobin</td>
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<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HRP2</td>
<td>Histidine Rich Protein II</td>
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<tr>
<td>IFA</td>
<td>Indirect Fluorescent Antibody</td>
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<td>IFNγ</td>
<td>Interferon gamma</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>IgG₁</td>
<td>Immunoglobulin G isotype I</td>
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<td>Immunoglobulin M</td>
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<tr>
<td>IQR</td>
<td>Interquartile Range</td>
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<tr>
<td>IRB</td>
<td>Institutional Review Board</td>
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</table>
iRBC  Infected Red Blood Cells
IRS  Indoor Residual Spraying
ITNs  Insecticide Treated Nets
kDa  Kilodalton
Kg  Kilogram
LDH  Lactose Dehydrogenase
LLINs  Long-lasting Insecticide Nets
LLPCs  Long-lived Plasma Cells
Log  Natural logarithm
MBC  Memory B Cells
MCWA  Malaria Control in War Areas
Mg  Milligram
MIGIA  Malaria Immunology and Genetics in the Amazon
MINSA  Peruvian Ministry of Health
MR4  Malaria Research and Reference Reagent Resource Center
MSP1  Merozoite Surface Protein I
NAI  Naturally-Acquired Immunity
NAMRU-6  U.S. Naval Medical Research Unit No. 6
Nm  Nanometer
OD  Optical Density
OPD  O-Phenylenediamine
OR  Odds Ratio
ORAS  Andean Organism for Health
PAHO  Pan-American Health Organization
PAMAFRO  Malaria Control Program in Andean-country Border Region
PBS  Phosphate-buffered Saline buffer
PBS-T  PBS Tween
PC  Plasma Cells
PCR  Polymerase Chain Reaction
PCV  Packed Cell Volume
Pf  Plasmodium falciparum
PfEMP1  P. falciparum Erythrocyte Membrane Protein I
PHS  Public Health Service
Pk  Plasmodium knowlesi
Pm  Plasmodium malariae
PMI  President’s Malaria Initiative
Po  Plasmodium ovale
PR  Parasite Rate
Pv  Plasmodium vivax
qPCR  Real Time Quantitative Polymerase Chain Reaction
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>RAVREDA</td>
<td>Amazon Network of the Surveillance of Antimalarial Drug Resistance</td>
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<tr>
<td>RBC</td>
<td>Red Blood Cells</td>
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<tr>
<td>RBM</td>
<td>Roll Back Malaria</td>
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<tr>
<td>RDTs</td>
<td>Rapid Diagnostic Tests</td>
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<tr>
<td>RFIHB</td>
<td>Rockefeller Foundation’s International Health Board</td>
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<tr>
<td>RT</td>
<td>Room Temperature</td>
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<tr>
<td>SD</td>
<td>Standard Deviation</td>
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<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
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<tr>
<td>SP</td>
<td>Sulfadoxine-Pyrimethamine</td>
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<tr>
<td>SR</td>
<td>Spleen Rate</td>
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<tr>
<td>TNFα</td>
<td>Tumor Necrosis Factor Alpha</td>
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<td>μL</td>
<td>Microliter</td>
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<tr>
<td>UNICEF</td>
<td>United Nations Children’s Fund</td>
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<tr>
<td>US</td>
<td>United States of America</td>
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<tr>
<td>USAID</td>
<td>US Agency for International Development</td>
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<tr>
<td>VSAs</td>
<td>Variant Surface Antigens</td>
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<tr>
<td>WBCs</td>
<td>White Blood Cells</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<td>WWI</td>
<td>World War I</td>
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I. Chapter One: Literature Review

1.1 Malaria History

Malaria is an ancient disease that has been referenced in many historical records of early civilizations such as China, Mesopotamia, Egypt, India and Greece (1). For centuries, it was believed that miasmas, mal’aria in Italian, rising from swamps caused malaria fevers. It was not until 1880 that Charles Louis Alphonse Laveran discovered the etiologic agent of the disease and 1897 that Ronald Ross discovered that mosquitoes were the vectors. Laveran observed that the spleens and fresh unstained blood of infected patients contained pigment. He found erythrocytic organisms that were removed with quinine from the blood, and that the course of the fevers coincided with the burst of ‘crescentic bodies’ (1). From his studies with malaria-infected and healthy subjects and microscopy work, Laveran realized that the etiologic agent of the disease was a parasitic protozoan. This was an unprecedented discovery, as no other protozoan had been found within human blood cells. For this discovery, the Nobel Prize for Medicine was awarded to him in 1907. Despite the accumulated knowledge about the disease, the mechanism by which malaria parasites spread between humans eluded malariologists. By the 1890s, several malariologists considered that mosquitoes were involved in the transmission of parasites. In 1897, Ronald Ross discovered that avian malaria parasites were transmitted by culicine mosquitoes and suggested that human malaria might be transmitted the same way, which he demonstrated by 1899. Subsequently, Ross and a team of Italian malariologists led by Giovanni Batista Grassi demonstrated the development of Plasmodium falciparum (Pf), Plasmodium vivax (Pv) and Plasmodium malariae (Pm) parasites in anopheline mosquitoes that fed from infected patients and their successful
transmission to healthy volunteers. The discovery of the mosquito lifecycle provided malarialogists with the possibility of controlling the disease by decreasing human contact with the vector. Different methods to prevent mosquito bites were developed over the following decades.

By the 20th century, the United States (US) government’s involvement in Cuba and the Panama Canal played another key role in history of malaria. The work that Dr. William Gorgas, the Chief Sanitary officer, carried out during that time inspired both the US Public Health Service (PHS) and the Rockefeller Foundation’s International Health Board (RFIHB) to perform surveillance and treatment campaigns to control malaria in the South. They carried out demonstration projects for the control of malaria, employing spraying of larvicides, water management, window screening, and mass administration of quinine, which were successful in reducing malaria morbidity by 70% across the South of the US (2). Large-scale efforts to control malaria began with World War I (WWI), where the disease afflicted a significant proportion of the troops that contracted malaria in and around training bases in southern US, and accentuated during World war II (WWII), during which the US formed the Malaria Control in War Areas (MCWA) agency (MCWA). During WWI, the PHS and RFIHB carried out drainage and larviciding operations, reducing malaria mortality by more than 60% by the end of the 1920s (2). During WWII, malaria caused more causalities than the enemy and had a significant impact on the operations and battles (3). The MCWA’s goal was to prevent the reintroduction of malaria into civilian population as well as the training of health department officials in the control of malaria. After WWII, the MCWA expanded and became the US Centers for Disease Control and Prevention (CDC), which and continued its mission to control malaria.
Based on the success of the regional malaria eliminations campaigns, in 1955 the World Health Organization (WHO) and the Pan American Health Organization (PAHO) formed the Global Malaria Eradication Program. The central component of the eradication campaign was the spraying of houses with dieldrin once a year, presumptive treatment of subjects with radical cure when warranted, and active and passive case detection (4). Due to the emergence of resistant vectors to dieldrin, the eradication campaign switched to dichlorodiphenyltrichloroethane (DDT), which was used biannually. The program succeeded in eliminating malaria from North America, Europe, the Caribbean and parts of Asia and South-Central America but did not achieve any major success in sub-Saharan Africa (5). By 1969, the eradication program was abandoned mainly due to technical challenges in the execution of the eradication strategy and failure to sustain the program. The following years, during the post-eradication era (1969-1991), the WHO and PAHO changed their goal of malaria eradication to malaria control efforts, focusing on technical issues and research and development of control tools (e.g. insecticide-treated nets) that led to the advancement in antimalarial drugs, vector control, and vaccine development. These new tools, together with a worsening malaria situation, resulted in a renewed focus of eliminating malaria as a public health problem. Different initiatives and programs to address and control malaria were formed at the end of the 20th century and the beginning of the 21st century, such as the Roll Back Malaria (RBM) Initiative, the US President’s Malaria Initiative (PMI), the Global Fund to Fight AIDS, Tuberculosis and Malaria (GFATM). The money invested in development assistance for health ($ 11.3 billion from 2000 to 2011) facilitated the scale-up of malaria prevention and control measures (e.g. long-lasting insecticidal nets, rapid diagnostic tests, artemisinin-based combination therapies), which have dramatically reduced the burden of
malaria in many places (6). Still, the challenge of controlling global malaria remains formidable, but new tools (e.g. vaccines) and advances in science have reinvigorated current efforts to control malaria and have put malaria eradication back on the global health agenda.

1.2 Disease Burden

1.2.1 Worldwide

Malaria remains among the top priorities on the global health agenda, despite decades of control efforts and eradication attempts, due to its significant disease burden and the lack of an effective malaria vaccine and sustainable malaria prevention programs (7, 8). Malaria is an acute febrile illness and major health concern that is widely spread in tropical and sub-tropical regions. Malaria affects low- and middle-income countries the most, of which the most affected are the poorest and marginalized communities. Global malaria cases and deaths increased rapidly since 1990 and reached its peak in 2003 with 232 million cases (uncertainty range: 143-387 million) and in 2004 with 1.2 million deaths (uncertainty range: 1.1-1.4 million) (6).

According to the latest WHO World Malaria Report, between 2000 and 2013, malaria incidence decreased by 37% and malaria mortality by 47%, with approximately 670 million cases and 4.3 million deaths averted during that period of time (9). The majority of the decreases in malaria cases were in the European Region, the Region of the Americas, and the Western Pacific Region, while the majority of decreases in malaria deaths were in the African Region (54% decline), especially in children under five years of age (58% decline).
Globally, 3.3 billion people are estimated to be at risk of infection, living in 97 countries and territories, of whom 1.2 billion people are at high risk of infection (Fig. 1), and with 198 million cases (uncertainty range 124-283 million) and 584,000 deaths (uncertainty range 367,000-755,000) in 2013 (9). The majority of the estimated malaria cases come from the African Region (~82%), followed by the South-East Asia Region (~12%), the Eastern Mediterranean Region (~5%), followed by the Western Pacific Region, the Americas Region, and finally by the European Region (9). In 2013, three countries accounted for approximately 50% of all malaria deaths: Nigeria (~30 million), DR Congo (~6 million), and India (>60 million); and four countries had the highest annual number of cases: India (>60 million), Nigeria (~6 million), and Mozambique (~6 million) (6). The majority of malaria-related deaths occur in the African Region (90%), of which the majority of all deaths are in children under five years of age (78%) (9). For \( P_v \) malaria, the majority of estimated cases (80%) come from only three countries: India, Indonesia and Pakistan (9).

1.2.2 Disease burden in the Americas

The origin of \( P_v \) malaria in the Americas is not yet clear with two different and non-exclusive possible models of introduction, one suggesting a recent post-European-contact introduction possibly involving multiple parasite populations and another that postulates a pre-Columbine human introduction that diversified \textit{in situ} (10). The origin of \( P_f \) malaria in the Americas dates back to the end of the 15\textsuperscript{th} century, most likely through the trans-Atlantic slave trade in the Americas (11). Between the 16\textsuperscript{th} and 19\textsuperscript{th} century, the introduction of malaria into South America was a recurrent process. By the 19\textsuperscript{th} century, malaria had spread globally putting over half of its population at risk (5).
Currently, there are 21 countries\(^1\) in the Americas with ongoing malaria transmission, with approximately 120 million people at risk of malaria, of whom 25 million are at high risk of infection (9). According to the latest WHO World Malaria Report, three countries (Brazil, Venezuela, and Colombia) accounted for the majority (72\%) of cases in 2013 (9). Also, thirteen countries\(^2\) reported a >75\% reduction in malaria incidence (microscopically confirmed). Two countries (Brazil and Colombia) are on track to achieve a 75\% decrease in case incidence by 2015, while three countries (Dominican Republic, Panama and Peru) are on track to achieve a 50-75\% decrease in case incidence by 2015. Three countries reported an increase in their reported malaria cases (Guyana, Venezuela and Haiti), although it is unclear if the increased malaria incidence in Haiti is real or due to increased diagnostic testing and reporting. The majority of cases in the American Region are due to \(Pv\) malaria (>70\%). The majority of \(Pf\) malaria is concentrated in the Dominican Republic and Haiti, where it accounts for almost 100\% of all reported cases, and Guyana and Suriname, representing over 50\% of all reported cases.

1.2.3 *Disease burden in Peru*

The Republic of Peru has a population of 31,151,643 inhabitants (12) and is organized in 25 departments and 196 provinces (Fig. 2). The three main geographical regions are: a) the Pacific coastal region, b) the highlands with the Andes mountains, and c) the jungle in the Amazon Basin, which are further subdivided into eight life zones or

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\(^1\) Argentina, Belize, Bolivia, Brazil, Colombia, Costa Rica, Dominican Republic, Ecuador, El Salvador, French Guiana, Guatemala, Guyana, Haiti, Honduras, Mexico, Nicaragua, Panama, Paraguay, Peru, Suriname, Venezuela

\(^2\) Argentina, Belize, Bolivia, Costa Rica, Ecuador, El Salvador, Guatemala, Honduras, Mexico, Nicaragua, Paraguay, Suriname, and French Guiana.
Natural Regions. It has a large variety of climates or microclimates, with 28 of the 32 world climates. Peru is undergoing an epidemiological transition with both communicable and non-communicable diseases affecting the country. Vector-born diseases are still a major health problem, with dengue and malaria as main causes (13). Overall, the Amazonian Departments of Loreto, San Martin, Ucayali, Madre de Dios, and Cusco have the highest burden of malaria, accounting for approximately 80% of all malaria cases in the country (14, 15).

Malaria has been present in Peru since at least the 1500s and remains an important public health problem despite longstanding efforts to control the disease during the 20th and 21st century (16). During the 1940s, the burden of malaria was high with approximately 95,000 reported cases, which subsequently declined to 1,500 cases by 1965 as a result of the malaria eradication campaign launched by the Rockefeller Foundation (17). In 1947, the Rockefeller Foundation introduced DDT to Peru and was used by the National Malaria Service in Peru’s coastal valleys and the Amazon region, resulting in a significant decline in malaria morbidity from approximately 945 per 100,000 cases (1941-1946) to 490 per 100,000 cases (1947-1958) (16). Subsequently, Peru and the Americas malaria shifted from a malaria control to elimination strategy, sponsored by the Interamerican Public Health Cooperative Service and supported by the United Nations Children’s Fund (UNICEF). As a result, malaria disappeared from some departments with uninterrupted transmission, being almost malaria free in the entire coastal region, Andean valleys and in the southern Peruvian Amazon by 1970 (18, 19).

Due to the appearance of mosquitos resistant to DDT and decreasing funds allocated to malaria control strategies, malaria gradually increased in 1970 (18) with malaria outbreaks and Pf malaria spreading across the country. In the 1990s, the burden
of malaria increased drastically, with the number of cases increasing 4-fold in Peru and 50-fold in Loreto (20), peaking to 250,000 reported cases by 1998. During this outbreak, Peru was the second country with the highest number of reported malaria cases, only after Brazil, with the majority of them in the Department of Loreto from communities located in the peri-Iquitos region (20, 21). Even though \( P_v \) malaria remained the predominant species in the country with approximately 160,000 reported cases \((P_f: 84,000 \text{ cases})\) (22), \( P_f \) malaria was the predominant species in the Peruvian Amazon region with a 2:1 ratio of number of reported cases compared to \( P_v \) (23). Following the scale-up of malaria control activities, the number of \( P_f \) cases decreased, accounting for less than one third of all reported malaria cases in the region (20, 24).

Various factors contributed to the reemergence of malaria, such as an extensive internal migration, the expansion of areas irrigated for rice and cotton farming, reintroduction and spread of the \( Anopheles darlingi \) vector (the predominant vector in South America), difficulties managing control activities in remote areas, illegal drug activities in the jungle, high malaria incidence in the neighboring countries of Ecuador and Colombia (25), and the effects of climate changes such as El Niño Southern Oscillation (ENSO). National malaria control activities intensified and multi-lateral initiatives such as the Amazon Malaria Initiative (AMI), the Amazon Network for the Surveillance of Antimalarial Drug Resistance (RAVREDA), and the Malaria Control Program in Andean-country Border Region (PAMAFRO) were launched with support from international agencies and foreign governments, and were able to stabilize the incidence of malaria in the country, with a steady decline from 2006 until 2011 with sustained low transmission intensity (15, 20, 22, 26). In 2012, heavy rains in the Department of Loreto resulted in floods that led to a malaria outbreak in the region.
National malaria cases increased from 23,060 confirmed cases (88.5% *Pv* and 21.5% *Pf*) in 2011 to 31,704 confirmed cases (87% *Pv* and 13% *Pf*) in 2012, to 48,839 confirmed cases (84% *Pv* and 16% *Pf*) in 2013, and to 65,235 confirmed cases (84% *Pv* and 16% *Pf*) in 2014.

### 1.3 Malaria Epidemiology

Humans are the only reservoir for the four human malarialas (*P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*), and are exclusively transmitted by mosquitoes from the *Anopheles* genus. Approximately half of the world’s population (3.4 billion) is at risk of malaria (9). Malaria morbidity and mortality are not evenly distributed around the globe, where some groups are more at risk than others. The at risk groups are: 1) young children who have not yet developed protective immunity to severe disease who live in stable transmission areas, 2) pregnant women living in transmission areas, 3) people infected with HIV/AIDS, 4) international travelers from non-endemic areas, and 5) returning immigrants from non-endemic to endemic areas (9).

Malaria endemicity is classified as: a) holoendemic, b) hyperendemic, c) mesoendemic, or d) hypoendemic according to its transmission intensity. In areas with stable malaria transmission the risk of severe disease is limited to infants, young children, pregnant women and visitors; the greatest mortality rates are in children under-two; and there are frequent asymptomatic cases that increase with age. Areas with unstable malaria transmission have much adult malaria with a broad range of disease outcomes in all age groups; and all exposed people are at risk of disease. Traditionally, malaria endemicity has been established through the spleen rate (number of palpable enlarged spleens in a sample of similar ages of the population, e.g. children under-five), or the parasite rate,
Malaria transmission in holoendemic settings occurs all year long, with high perennial transmission (stable), where malaria mortality and anemia are highest during the first years of life, and where the majority of children under-five years has enlarged spleens (>75%) and are parasitemic (>60%-70%) (27). Hyperendemic settings have intense but seasonal malaria transmission (stable) with periods of no transmission during the dry season, where more than half the children under-five have enlarged spleens (>50% but <70%) and are parasitemic (>50% but <70%). Mesoendemic settings have regular seasonal malaria transmission of varying intensity (unstable) depending on the local circumstances, such as rainfall conditions, where 20%-50% of children under-five have enlarged spleens and <20% are parasitemic. Hypoendemic settings have very intermittent transmission (unstable) with periodic transmission succeeding unusual rainfall, where less than 10% of children under-five have enlarged spleens and are parasitemic.

Risk factors associated with malaria morbidity and mortality are related to both the host and the parasite, such as age, prior exposure, immune status, genetic background, malaria species and parasite strain, and transmission intensity. Risk factors associated with the acquisition of malaria are related to the environment, such as the proximity to mosquito breeding sites and water bodies, agricultural lands or forests where mosquitoes breed (28, 29), as well as behavioral and social risk factors, such as poor housing conditions, population movement, irregular or non-use of mosquito nets, treatment-seeking patterns, practices of drug utilization, and other (30). The distribution and abundance of mosquito vectors, and therefore the transmission of malaria, are affected by
different factors such as: 1) temperature, 2) presence of water for larval breeding, 3) seasonal fluctuation of mosquito populations, 4) duration of conditions suitable for mosquito survival, and 4) vectorial capacity (measurement of efficiency of vector-born disease transmission) (27).

The entomological inoculation rate (EIR), which is the number of infectious mosquito bites received per person per unit of time, is used to measure malaria transmission intensity. It is the product of the human biting rate (number of mosquitoes biting a person over a fixed period of time) and the sporozoites rate (fraction of mosquitoes that are biting and infectious). The main advantage of the EIR is that it directly reflects the effect of anti-vector actions and antigametocytocidal drugs, while its disadvantages are the lack of a standardized protocol, variability in methodologies, and the scarcity of trained specialists (31). In areas where the annual EIR are below 10, malaria transmission tends to be unstable and of low-moderate intensity, with a prevalence rate almost directly proportional to the EIR. In areas where annual EIR are above 10, malaria transmission tends to be stable and of high intensity. There are other indices of malaria transmission, each with their own advantages and disadvantages, as the parasite rate (PR) that estimates the proportion of the population carrying infected RBCs, the Annual Parasite Index (API) that estimates the number of parasite infections in a defined area per 1,000 per year, and the spleen rate (SR) that estimates the proportion of children 2-9 years of age with a palpable spleen. Most recently, it has been recognized that EIR is a more direct measure of transmission intensity and has been used to establish malaria epidemiology (32).
1.4 Malaria Diagnosis

Prompt and accurate malaria diagnosis is fundamental for the initiation of effective disease management. Clinical suspicion and parasitological diagnosis through detection of parasites in blood are the basis of malaria diagnosis. Malaria suspicion is mostly based on the presence or history of fever, but diagnosis solely based on this feature has very low specificity because malaria signs and symptoms are nonspecific, and can result in malaria over-treatment. Parasitological diagnosis can be performed through light microscopy (thin and thick smears), as well as with rapid diagnostic tests (RDTs), or molecular assays as polymerase chain reaction (PCR) test. The choice of diagnostic modality depends on local conditions, such as available technical skills; patient case-load; malaria epidemiology in the area; the possibility of using microscopy for the diagnosis of other diseases; as well as costs. For example, in settings with high caseload of febrile patients, microscopy may cost less than RDTs, but may be less logistically feasible.

Even though the diagnosis of malaria by microscopy allows for the quantification of parasites in blood as well as detection of other blood-born diseases, it is time consuming and relies on good microscopes, reagents and skilled technicians. Still, microscopic diagnosis of malaria has been in use for over a century given its low cost and relatively good sensitivity when appropriately used. In reference laboratories, microscopy diagnosis can reliably detect parasite densities >10 parasites per μl of blood (33), whereas in areas outside research settings and reference laboratories it reliably detects parasite densities in the range of 50-100 parasites per μl of blood (34).

Malaria infection can also be detected through RDTs, which detect specific malaria antigens that are present in blood. RDTs were mainly designed to be used in malaria endemic areas that are beyond the reach of quality microscopy diagnosis. These
point-of-care tests are sensitive, fast, and simple to perform, and are being used by several national malaria control programs around the globe, mostly in the African Region and South-East Asia Region. Their performance in the field is influenced by different factors, such as the storage conditions (35, 36), their correct interpretation (37), and parasite density (38, 39). The main disadvantage of using RDTs to diagnose malaria is that they are not a quantitative test, are expensive, do not perform well at low parasitemias, and not all RDTs are suitable to detect non-Pf species. Histidine-rich protein II (HRP2) based RDTs, which detects Pf histidine-rich protein II (PfHRP2), can give false positive diagnosis weeks after parasites are eliminated (40-42). Furthermore, a study in the Peruvian Amazon Basin documented that Pfhrp2- and Pfhrp3-deleted Pf parasites had false-negative RDTs results and therefore this RDT might fail to detect a high proportion of Pf malaria in areas with these genes deletions (43). Other malaria RDTs are based on the detection of the enzyme lactate dehydrogenase (LDH) or aldolase. LDH is a protein produced by both the sexual and asexual stages of the parasite of all human malaria species. Therefore, LDH-based RDTs can detect all human malaria species, including mixed infections. However, this RDT does not preform well at low parasite densities (44-46) but, unlike HRP2 RDTs, the LDH protein does not persist in the blood after the clearance of parasites (47, 48). Aldolase, a glycolytic enzyme, is another target for malaria RDTs. This enzyme is found in tissues of the host and malaria parasites, and is used to detect Pf and Pv malaria. The sensitivity of this RDTs is also parasite-density dependent.

The PCR test can diagnose malaria disease by amplifying the parasite’s DNA. This test can detect low concentrations of parasites with higher sensitivity and specificity than microscopy (49), but it is time consuming and requires specialized equipment.
Therefore, PCR is not suitable for most field settings. It can detect up to 0.1 parasites per microliter of whole blood or 2 parasites per microliter of blood collected by filter paper, is capable of give a species-specific diagnosis, and can identify mutations that might be correlated to drug resistance.

1.5 *Plasmodium vivax*

1.5.1 *Epidemiology*

*Plasmodium vivax* (*Pv*) is currently the major cause of malaria outside of Africa, threatening almost 40% of the world’s population (50). It is the human malaria species most widely distributed in the world and the most common species in temperate regions of the world. This *Plasmodium* species has probably evolved in Southeast Asia approximately 217,000 to 304,000 years ago in small nomadic groups (51). Globally, there are up to 2.6 billion people at risk for *Pv*, and 80-300 million estimated clinical cases every year (52, 53). In 2013, the World Malaria Report estimated 15.8 million cases (uncertainty range 11.9 – 22 million cases), representing 8% of all the malaria cases that occur worldwide (9). It is estimated to represent 47% of all malaria infections outside sub-Saharan Africa, with the majority of cases originating from the South East Asia Region (11 million estimated cases, uncertainty range 7-17 million cases) where it accounts for 44% of all malaria cases, and the Eastern Mediterranean region (3 million estimated cases, uncertainty range 2.3-3.8 million cases) where it accounts for 33% of all reported cases, followed by the African region (1.4 million estimated cases, uncertainty range 1-1.7 million cases) where it accounts for 1% of all reported cases, the Americas (5000,000 estimated cases, uncertainty range 400,000-600,000 cases) where it accounts for 62% of all reported cases, the Western Pacific region (200,000 estimated cases,
uncertainty range 100,000-400,000 cases) where it accounts for 16% of all reported cases, and finally the European region (2,000 estimated cases) where it accounts for 43% of all reported cases (9). It is rarely found in sub-Saharan African countries, where it represents less than one percent of the recorded infections (54). Over time, \( P_v \) persists across most of its historic territory, with the exception of some regions in the Northern Hemisphere where it has receded completely.

### 1.5.2 Lifecycle

\( P_v \), as the other four human malaria parasites has a complicated life cycle that is spent between a mosquito vector (sexual cycle) and a human host (asexual cycle) (Fig. 3). The disease is transmitted through the bite of an infective mosquito that inoculates sporozoites into the host, which enter the bloodstream and reach the liver, invading hepatocytes. Within the hepatocytes, sporozoites differentiate into tissue schizonts and undergo mitotic replication (exoerythrocytic schizogony) producing many merozoites. Within the liver stage, \( P_v \) (as well as \( P. ovale \)) has an additional life-stage called hypnozoite (Fig. 4). Hypnozoites are formed in the liver and can remain in a dormant state upon subsequent re-activation, weeks, months or up to years after the initial infection. This re-activation of hypnozoites is known as relapse (55). After 7-10 days, parasitized hepatocytes burst open releasing merozoites into the bloodstream and initiate the erythrocytic cycle. In the blood stream, merozoites invade red blood cells (RBC) and differentiate into trophozoites, which undergo mitosis (erythrocytic schizogony) and turn into schizonts containing large numbers of merozoites within. Infected RBC (iRBC) burst open, releasing merozoites back into the bloodstream, thus perpetuating the erythrocytic cycle. Overall, the erythrocytic cycle takes approximately 48 h to be completed for \( P_v \), as
well as for Pf and P. ovale (Po), while Pm takes 72 hours and P. knowlesi (Pk) takes 24 hours (56). Disease results from the multiplication of parasites within red blood cells (RBC) and their subsequent rupture, causing symptoms like intermittent fever, headache, and anemia.

During the erythrocytic cycle some trophozoites differentiate into male and female gametocytes, the sexual stages of the parasite in the human host, which circulate in the peripheral blood until they are taken up by female Anopheline mosquitos with a blood meal. Within the mosquito vector, gametocytes undergo their sexual life stage in the midgut where they mature and are fertilized, form a zygote and differentiate into an ookinete (57). Ookinetes cross the midgut epithelium and differentiate into oocysts under the basal lamina (58). Within oocysts another phase of multiplication occurs where sporozoites are formed (sporogony). Once oocysts burst, sporozoites are released into the hemocele, migrate through the body cavity and finally invade the salivary glands through ligand-receptor interactions (59). Sporozoites are ultimately inoculated into new hosts whenever the vector takes a new blood meal (60). Approximately 20% of all sporozoites will successfully invade the vector’s salivary glands (61). The total amount of time required for the development of the parasite within the vector, known as the extrinsic incubation period, ranges from 10-21 days. Because mosquito vectors play such a crucial role in the transmission cycle of malaria, tropical and sub-tropical climates are favorable to sustain endemic malaria.

1.5.3 Microbiology

Pv has unique biological features that distinguish it as a species (52). One of the most important features is its ability to relapse due to the activation of hypnozoites.
Another feature of *Pv* malaria is the length of its incubation period, ranging from 12 days to up to several months. Also, *Pv* parasites can differentiate into gametocytes before clinical infection is evident and subsequent treatment is administered, allowing for a continued host-vector transmission (62). Furthermore, *Pv* invades preferably reticulocytes, which limits its growth both in vivo and in vitro. This preference for young red blood cells results in lower levels of parasitemia capable of inducing symptoms, as well as a lower pyrogenic threshold. *Pv* infections in turn result in more severe cycles of symptoms (i.e., fever, sweats and chills) and higher levels of proinflammatory cytokines, such as tumor necrosis factor alpha (TNFα), compared to *Pf* infections with similar parasitemia (63, 64). Moreover, vivax infected RBC (iRBC) tend to present all parasite stages in peripheral blood and therefore become more deformed as they mature (65), which is believed to explain its inability to sequester in the microvasculature or cytoadhere and result in severe infections. However, this paradigm has been recently challenged by different research groups who have observed the sequestration of *Pv* parasites in organs, such as the lung (66).

### 1.5.4 Pathology

As a result of the rupture of iRBC, a variety of products are released resulting in inflammation, which is associated with pathology. The pathology of *Pv* malaria can range from asymptomatic to symptomatic infections, with the presentation of diverse symptoms, and can progress from a mid uncomplicated infection to severe disease and even fatal outcomes. Early infections are usually symptomatic, which can become asymptomatic over time with re-exposure to parasites. Clinical symptoms of malaria are caused by infected iRBC that release toxic elements when they burst, such as hemozoin,
which stimulate the production of cytokines and other soluble factors (67). These immune factors are responsible for inducing fever and rigors, and might also influence other pathophysiological consequences associated with malaria. The most common symptoms of uncomplicated malaria are fever, chills, sweats, headaches, nausea and vomiting, body aches, and general malaise (67), which are non-specific symptoms that can be confused for different febrile illnesses. Physical signs of uncomplicated malaria encompass elevated temperatures, perspiration, weakness, enlarged spleen, mild jaundice, enlargement of the liver, and increased respiratory rate (67). Intermittent fever is a typical symptom for malaria infections, which occur every second day in *Pv*. Uncomplicated *Pv* infections have been associated with mild anemia, mild thrombocytopenia, elevation of bilirubin, and elevation of aminotransferases (67). Thrombocytopenia (platelet counts <150,000/μL) appears to be frequent among *Pv* malaria patients, and it is thought to be even more frequent than *Pf* patients (68).

Studies in Papua New Guinea (69), Indonesia (70, 71), India (72, 73), and Brazil (74), reported a significant proportion of hospitalized severe malaria cases had *Pv* monoinfection without the presence of other comorbidities associated with the observed disease severity. For *Pv* monoinfections, the major severe clinical symptoms are severe anemia, thrombocytopenia, pancytopenia and coagulopathy (72, 75-77), seizures and cerebral malaria (72, 78-80), acute renal dysfunction (75, 81-83), acute hepatic dysfunction and jaundice (72, 75, 82), acute pulmonary dysfunction (66, 84), acute respiratory distress syndrome (84-86), splenic rupture (87), and even death (71, 88). Furthermore, the inflammatory response to *Pv* seems to be greater than that seen with *Pf* infections of similar or even greater parasite burden (63, 89, 90). *Pf* only invades
reticulocytes (91, 92) thus resulting in lower parasite burden, even during severe disease (93), and has a lower pyrogenic threshold (50, 94).

*Pv* has been recognized as a common and highly significant health issue in pregnant women with serious consequences for maternal and infant health. Infected pregnant women tend to be more anemic and deliver more low-birth weight infants compared to uninfected women (95, 96), but less compared to pregnant women infected with *Pf* malaria. *Pv* infected reticulocytes do not express surface proteins that allow for their sequestration in the host vasculature such as the placenta (97). This suggests that rather than placental sequestration of parasites, the systemic infection in the mother may be responsible for the effects of *Pv* during pregnancy (97). Furthermore, the increased severity of *Pv* infection in subsequent pregnancies suggests that the mechanisms that are associated with parity-dependent immunity observed in *Pf*, such as antibodies against proteins involved in placental sequestration, might not be relevant to the pathogenesis of *Pv* infections during pregnancy (97).

### 1.5.5 Relapse

*Pv* disease can re-appear months to years after the initial infection due to the reactivation of hypnozoites, which is known as a relapse (55). The hypnozoite stage and relapse gives the parasite several advantages by maximizing the possibility to reach the vector for sexual reproduction. The mechanisms behind *Pv* dormancy and reactivation are still unknown, as well as the amount of hypnozoites that result from an infected bite. The ability of *Pv* to relapse represents a challenge for differentiating relapse infections from new infections or recrudescence. Furthermore, *Pv* relapse can result in either a new clinical episode or an asymptomatic infection. Genotyping can distinguish a
relapse/recrudescence from a new infection, but sometimes it cannot distinguish reliably relapse from recrudescence since they might have derive from the same initial infection (98). Nonetheless, the “rule of thumb” used in the field to discriminate between relapse from recrudescence is that if the recurrence takes place within 16 days of chloroquine initiation of the primary infection, it is most likely due to recrudescence due to drug failure; if it occurs between 17 to 28 days, it can either be due to recrudescence or relapse; if it occurs beyond day 28, it is probably due to relapse (99).

1.5.6 Treatment

The objective of *P. vivax* treatment is to cure the blood stage as well as the liver stage infection (radical cure), that is, treating the infection as well as preventing potential relapse. *P. vivax* is mostly still sensitive to chloroquine, although resistance to the drug has been reported in some areas such as Indonesia, Peru and Oceania (36). The WHO recommends chloroquine monotherapy as the standard treatment for *P. vivax* malaria in combination of primaquine to achieve radical cure. Blood stage *P. vivax* infections are treated with chloroquine (36). When chloroquine is contraindicated or resistance to it is common, artesunate is the drug of choice. In areas where artemisinin-based combination therapy (ACT) has been adopted as the first-line treatment against *P. falciparum*, it can also be used to treat *P. vivax* infections in combination with primaquine (PQ). Combination therapy of artemisinin and sulfadoxine-pyrimethamine (SP) is not effective against *P. vivax* infection due to increasing resistance to SP (36). Mefloquine and atovaquone-proguanil are effective alternatives to treat *P. vivax* malaria in patients who are unable to tolerate chloroquine or have chloroquine-resistant *P. vivax* malaria infections. Lastly, quinine may also be used to treat *P. vivax* malaria, but has been associated with inferior treatment outcomes. Severe *P. vivax* malaria case
management should be the same as for severe/complicated *Pf* malaria with parenteral administration of quinidine gluconate (36).

Because PQ kills hypnozoites, the use of this drug is key for the effective treatment of *Pv* malaria, and is only administered to adults and children six months of age or older, and to pregnant women only after delivery. A minimum of 14-day course of primaquine is needed to achieve radical treatment. Before administering primaquine to *Pv* cases, glucose-6-phosphate dehydrogenase (G6PD) status should to be assessed in order to reduce the risk of hemolysis, although some areas as Peru do not perform this test before administering the drug. G6PD deficiency results in acute hemolysis after primaquine treatment. Individuals with mild forms of G6PD deficiency, primaquine should be given in a dose of 0.75 mg base/kg body weight once a week for 8 weeks (36). If hemolysis is significant during treatment, it should be stopped. The risks and benefits of administering primaquine differ by high- vs. low-transmission areas. In high-transmission areas, the benefits of widespread primaquine use do not necessarily outweigh the risks associated with its use. In low-transmission areas, the benefit of its use on patients who are not G6PD-deficient will exceed its risks. Also, primaquine is contraindicated in pregnant women and children less than 4 years of age (36). Other drugs with significant activity against hypnozoites are buloquine and tafenoquine (36). Mixed infections are to be treated with ACTs, since they are effective against all malaria species.

Regarding G6PD deficiency, a study conducted in Peru by Ruebush *et al.* (2000) did not observe subjects with G6PD deficiency in the region of Padrecocha in Iquitos, Loreto. Additionally, a Naval Medical Research Unit No. 6 (NAMRU-6) primaquine clinical trial in Peru only observed 2 cases of G6PD deficiency out of 546-screened
subjects and failed to observe moderate or severe adverse effects after primaquine administration (100).

1.6 Naturally-Acquired Immunity to Malaria

Our understanding of the development of an immune response effective to prevent clinical malaria is limited at best. This inadequate understanding of the immune mechanisms associated with naturally-acquired immunity (NAI) to clinical malaria has contributed to our failure to develop an effective vaccine. Most of the existing evidence of NAI comes from studies conducted in high endemic settings with perennial intense transmission and are based on \( Pf \) infections. These studies have reported that NAI appears to take years to develop despite repeated exposures to parasites.

In humans, three different definitions of NAI have been described: i) anti-disease immunity (reduces the risk of malaria morbidity and protects against clinical disease), ii) antiparasite immunity (affects parasite density and protects against parasitemia), and iii) premunition (protects against new infections by maintaining a low-grade and usually asymptomatic parasitemia). NAI to clinical malaria is defined as a lower risk of clinical disease, as indicated by both the absence of fever with parasitemia and lower densities of parasitemia (101). The features of the current state of understanding of NAI to clinical malaria are that 1) it is acquired only after cumulative uninterrupted heavy exposure to the parasite, 2) is lost upon interruption to the exposure, 3) it is species specific, 4) stage specific, and 5) the rate of acquisition is dependent on the degree of exposure to the parasite.
1.6.1 **History of naturally-acquired immunity**

Robert Koch was the first scientist to report the existence of naturally-acquired protection against malaria through cross-sectional studies in Papua New Guinea. In the early 1900s, Koch examined parasite distribution (frequency and density) using stained blood films in subjects living in either low endemicity or high endemicity areas. He observed distinct age-dependent parasite distribution patterns in subjects living in high endemic areas compared to the more uniform pattern across age groups in subjects living in the low endemic areas (102-105). From his studies, Koch concluded that protection against malaria only developed after lifelong uninterrupted heavy exposure to the parasite. Studies conducted on *P.* *k.* in rhesus macaques demonstrated the existence of antigenic variation have also contributed to the hypothesis that immunity to malaria develops slowly after repeated infections.

Malaria therapy of neurosyphilis patients also shed some light on the development of NAI. Julius von Wagner-Jauregg demonstrated that an effective anti-malaria immunity could be induced in humans. Additionally, several clinical studies confirmed von Wagner-Jauregg’s observations. These studies reported that partial immunity to malaria could be induced in humans after a single infection, that repeated infections were capable to confer a more adequate protective immunity, and that protection against *Pf* seemed to be acquired more slowly than to *Pv* or *Pm* (106, 107). Immune protection to malaria parasites was reported to be species specific but not necessarily strain specific, that is parasites clones within a single species that have genetic polymorphisms that give rise to antigenically distinct proteins (107-109). Researchers observed that patients challenged with heterologous strains also exhibited shortened clinical episodes and reduced levels of parasitemia but of lesser magnitude compared to patients challenged with homologous
strains (107, 110-112). Even though malaria therapy reported the rapid onset of protective immunity in neurosyphilis patients, this observation was used to support the idea of a cumulative acquisition of NAI given the superior protection provided by homologous strains.

In the 1960s, Brown and Brown demonstrated frequent antigenic variation in *Pk* infected monkeys (113). They observed that the agglutination of schizont-infected cells was highest with homologous parasites, that relapses in chronic infections tended to result in low parasitemias, and that parasites causing these relapses resulted in virulent fatal infections when inoculated into normal ‘non-immune’ monkeys. They also argued that since adults do develop a reasonably immunity to *Pf* but children exposed to endemic malaria show high parasitemias, resistance to malaria depends on a cumulative experience to a diverse repertoire of antigenic variants. Therefore, they concluded that repeated infections allow for the accumulation of a diverse repertoire of antigenic memory capable of detecting and defeating most parasite variants. Additionally, Brown and Brown proposed the existence of two simultaneous levels of immunity, one variant specific and another that transcended antigenic variation and partially inhibited all relapses.

### 1.6.2 Naturally-acquired immunity epidemiology

NAI appears to develop in two stages: i) resistance to severe malaria, which is acquired quickly requiring only a small number of infections (114) and ii) partial immunity to blood-stage parasites and mild malaria, which is acquired more slowly after repeated exposure to the parasite (101, 114-121). Epidemic malaria reports, systematic surveys and studies in low and unstable transmission areas have shed light on the rapid
acquisition of NAI to severe disease, showing that prior exposure to malaria had a significant protective effect, even when it took place decades earlier (120, 122). Some systematic surveys have observed that previously immune adults had lower parasite densities and had lower risk of severe/fatal outcomes, compared to malaria-naïve individuals (123, 124). Studies in low and unstable malaria transmission areas have reported that early infections contributed to the protection of malaria infection later in life. Together, these observations support the idea that the acquisition of NAI against severe/fatal malaria is acquired relatively quickly and can be maintained across time, even without periodic memory boosting by re-infections (117).

There are two key factors associated with malaria morbidity and therefore with NAI, the intensity of exposure to the parasite and the age of the subject. In high-endemic areas, NAI results in lower prevalence of parasitemia with age and lower rates of disease (115). Infections are often severe in young children, with partial immunity to disease not being established until adolescence. Different studies in high-endemic areas have reported that Pf parasitemia peaks in children under-five, subsequently declining in an age-dependent manner (125, 126). Instead, in areas of lower malaria endemicity, parasitemia peaks in older individuals with lower parasite densities (127, 128).

In areas of high endemicity, it is difficult to separate the independent effects of age and exposure. For example, the NAI observed in adults could be interpreted as the cumulative exposure to malaria infections, and the vulnerability observed in infants and young children could be presumed to be the result of the reduced number infections. Research studies on Javanese migrants under abrupt and chronic exposure to infection have been able to study the development of NAI independently from the effect of exposure and age for both Pf and Pv (119). In general, these studies observed a rapid
acquisition of NAI to severe disease, as well as against parasitemia and mild disease. The age-specific prevalence of parasitemia was uniform among age groups at the beginning of the abrupt exposure. Subsequently, after 18 to 24 months a distinct age-dependent pattern was observed similar to what was observed in local residents with a life-long exposure to malaria, where adults experienced reduced clinical symptoms and lower-density parasitemias. Therefore, the migrant’s NAI did not result from a cumulative heavy exposure to malaria but resulted from their recent exposure. Other longitudinal studies on Javanese migrants have also observed an equal risk of infection in both children and adults upon abrupt exposure to \( Pf \) malaria (129).

Interestingly, studies on Javanese migrants, as well as observations from epidemic malaria, have described an increased risk of severe disease and death in adult subjects compared to equally exposed children (130-133). Together, these observations point to an age-dependent NAI with an inverse pattern in acute versus chronic exposure to infections, where resistance to severe malaria is acquired quickly after an acute exposure, where adults are at higher risk of severe outcomes, but under chronic exposure to infections adults develop a more robust anti-parasite and clinical NAI more quickly compared to children (134). Therefore, the susceptibility observed in young children living in high endemic areas may be due to innate differences in their acquired immune response to malaria infections, while the rapid acquisition of NAI in adults would suggest the development of a strain-transcending NAI (134).

Pregnancy has also been reported to have an effect on NAI, increasing the susceptibility to debilitating to severe disease and parasite densities. This susceptibility is associated to the immunosuppression experienced during gestation due to elevated
corticosteroids (135) that impair cell-mediated immunity, as well as the accumulation of PfIRBC in the placenta (136).

Finally, there are two hypotheses on the acquisition of NAI, one attributing the development of clinical immunity to the acquisition of a sufficiently diverse repertoire of strain-specific immune responses, and another one based the observations of the Javanese migrant studies that suggests that clinical immunity results from recent heavy malaria exposure. The first hypothesis would explain the slow onset of clinical immunity in high-endemic areas due to parasite diversity/antigenic variation resulting in strain-specific immunity, while the second hypothesis explains differences in NAI by age-specific characteristics that are independent of parasite exposure (101). The rapid onset of immunity in neurosyphilis patients versus the slower development in field studies was attributed to the use of homologous parasite strains versus heterologous challenges, supporting the concept that continuous exposure to a diverse repertoire of parasite strains is necessary to generate memory effector cells in naturally-exposed individuals. However, Doolan et al. argued that because the neurosyphilis studies did not include children, the observed rapid onset of immunity could also be an effect of age-dependent intrinsic differences in the immune response, which was observed in the migrant Javanese population where non-immune adults naturally exposed to malaria acquired a strain-transcending immune response quickly (101). Moreover, other studies on experimental malaria therapy observed that after a single inoculation with Pv parasites, patients induced a strong clinical protection to homologous re-infection and partial protection to heterologous re-infection (108).
1.6.3 Naturally-acquired immunity in low-endemic settings

Submicroscopic parasitemia and mild disease in hypoendemic areas could be explained as: i) the product of residual immunity acquired after repeated malaria exposure that maintains parasite density at a submicroscopic levels, ii) the result of an effective immunity mechanism that controls parasite density despite limited malaria exposure, suggesting that a low-level parasite exposure can generate a robust and prolonged immune response. Studies in the Solomon Islands (137), Brazil (138), Thailand (139) and Peru (140) suggest that residual immunity to *Plasmodium* parasites cannot be entirely responsible of the high proportion of submicroscopic infections, and report the acquisition and maintenance of *Pf*-specific memory B cells (MBC) and antibodies in both children and adults with few or no previous infections. Experimental *Pf* malaria infections in malaria-naïve volunteers by infected mosquito bites also describe long-lasting induction of protective immunity after limited exposure to parasites (141).

In a low-transmission malarious area in the Peruvian Amazon Basin, a cross-sectional study conducted in four villages located near the Peruvian Amazon region of Iquitos observed a substantial proportion of asymptomatic patients with patent *Pf* and *Pv* subjects, with cases detected through active and passive surveillance by light microscopy and PCR (49). Clinical immunity to *Pv* in low-transmission areas has also been observed in the Rondonia and Amazons states of Brazil (142, 143). These observations support the hypothesis that low transmission intensity may be sufficient to support the development of NAI to malaria infections and that infrequent annual malaria infections could effectively boost the immune response into developing and maintaining an adequate antimalarial immunity. Perhaps the capacity to mount an efficient memory response
contributes to the frequent asymptomatic Pf and Pv infections with low-density parasitemia in the Peruvian population.

1.6.4 Naturally-acquired immunity to P. vivax malaria

The development of NAI to Pv malaria results from both the exposure to malaria parasites during blood-stage infections and infection relapse. As described by Mueller et al., NAI to Pv malaria appears to be manifested as protection against high-density parasitemia and uncomplicated clinical disease, such as fever, malaise and anemia (144). Because Pv NAI has been observed in individuals without continuous boosting of their immune response through frequent or persistent malaria infections (premunition), such as in syphilis-infected individuals who underwent malaria-therapy (108, 145) and individuals who reside in low transmission settings (e.g. Solomon Islands, Peruvian, Brazil) (138, 146), it is believed that NAI to Pv develops more rapidly than to Pf and requires fewer infections. It is also possible that NAI to malaria develops differently in seasonal or low-transmission settings, involving different immunologic mechanisms, compared to in high-transmission settings (147). Nonetheless, observations that NAI develops more rapidly for Pv than Pf has also been observed in highly endemic settings as in Papua New Guinea, where children five years of age or older develop, after continuous exposure, almost complete clinical immunity capable of controlling blood-stage parasitemia (52, 147). Interestingly, studies on Javanese non-immune migrants that observed age-intrinsic differences in the development of NAI to Pf, with adults acquiring clinical immunity more rapidly than children after comparable exposure (130, 148), did not acquire more rapid immunity to Pv malaria, where both adults and children acquired
clinical immunity at a similar rate (149) suggesting species-specific differences in NAI development.

Different studies on syphilis-infected patients treated with *Pv* malaria have shed light on the biology and immunology of *Pv* malaria. Patients treated with *Pv* parasites observed that parasitemia could persist in low densities for 2-3 months in the absence of fevers and that relapse occurred after parasite clearance, producing lower parasitemias and resulting in more attenuated clinical disease, suggesting tolerance or immunity to disease (108, 150-152). Not only did these studies observe that NAI to *Pv* malaria develops more rapidly than *Pf* malaria, but they also reported almost complete strain-specific clinical immunity in up to two-thirds of the treated patients upon re-challenge, and partial protection against heterologous strains with significantly reduced mean daily parasite count and reduced incidence of high fevers (108, 145). After similar experimental malaria therapy studies, Ciuca *et al.* concluded that a single *Pv* infection is capable of inducing strong clinical protection to homologous strains, partial protection to heterologous strains, and no cross-species protection (108). Also, *Pv* NAI was similar when experimental infections were induced by intravenous inoculation or by bites of infected mosquitoes, suggesting that NAI was directed to blood-stage infection (153).

*Pv* malaria has unique biological characteristics that could contribute to the faster development of NAI, compared to *Pf* malaria. Differences in the acquisition of immunity could be explained by the ability of *Pv* to relapse, where latent stages in the liver could boost immunity even in the absence of or low malaria transmission. The periodicity of relapsing parasites and the population diversity of hypnozoites could facilitate the development of NAI in high-transmission settings compared to low-transmission settings with longer relapsing periodicity (154). Mueller *et al.* also suggested that relapsing
parasites could help explain the faster acquisition of NAI compared to Pf malaria, in that the periodicity of relapse and diverse hypnozoite populations account for more new Pv blood infections than for Pf malaria, despite the latter having similar or higher sporozoites rates (153). Therefore, hypnozoites could contribute to a greater force of blood infection for Pv than Pf malaria. Another biological characteristic contributing to Pv NAI development could be the larger vir gene family producing variant surface antigens that could facilitate host immune evasion (153). Finally, it is possible that the selective invasion of reticulocytes using the Duffy antigen chemokine receptor (DARC) helps the rapid development of NAI to Pv malaria by targeting this critical and perhaps non-redundant RBC invasion pathway, compared to the more redundant Pf invasion ligands (153). Non-redundant receptor proteins, which will most likely be highly conserved proteins given their critical role in RBC invasion and parasite survival, might be presented more consistently to the immune system, which will lead to a more rapid acquisition of naturally-acquired antibodies against them.

1.7 Malaria Immune Response

Immunity to malaria depends on a coordinated and timely innate and adaptive immune response, the parasite’s ability to evade the immune system, and the host’s susceptibility to the parasite.

1.7.1 Immune evasion

Plasmodium parasites have strategies to evade and disable the natural immune response. This immune evasion is achieved through antigenic variation and polymorphisms of variant surface antigens (VSAs). Antigenic variation is possible for Pf
due to its multi-gene family “var”, which are expressed on the surface of infected RBC. The var gene family encode for approximately 60 proteins known as erythrocyte membrane protein 1 (PfEMP1) as well as many additional VSAs (155) These var gene products are fundamental for binding to the endothelium and the sequestration of infected RBC (156, 157). Various var genes exist, which are individually expressed and get switched throughout clonal parasite populations over the course of an infection (158). Since existing malaria-specific antibodies do not recognize the newly switched antigens immediately, they allow for immune evasion and the establishment chronic infection. Malaria adaptive immunity that controls parasitemia is the result of the recognition of proteins encoded by var genes, and is a slow process that requires continuous exposure to the parasite (159). Variant genes have also been found in Pv malaria, named vir genes, which are also proteins that are exposed on the surface of iRBC (160) and seem to partly mediate cytoadherence (161).

1.7.2 Inflammatory response

The initial inflammatory response to malaria antigens and byproducts, such as the release of interleukin (IL)-1β, IL-6, IL-12 tumor necrosis factor alpha (TNFα), and interferon gamma (IFNγ), is crucial for controlling blood-stage malaria. Pro-inflammatory responses are associated with protective immunity to malaria during early infection, but an overproduction of IFNγ or TNFα can affect the development of severe immunopathology (115, 162). Malaria infection also elicits a strong pro-inflammatory response that is quickly down regulated by anti-inflammatory responses, such as IL-10 (163, 164). The development of pathology in malaria infection has been found to be associated with an imbalance of cytokines involved in the regulation of the inflammatory
response (162). Studies on *P. vivax* malaria in the Brazilian Amazon on individuals with asymptomatic, symptomatic and severe infection, observed that disease severity was strongly associated with the activation of pro-inflammatory responses and a cytokine imbalance, as well as a strong linear trend between increasing disease severity and increasing plasma levels of TNFα, IFNγ, and IFNγ/IL-10 ratio (90, 165).

### 1.7.3 Humoral response

Antibodies are thought to be the most critical mechanism of protection (101, 115, 139, 169), with several studies showing that passive transfer of antibodies is sufficient to control blood-stage disease by reducing parasitemia and clinical disease (101, 115, 170, 171). Antibodies are crucial in mediating acquired- and vaccine-induced immunity in humans (172). Human studies observed that antibody transfer with immunoglobulin gamma (IgG) from immune adults to *P. falciparum*-infected children resulted in significant reductions in parasite burden and disease (173), and that whole blood from highly immune subjects helped patients with acute infection (174). This control of blood-stage disease may be associated with the inhibition of merozoite invasion into new RBCs, blocking cytoadherence of iRBCs, and enhancing phagocytic activities.

Malaria induces a strong humoral immune response, primarily involving IgM and IgG immunoglobulin production. A large proportion of the antibodies produced during malaria are actually non-malaria specific, which are produced as the result of polyclonal B cell activation (173). Most of the existing studies on anti-malarial antibodies and disease protection have been conducted in high endemic areas, with very few studies in low-endemic settings. Currently, the existing evidence on the longevity and kinetics of anti-malarial antibodies is contradictory, with some studies reporting stable antibody
responses (139, 175-178) while other report short-lived responses (176, 179, 180). Some longitudinal studies in high endemic areas observed that anti-malarial antibody titers decline quickly after parasite clearance in children (162, 179-182), that over time they become increasingly stable with increasing age (183), as well as with persistent asymptomatic infections (180), and are long-lived in adults (139, 175-177). Studies in low-endemic settings reported that *Pf* malaria infections were capable of inducing long-lived antibody responses despite the infrequent exposure to malaria parasites (178, 181, 184).

Little information exists regarding differences in antibody levels between symptomatic and asymptomatic malaria cases. The few studies that have looked at differences in antibody levels by clinical presentation group have been on *Pf* malaria. A study by Iriemenam et al. in Sudan observed that asymptomatic individuals were older, had lower parasitemia, and had higher mean ratios anti-malarial antibodies with stronger relativities towards IgG1 and IgG3 (165). These suggests that anti-parasite immunity in asymptomatic malaria subjects appears to reflect a quantitative switch to specific IgG subclasses, especially IgG1 and IgG3, which are the highest complement activators and the IgG subclasses with the highest affinity to Fc-receptors on phagocytic cells. It also suggests that increased antibody levels might be associated with the absence of symptoms, through their ability to neutralize malaria parasites and antigens. Concerning the association of antibodies and clinical protection, antibodies can be both a marker of protection and exposure. A study in Papua New Guinea with *Pf* infected children under five years of age observed that antibodies were associated with increased risk of malaria disease and was considered a biomarker of exposure in populations with low immunity,
while it was associated with clinical protection in older children who had experienced greater malaria infections over the course of their life (183, 185).

1.7.4 **Immune memory**

The long-term protective humoral immunity against malaria parasites requires the generation and maintenance of memory B cells (MBC) and long-lived plasma cells (LLPCs). Upon antigen recognition, naïve B cells with high-affinity antigen binding differentiate into short-lived, isotype-switched plasma cells (PCs) that help control the initial malaria infection, while naïve B cells with lower affinity binding are selected to enter follicles and form germinal centers (186). Within germinal centers, naïve B cells undergo affinity maturation and immunoglobulin class-switching through a CD4+ T-cell dependent process, yielding LLPCs and MBCs. While LLPCs relocate to the bone marrow where they secrete antibodies that are critical during re-infection serving as a first line of defense, MBCs recirculate and upon antigen re-exposure and recognition initiate antibody recall responses proliferating and differentiation into acting PCs. Compared to other pathogens and vaccine antigens, which are efficient and durable (187, 188), MBC and LLPC responses to *Pf* infection seem less efficient. *Pf* studies in both high and low transmission settings observed MBCs only in a minor fraction of the population and appear to be dominated by short-lived PCs, describing it as an inefficient B cell response to infection (189, 190). Studies on the phenotype of malaria-induced MBC have observed an ‘atypical’ phenotype that expresses the inhibitory receptor Fc-receptor-like-4 (FCRL4), as observed in HIV- and hepatitis C-infected individuals, and expressing a profile of lymphoid-homing receptors similar to exhausted CD8+ T cells during chronic viral infections (191). Studies on MBCs during *Pf* infection in adults from
Mali, Peru, and non-exposed naïve US adults observed that atypical MBCs were expanded in individuals living in a low (Peru) and high endemic area (Mali), compared to U.S. *Pf*-naïve adults, and increasing transmission intensity was associated with the expansion of atypical MBCs (186).

Only a few studies on malaria immune memory have taken place in low endemic countries (178, 181, 184). A study by Wipasa *et al.* observed that infrequent *Pf* and *Pv* infections were capable of inducing long-lived antibody and MBC responses, that the “breadth” of these responses increased with age, and frequencies of MBCs were similar in order of magnitude as responses to commonly used vaccine antigens (e.g. diphtheria, tetanus toxoid) (139). These observations suggest that low exposure to malaria in low-endemic settings is capable of inducing long-lasting immune memory. Another study on *Pf* malaria conducted in the Peruvian Amazon Basin also observed a functional memory response when analyzing the MBC response to PfMSP1 by enzyme-linked immunospot (ELISPOT) assay. This study reported that even adult subjects with no prior malaria exposure, who were experiencing their first infection, had anti-*Pf*MBC present at various post-infection time points, and that the amount of prior exposure did not predict the magnitude of the antimalarial MBC response. These findings also suggest that immunological memory is achievable in individuals residing in low-endemic areas, and could be potentially extrapolated to the development of NAI to *Pv*, but this remains to be proven.

1.8 Merozoite Surface Protein 1

The merozoite surface protein subunit 1 (MSP1) is a protein that is expressed in all *Plasmodium* species abundantly on the surface of mature merozoites (192). MSP1 is a
200 kDa glycophosphatidylinositol (GPI)-anchored protein produced during schizogony that is expressed on the merozoite surface during the hepatic and the erythrocytic stages of the parasite (193). It is composed of several blocks of conserved and variable regions, specifically six highly polymorphic regions interspersed with seven conserved blocks for \( P.v \) MSP-1 (PvMSP1) protein (194). The protein is proteolytically processed into different fragments (83-, 30-, 38-, and 42-kDa) just before egressing from the schizont (195-197), where only the MSP142 remains membrane bound (Fig. 5). Subsequently, the MSP-142 fraction is further cleaved into MSP133 N-terminal and MSP119 C-terminal fractions immediately before merozoite invasion, where MSP119 C-terminal fragment remains bound to the merozoite membrane through a glycophosphatidylinositol (GPI) anchor (198, 199), which is required for erythrocytic invasion (200). During RBC invasion, another proteolytic cleavage takes place, separating the GPI-anchored C-terminus of MSP119 from the rest of the molecule. Only the membrane-bound portion of the molecule is transferred to the invaded RBC, and the rest is shed from the surface of the parasite (198, 199).

Knock-out studies have concluded that PfMSP1 is essential for parasite invasion and growth (201, 202). PfMSP1 has been shown to participate in the parasite invasion to RBC (203), where antibodies specific to this protein, especially against PfMSP119, have been able to block parasite invasion in vitro (200, 204, 205) and in vivo (206-210) inducing protective immunity in animal models. Studies by del Portillo et al. and Han et al. suggest that PvMSP1 has similar biology and function as PfMSP1 (211, 212).
1.8.1 **MSP1-19**

The MSP1\textsubscript{19} fragment is a leading malaria vaccine candidate protein for both \textit{Pv} and \textit{Pf} malaria and corresponds to the C-terminal region of the MSP1 protein. It is comprised of two cisteine-rich epidermal growth factor (EGF)-like motifs, which are the target of both monoclonal antibodies and a major target of naturally-acquired human antibodies capable of blocking erythrocyte invasion in vitro (198, 213-215). Antibodies can efficiently inhibit RBC invasion in vitro (213) and prevent secondary cleavage of MSP1\textsubscript{42} during RBC invasion (200). Even though the MSP1 protein is highly polymorphic, the MSP1\textsubscript{19} fragment is highly conserved within \textit{Plasmodium} species (198), including both \textit{Pf} (202, 216) and \textit{Pv} (194, 217) malaria. However, \textit{Pv} and \textit{Pf} MSP1\textsubscript{19} homolog fragments have a sequence divergence of approximately 46% amino acid mismatch, which prevents significant between-species cross-recognition (218).

1.8.1.1 **Animal Studies**

Different animal studies have assessed the immunogenicity of the MSP1 protein and the correlates of protection in vivo in rodent and non-human primate models to provide evidence that support its use as a vaccine antigen candidate. A study by Valero \textit{et al.} that transferred monoclonal antibodies to mice against three different \textit{P. yoelii} MSP1 epitopes, including MSP1\textsubscript{19}, conferred passive immunity against infection (219), while a study using a recombinant 15-kDa \textit{P. yoelii} C-terminal MSP1 fragment was observed to confer protective immunity to mice (220). Also, different non-human primate studies with active immunization of recombinant \textit{P. cynomolgi} (\textit{Pv} primate analog) (210, 221), recombinant PfMSP1\textsubscript{19} (222), or recombinant PfMSP1\textsubscript{42} fragment (223) resulted in protective immunity against experimental infection with blood stages. A vaccine trial on
P. cynomolgi anti-blood stage malaria delivered recombinant MSP1$_{19}$ subcutaneously to Macaca sinica in three doses at 4-week intervals and observed a 10-fold increase in antibody titers capable of controlling parasitemia after vaccination as well as re-challenge six months later (210). Conversely, other studies have observed that immunization with PvMSP1$_{19}$ did not induce significant protection, such as a study with splenectomized Saimiri boliviensis monkeys (208, 224).

1.8.1.2 **Human Studies**

Human studies have also observed how the immune response targets the MSP1 protein, particularly subunits MSP1$_{33}$ and MSP1$_{19}$, with MSP1$_{19}$ as a major target of protective antibodies while MSP1$_{33}$ as an antigen target of both CD8$^+$ and CD4$^+$ T helper cells (225-227). Some studies with African cohorts reported that increasing levels of naturally-acquired anti-PfMSP1$_{19}$ antibodies were positively associated with protection from Pf infection (228, 229), while other studies did not observe this association (230, 231).

A study conducted in the Peruvian Amazon Basin observed that even one reported prior Pf infection was sufficient to produce an adequate antimalarial antibody (anti-PfMSP1$_{19}$) response in both children (>40%) and adults (>60%) for over a 5-month period in the absence of reinfection (178). This study also observed that the prior number of Pf infections, as well as age, influenced the duration of the antimalarial antibody response. Children with no prior malaria exposure had a median seropositivity time of 86 days, while adults with either no prior exposure to more than 2 prior exposures and children with one or more prior exposures were seropositive for at least 180 days. Clark et al. also observed that initial antibody levels did not explain a shorter duration of the
antibody response (178). A study by Braga et al conducted in the Brazilian Amazon reported that the prevalence of PfMSP1\textsubscript{19} IgG antibodies increased with malaria transmission intensity, being more prevalent in areas with more intense transmission, and length of exposure, with increasing antibody levels in individuals with long-term exposure, as well was positively correlated with asymptomatic clinical presentation in parasitemic individuals (233, 234).

Concerning Pv malaria, the only existing study to date, conducted by Morais et al. observed a significant differences in mean antibody anti-PvMSP1\textsubscript{19} levels by transmission intensity, with aparasitemic individuals living in a mesoendemic area in Apiacas, Brazil, having significant lower antibodies when compared to individuals who had less frequently or sporadically-exposed subjects residing in low-endemic areas (232).

1.9 Rationale

Malaria remains among the top priorities on the global health agenda despite decades of control efforts and eradication attempts due to its significant disease burden and the lack of an effective malaria vaccine and sustainable malaria prevention programs (7, 8). The prospect for the development of a malaria vaccine is dependent on our understanding of the natural malaria immunity, its development and maintenance. For Pv, the most widely distributed human malaria species in the world and the most prevalent malaria in the Americas (101, 115, 235). This motivation has been hampered by its under-appreciated morbidity and mortality, as well as the scarcity of literature dedicated to this particular species. One of the main challenges for the control and elimination of malaria in the Americas is the presence of Pv and its ability to relapse, as well as the
presence of asymptomatic individuals who go undiagnosed, facilitating the persistence of parasites across time and space and perpetuating the disease.

Despite the decline in global malaria incidence since the year 2000, there are great challenges in malaria surveillance, control, and elimination initiatives, such as the presence of asymptomatic and submicroscopic cases and limited sensitivity of conventional microscopy and rapid diagnostic tests (RDTs) compared to other molecular testing tools. Malaria surveillance is vital to determine the burden of disease, its distribution, and guide control and elimination efforts. Currently, more sensitive molecular diagnostic methods, such as PCR, are detecting greater number of infected individuals, thus questioning the current view on malaria burden and strategies required to reduce it. A cross-sectional study conducted in the Peruvian Amazon Basin observed that PCR-based detection of malaria parasite detected more infected individuals than by light microscopy, with a PCR-positive prevalence of 17.6% and a slide-positive prevalence of 4.2% (49). Also, the study observed that two-thirds of slide-positive and one-fourth of PCR-positive individuals were symptomatic. However, it is important to mention that malaria diagnosis by PCR is time consuming and requires specialized equipment and training, making PCR not suitable for most field settings.

Asymptomatic submicroscopic malaria infections, which are missed by conventional diagnostics methods as light microscopy and RDTs, can act as parasite reservoirs that enable the persistence of the disease. Additionally, asymptomatic patent cases might not seek for treatment at their local health facility, thus remaining untreated and parasitemic. Therefore, studies on asymptomatic parasite carriers are necessary to inform and facilitate malaria control and elimination efforts. A study by Okell et al. estimated that when Pf malaria transmission reaches very low levels (slide prevalence
<10-20%), submicroscopic infections are important in sustaining transmission, and are estimated to be a source of 20-50% human-vector transmissions, therefore challenging the idea that people living in low-endemic areas with little exposure to the disease have insufficient immunity to control parasitemia (236).

The immune mechanisms responsible for the development and maintenance of NAI, the host factors that affect the clinical response to malaria, and the effect of low transmission intensity, are poorly defined for Pν (52, 101, 115). Such information may provide insights into the epidemiology of Pν malaria in low endemic settings, aid the rational design of effective malaria vaccines and other control strategies, as well as improve public health strategies that address this important public health problem. Conducting research studies on the epidemiology and immunology of Pν are crucial steps for the successful control of malaria in the Americas and to achieve its elimination from the region.

1.10 Overall Goals and Specific Aims

1.10.1 Overall goal of the study

The overall goal of this dissertation was to study the humoral response to PvMSP119, a leading Pν vaccine antigen candidate, in a low endemic setting to better understand naturally-acquired humoral responses to infrequent malaria infections as well as clinical immunity to disease. Assessing antibody dynamics in this population will be important to better understand the development of NAI as well as to study the relationship between exposure and protection to clinical presentation in low-transmission conditions, which in turn will be important to understand the effect that elimination efforts may have in local communities.
1.10.1.1 Specific aim 1

To conduct a review of historical malaria surveillance data to determine the burden of disease due to malaria in the rural community of Zungarococha, in the Peruvian Amazon Basin, identifying significant changes in disease burden and malaria risk factors over an eight-year time span.

1.10.1.2 Specific aim 2

To determine if asymptomatic *P. vivax* infected individuals who reside in a low-endemic malarious area in the Peruvian Amazon Basin have higher concentrations of anti-PvMSP119 IgG antibodies than symptomatic individuals, which could partially explain the absence of symptoms in this group.

1.10.1.3 Specific aim 3

To investigate the maintenance of anti-PvMSP119 IgG antibodies in *P. vivax* infected individuals who reside in a low-endemic malarious area in the Peruvian Amazon Basin in an effort to better understand antibody dynamics to *Pv* malaria under infrequent malaria exposure.
1.11 References


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1.12 Figures

Figure 1. Countries with ongoing transmission of malaria, 2013

Source: WHO World Malaria Report 2014
Figure 2. Map of Peru

Figure 3. The lifecycle of human *Plasmodium spp.* species

Sporozoites are taken up by Kupffer cells in the liver and pass through several hepatocytes before establishing infection. For all human malaria species, sporozoites replicate as exo-erythrocytic schizonts, which then form into merozoites that are released into the plasma after 7-10 days. *P. vivax* and *P. ovale* are capable of producing hypnozoites that remain in the hepatocyte. Hepatocytes can be reactivated to develop as exo-erythrocytic schizonts, thus provoking relapse.

Figure 5. Schematic structure of merozoite surface protein 1 (MSP1) protein

Diagram of the different fragments and processing of MSP1 protein. SP, signal peptide; GPI, glycoprophatidylinositol anchor signal; kDa, kilodalton.

II. Chapter Two: Paper I - The epidemiology of declining malaria burden in a rural community in the Peruvian Amazon Basin: trend and risk factors over an eight-year time span

2.1 Abstract

*Background:* Peru reported a nationwide decline in malaria case burden from 2005 to 2011, most likely the result of the scale-up of multi-lateral, comprehensive interventions. This study assessed the changing malaria epidemiology in Zungarococha, a rural community in the Peruvian Amazon Basin, presenting an eight-year (2004-2011) descriptive analysis of malaria burden and risk factors in relation to the changing national malaria control programs.

*Methods:* Repeated community-based, cross-sectional surveys recruited individuals of all ages from the villages of the Zungarococha, collecting blood slides to detect malaria parasites by light microscopy. Overall and species-specific malaria parasite prevalence was estimated and compared across years and within age groups. The association between malaria and individual-level covariates was studied using logistic regression and the prevalence of asymptomatic microscopy-positive malaria was estimated.

*Results:* In Zungarococha, malaria parasite prevalence declined significantly overall and for species-specific malaria from 2004 to 2005 (from 14.2% to 4.6%), further declined by 2006 and 2007 (with 2.5% and 1.2% respectively), subsequently stabilizing in 2008 (1.8%), 2009 (0.6%), 2010 (0.5%) and 2011 (1.2%) with low parasite prevalence.
The sharpest decline in parasite prevalence was observed in under-fives between 2004 (15.9%) and 2005 (0.5%). The proportion of asymptomatic microscopy-positive participants was greater than symptomatic cases (47.7% and 34.8%, respectively), with asymptomatic participants significantly older (median 21.8 years) than symptomatic ones (median 16.4 years). After adjusting for sex, malaria risk was found to be predominately occupational, with farmers and laborers at higher risk of infection.

**Conclusions:** A decline in malaria burden in Zungarochocha, Peru occurred between 2004 and 2011, most likely a result of multi-lateral malaria control initiatives and interventions implemented in the area since 2001. The presence of asymptomatic microscopy-positive participants during this low-malaria transmission period suggests the existence of an acquired, clinical immunity to malaria and potential relevance for the successful control and elimination of malaria in the community. Future studies on malaria epidemiology and naturally-acquired immunity in low endemic areas as the Peruvian Amazon Basin will be important to aid current public health control and elimination strategies.

### 2.2 Background

Malaria has been present in Peru since at least the 1500s and remains an important public health problem despite longstanding efforts to control the disease (1). The majority of national cases occur in the Department of Loreto in the Amazon region, accounting for most of the malaria burden in the country (2). Most malaria cases in Loreto are concentrated around the city of Iquitos, the capital of Loreto (Fig. 1). Loreto is considered to be a low endemic area with seasonal malaria transmission, and most cases occur during the months of January to July. Nonetheless, previous studies around Iquitos
Historically, malaria epidemics and outbreaks in Loreto have been influenced by changes in the support of malaria control programs, environmental factors such as El Niño Southern Oscillation (ENSO), floods and deforestation, as well as population movements that exposed naïve hosts to malaria parasites due to socio-economic reasons, such as migration to remote areas to engage in oil and mining exploration activities, migration driven by labor-intensive agricultural activities, and resettlement from areas occupied by narco-terrorist groups (1). In the early 1990s, malaria resurfaced in Peru with major outbreaks in the periphery of Iquitos city due to heavy rains and flooding caused by ENSO, which resulted in increased vector populations, and well as the result of rural expansion and deforestation in the region and reintroduction of the highly efficient malaria vector *An. darlingi* (5, 6) (Fig. 2). During the outbreak, the annual number of malaria cases increased from over 1,000 cases in 1990 to over 100,000 cases in 1996, with periods of mesoendemicity (5, 6).

In 1991, indigenous *Plasmodium vivax* (*Pv*) malaria was initially reported in Loreto in neighboring communities of its capital Iquitos. By 1997, Loreto accounted for 67.2% of all national malaria cases, with most outbreaks taking place in the periphery of Iquitos in communities located along the Nanay River and along the Iquitos-Nauta highway, the first asphalt road from the city (Fig. 1). In 1998, two years after the epidemic, Roper et al estimated that annual malaria incidence in Padrecocha, a community north of Iquitos, was 826/1000 person/year for *Pv* and 166/1000 person/year for *P. falciparum* (*Pf*), with no difference in clinical manifestations by age (7). By the end
of the 1990s, malaria cases were concentrated in the Amazon Basin and the northern Pacific coast, accounting for 85% of all malaria cases and 95% of all Pf cases in Peru (6). The National Malaria Control Program and the Loreto Public Health Department implemented different strategies to control the malaria outbreak, which included source strategies with community participation (e.g., disruption of larval sites), chemical strategies (e.g., spatial fogging, indoor residual spraying, and insecticide treated bed net distribution), health system strengthening for prompt diagnosis and treatment of cases, and the adoption of artemisinin-combination therapy (ACT) to treat uncomplicated Pf malaria (1). Through intense vector control programs, principally domiciliary spraying of insecticides and distribution of insecticide-impregnated bed nets, and changes in drug policy, with the adoption of ACT for uncomplicated Pf malaria, malaria cases began to decline since the beginning of the 21\textsuperscript{th} century with a drastic reduction and stabilization of cases by 2005 with 45,000-55,000 cases reported annually (Fig. 2) (2). The national malaria burden continued to decline until 2012 when heavy rains led to one of the strongest Amazon floods in Loreto since 1986 (8). Heavy rains started in November 2011 and continued into early 2012, resulting in a state of emergency in the region. Major floods facilitated vector breeding, as well as the collapse of the sewer systems, that led to overcrowding, problems with sanitation and access to essential service (e.g. electricity, potable water), and negatively affected the public health infrastructure (9). These factors contributed to disease outbreaks, including malaria. Regional malaria burden in Loreto began to rise with a 2.1-fold increase in annual cases between 2011 (11,779 confirmed cases) and 2012 (25,149 confirmed cases), accounting for over 80% of all malaria cases in Peru (2).
The Malaria Immunology and Genetics in the Amazon (MIGIA) cohort study in the rural community of Zungarococha provided the basis for the present study to better understand the changing epidemiology of malaria in the Amazon Basin during an eight-year span from 2004 to 2011. We describe the declining trend of malaria parasite prevalence in the area throughout the hypoendemic period and following the most recent malaria epidemic, assess risk factors for malaria, and discuss the potential contribution of different interventions implemented in the area during the post-epidemic time period.

2.3 Methods

Study area and population: The Malaria Immunology and Genetics in the Amazon (MIGIA) cohort study was conducted from 2003 to 2011 in the San Juan district, south of Iquitos City, in the Department of Loreto in the Peruvian Amazon Basin, specifically in the rural community of Zungarococha located approximately 5 kilometers (km) from Iquitos city (Fig. 3). Both Pf and Pv contribute to malaria transmission, which occurs year-round with a prominent peak during the rainy season (January – July), with Pv at the predominant species. A study by Branch et al. reported a prevalence of 0.13 and 0.39 infections/person/malaria-season for Pf and Pv, respectively in Zungarococha during 2003, using a combination of longitudinal active case detection and passive case detection at the health clinic (3).

Zungarococha, with a population of approximately 2000 residents, is composed of four main villages that are approximately two kilometers apart and are served by the same MINSA health post. The four main villages are Zungarococha town (N ≈ 1300), Puerto Almendra (N ≈ 200), Ninarumi (N ≈ 470), and Llanchama (N ≈ 170) (Fig. 3). There are other smaller villages, such as King Kong and Correntillo, which are located
between the main villages of the community. Overall, the environment and income level of the main villages in Zungarococha are similar, with the exception of Zungarococha village, which is more developed and harbors the community MINSA health post. All villages are connected by a dirt road with a daily bus service that provides transportation. The main economic activities in the area are agriculture, fishing, and construction, performed mostly by men. Women tend to work in or near their homes. The majority of houses in the community are made of wood with resin-rich thatch roofs, with approximately 10% made of cement-block. The houses often do not have screens that could block mosquitoes from entering the household. Houses are on average 8 x 12 meters in size, are often close together from each other (< 6 meters apart) and in line with the streets, facing the dirt road. The back of the house is often exposed to more foliage-dense riverine areas, and tends to be close to swampy areas of rivers or ponds (within ~100 meters). Many activities, such as cooking, take place in the back of the house. Only Ninmarumi village has a different village design, with half the village households distributed close together and the other half more dispersed. Some houses and streets in Zungarococha and Puerto Almendra villages had electricity.

Malaria cases were diagnosed, treated and documented by Ministry of Health (MINSA) personnel by passive-case detection. Individuals with fever who attended a local MINSA health post or health clinic were tested for malaria by light microscopy and received treatment at no cost to the patient. Malaria therapy was given as directly observed treatment to those infected (Table 1) and the anti-malarial drug supply was tightly controlled by MINSA.
Post-epidemic malaria control strategies in Peru: Following the malaria epidemic in the 1990s, the U.S. Agency for International Development (USAID) launched the Amazon Malaria Initiative (AMI) in 2001 to support the regional Roll Back Malaria Program. AMI was implemented by a consortium of technical partners including the Peruvian Ministry of Health (MINSA), the Pan American Health Organization (PAHO) and the U.S. Centers for Disease Control and Prevention (CDC) to prevent and control malaria in Peru through monitoring drug efficacy, building human capacity, decentralizing laboratory capacity, and implementing integrated vector control activities (10-12). Also in 2001, the Amazon Network for the Surveillance of Antimalarial Drug Resistance (RAVREDA) international initiative was launched by USAID and sponsored by the PAHO to address the emerging antimalarial drug resistance in the Amazon region, which according to World Health Organization (WHO) recommendations promoted the introduction of ACT for non-complicated Pf malaria in Peru in 2001 (13). From October 2005 to September 2010, additional support from international donors allowed the scale-up of comprehensive malaria control strategies through the multi-country program “Malaria Control Program in Andean-country Border Region: A Community Approach” (PAMAFRO), a multi-lateral initiative implemented by the Andean Organization for Health (ORAS) and sponsored by the Global Fund to Fight AIDS, Tuberculosis and Malaria (14-16). Following the WHO recommendation, PAMAFRO implemented the first long-lasting insecticide bed net (LLINs) national intervention in 2007, distributing over 25,000 Olyset Net® LLINs free of charge to priority communities from Loreto (15). Since September 2010, malaria control activities have been mainly supported by the MINSA national budget, focusing on passive control surveillance with sporadic active
and reactive case detection strategies using standard light microscopy and prompt
treatment of confirmed cases with chloroquine + primaquine for uncomplicated \(Pv\)
infections and artemisinin + mefloquine for uncomplicated \(Pf\) infections (Table 1).

Control measures are built-in the general local health services within the MINSA
National Malaria Control Program, which is a partially decentralized program.
Additionally, the MINSA conducted annual fumigation campaigns (17) performing
indoor residual spraying (IRS) by applying pyrethroid insecticides (e.g.,
alphacypermethrin 10%, deltamethrin 5%) to houses (18).

**Study design and data collection:** The MIGIA cohort study used active and
passive case detection of infants, children, adults, and pregnant women. The MIGIA
sampling strategies were: i) passive case surveillance with a full-time clinical team
stationed at the Zungarococha health post throughout the year; ii) active case detection
through community-based, cross-sectional surveys at the start (January-March) and end
(July – August) of the malaria season; and iii) active case surveillance for one month,
with weekly household visits throughout the malaria season (February – August) (3).

The weekly household visits took place within a 100-meter radius from a
“sentinel” house, defined as houses that had at least one \(Pf\) case (index case) that had
either visited the health post in the previous month for malaria diagnosis (passive
detection), or was detected through the community-wide survey or weekly household
visits in the previous month (active detection). Whole blood was collected by fingerpick
or venipuncture for microscopy. Upon the identification of a \(Pf\) index case, a 100-meter
radius around that index case’s house was delimited and defined as the “\(Pf\) at-risk area”
(Fig. 4). If at-risk-areas overlapped in a given month, the parent study assigned the
randomly selected house to one of the areas. Generally, there were 20-50 houses within a
given 100-meter radius area. Within the “Pf at-risk area”, houses were randomly selected,
using a random number generator, to conduct weekly visits in order to capture the onset
and dynamics of infection with Pf and Pv in individuals living in proximity to the Pf
index case (Fig. 4). The parent study team visited all randomly selected houses within
each at-risk-area and recruited all household members until at least 30 individuals (~ 5 –
9 houses) were enrolled. All consenting participants, including the sentinel household
with the index case, were included in the weekly visits, with each participant visited once
per week for four weeks. Overall, there were five to six at-risk-areas per month of active
surveillance. Because of the relatively small size of each village, less than 600 square-
meters, it was not unusual to sample the same at-risk areas in successive months of active
surveillance. In 2003, Branch et al. reported sampling 70-90% of all the houses within
Puerto Almendr and Ninarumi villages, and 50-70% of all houses within Zungarochocha
village during the active surveillance period (3). Llanchama village was not included in
active household follow-ups because it was not consistently accessible.

During each visit, whether in the health post, community-wide survey, or weekly
home-visit, clinical (e.g. temperature, malaria-related symptoms, hematocrit),
epidemiological (e.g. number of past malaria infections, years lived in the area), and
demographic information (e.g. age and sex) were recorded by the study team. The study
physician measured axillary temperature using a digital thermometer and collected 0.25
to 3 ml of blood by fingerpick or venipuncture. Blood samples were used to prepare thin
and thick blood smears, as well as capillary hematocrit tubes. During active case
detection visits, blood slides, hematocrit tubes and vacutainers were transported to the
laboratory for microscopy and processing. Vacutainers were centrifuged to separate plasma from packed blood cells, and frozen within 18 hours at -85°C. Capillary hematocrit tubes were centrifuged and the volume percentage of red blood cells (packed cell volume, PCV) was measured. All remaining blood was stored in EDTA-containing vacutainers for further processing and storage. Additionally, microscopy-positive participants were asked to provide an additional 3-6 ml of blood collected by venipuncture to perform molecular (i.e. PCR) and biochemical (i.e. glucose, lactose, creatinine) tests. The MINSA authorities administered treatment following national drug policy guidelines through directly observed treatment (Table 1) (19). To confirm treatment success, a fingerpick blood sample was collected and analyzed 7 and 14 days after malaria diagnosis.

**Malaria diagnosis:** Malaria infections were documented by microscopic analysis of Giemsa-stained thick and thin blood smears, following standard procedures. Two expert microscopists with over 15 years of experience counted parasites, reading 200 microscopy fields. Both *Pv* and *Pf* trophozoites and gametocytes were counted separately. At least 500 white blood cells (WBCs) were counted before an individual was diagnosed as negative by microscopy. Species-specific parasite density (parasite/uL) was determined by the number of parasites divided by the total number of WBCs counted and multiplied by 6,000. The conversion factor of 6,000 RBCs per one WBC to determine parasite density per microliter is regularly used in the area (20).

During active case detection, priority was given to symptomatic subjects (axillary temperature >37.5°C or reported fever within 2 days), pregnant women, children (<5 years), and anemic subjects (hemoglobin <10 g/dL measured by HemoCue or hematocrit
PCV <30%), who had their slides read within one day of the household visit and received species-specific antimalarial drug treatment as described above within one day of diagnosis. Asymptomatic subjects had their slides read within 6 days after the household visit. Asymptomatic participants who were positive in the earlier blood slide and were found to be symptomatic at the following weekly visit were given antimalarial drug treatment within one day of diagnosis. Asymptomatic participants who remained without symptoms at the one-week follow-up visit were re-visited the following day to perform a confirmation slide. All asymptomatic microscopy-positive participants at follow-up were given antimalarial drug treatment.

**Statistical Analysis:** The MIGIA cohort study used a Microsoft Access database for data management and entry, validation, and cleaning. Changes in the epidemiology of malaria in the Zungarococha community were assessed by estimating both overall and species-specific malaria parasite prevalence at the beginning of each malaria season (January – March) from 2004 to 2011 using MIGIA’s community-based, cross-sectional survey data. Clinical groups were defined as: i) symptomatic malaria: any subject with positive microscopy who presented with a fever (axillary temperature >37.5°C) at the time of diagnosis or a self-reported fever during the two previous days; and ii) asymptomatic malaria: any subject with a malaria positive microscopy slide who did not have fever at the time of microscopy diagnosis and did not report having fever during the two previous days. Age was categorized in three groups: i) individuals under five years of age (<5 years), ii) individuals between 5 and 14 years of age (5-14 years), and iii) individuals 15 years of age or older (≥15 years). Anemia was defined following the WHO hemoglobin (Hb) established thresholds with: Hb <11 g/dL in children between 0.5 and 5
years of age, Hb <11.5 g/dL in children between 5 to 12 years of age, Hb <12 g/dL in individuals between 12-15 years of age, Hb <11 g/dL in pregnant women over 15 years of age, Hb <12 g/dL in non-pregnant women over 15 years of age, and Hb <13 g/dL in men over 15 years of age (21).

The analysis was performed using STATA version 13 (STATA Corp Inc., TX, USA) and consisted of comparisons of different epidemiological variables, such as parasite positivity, parasite density (trophozoites and gametocytes), anemia, mean hemoglobin levels, parasitemia, ITN use, sex, age, number of self-reported life-time malaria events, main occupation (i.e., farmer, laborer, fisherman, student, and other occupations such as student and homemaker), and clinical group. Continuous variables that were not normally distributed, such as parasite density (trophozoites and gametocytes), were log transformed for statistical analysis. Categorical data were compared using either the chi-square test, with Bonferroni correction for multiple comparisons, or Fisher’s exact test, and continuous variables were compared using Student’s t-test or Wilcoxon Rank Sum test and one-way analysis of variance (ANOVA) or Kruskal-Wallis test for multiple comparisons. A generalized estimating equations (GEE) logistic regression analysis with an unstructured correlation matrix and robust variance estimation was used to estimate the overall and species-specific risk of being infected with malaria parasites over the study period, adjusting for covariates and addressing the non-independence of the data between survey years.

Research Ethics: This secondary data analysis study was approved by the U.S. Naval Medical Research Unit No. 6 (NAMRU-6) Institutional Review Board (IRB approval code: NAMRU6.2013.0009). Ethical approval of the MIGIA study was
obtained from the Instituto Nacional de Salud (Human use approved protocol: 08-982),
the University of Alabama at Birmingham, the Universidad Peruana Cayetano Heredia,
and the New York University School of Medicine Institutional Review Boards. Prior to
enrollment, written informed consent was obtained from all study participants eighteen
years of age or older. For participants between seven and eighteen years of age, assent
from the minor and consent from the parents or guardians were obtained. The Johns
Hopkins Bloomberg School of Public Health granted an IRB waiver given that this study
used de-identified data for the analyses.

2.4 Results

Study population

The MIGIA cohort study performed eight community-based, cross-sectional
surveys at the start of the malaria season (January – March) from 2004 to 2011 in the
Zungarococha community (Table 2). A total of 3,402 individuals of all ages were
enrolled, of whom 1,448 (42.6%) had only one study visit and 1,954 (57.4%) had more
than one study visit, with a total of 9,688 study visits over the eight years. Throughout the
study period, there were no significant differences in sex, with the majority of
participants female (54.9%). The study population’s median age was similar throughout
the eight surveys with median of 16.3 years (range 0 – 97 years) (Table 2). The majority
of the study population was consistently in the 15 years or older age group, followed by
the 5-14 year old group, with the minority consistently in the under-five group (p=0.8).
The majority of study visits were in Zungarococha (n=3,317, 34.2%) and Ninarumi
(n=3,136, 32.4%) villages, and to lesser extent from Puerto Almendra (n=1,096, 11.9%),
Llanchama (n=1,148, 11.3%), and other minor villages (n=991, 10.2%).
The proportion of the study population who reported sleeping under a bed net was high, increasing significantly from over 80% in 2004 to 99% in 2005 (p < 0.001), and remained high from 2006 to 2011 (Table 2) reaching 100% reported usage by 2009 until the end of the study. The prevalence of anemia in this community fluctuated over the years but remained below 30% throughout the study period. The median number of self-reported lifetime malaria episodes during the eight-year span was significantly different (p < 0.001) between years, with a median of 3 lifetime events from 2004 to 2007, a median of 2 lifetime events from 2008 to 2010, and one median lifetime event in 2011.

Overall malaria prevalence in Zungarococha

In Zungarococha, throughout the eight surveys conducted from January to March of 2004 to 2011, there were 287 (2.9%) microscopy-positive cases, of which the majority were men (54.4%, p = 0.001). Additionally, there were significantly more slide-positive cases in the 15 or older age group (59.6%), followed by the 5-14 year-old group (26.5%), and to a lesser extent in the under-five group (13.9%) (p = 0.04). The overall village-specific parasite prevalence was highest in Puerto Almendra village (4.4%), followed by Ninarumi (4.1%), Llanchama (2.8%), Zungarococha (1.9%), and finally the other minor villages (1.5%). The parasite prevalence changed significantly from 2004 to 2011, consistently declining between survey years (p<0.0001) (Table 2). The greatest decline in malaria prevalence was between 2004 and 2005, with a significant three-fold decline from 14.2% to 4.6%, followed by a two-fold decline to 2.5% in 2006, and an additional two-fold decline to 1.2% in 2007. Subsequently, the parasite prevalence remained low
until the end of the study period, and was 1.8% in 2008, 0.6% in 2009, 0.5% in 2010, and 1.2% in 2011 (Fig. 5).

**Species-specific prevalence of malaria infections**

Throughout the eight-year study there were more microscopy-positive *Pv* cases (n = 75/287, 73.5%) than *Pf* cases (75/287, 26.1%), and only one slide-positive mixed infection was identified. Moreover, the majority of malaria cases in each survey year were due to *Pv* malaria (Table 3). As with the overall *Plasmodium spp.* malaria slide-positive cases, most *Pv* and *Pf* cases were diagnosed in the ≥15 year-old group (57.4% and 65.3%, respectively), followed by the 5-14 year old group (27% and 25.3% respectively), and the under-five group (15.6% and 9.3%, respectively). Also, the village-specific species-specific malaria parasite prevalence was significantly different (p < 0.0001), with Ninarumi (3%) and Puerto Almendra (2.7%) having the highest *Pv* parasite prevalence (3%), and Puerto Almendra (1.6%) and Llanchama (1.2%) having the highest *Pf* parasite prevalence.

To better understand the declining malaria parasite prevalence from 2004 to 2011, species-specific prevalence over time were estimated (Table 2). Over the eight-year period, both *Pv* and *Pf* parasite prevalence declined (p < 0.0001, respectively) (Fig. 5). For *Pv*, parasite prevalence showed a significant 2.7-fold decline between 2004 (9.8%) and 2005 (3.6%) (p < 0.0001), followed by a non-significant 1.6-fold decline in 2006 (2.2%), and an additional significant 2.4-fold decline in 2007 (0.9%). Subsequently, *Pv* parasite prevalence remained low during 2007 (0.9%), 2008 (1.6%), 2009 (0.4%), 2010 (0.4%), and 2011 (0.5%). For *Pf*, parasite prevalence declined significantly be 4.9-fold
between 2004 (4.4%) and 2005 (0.9%) (p < 0.0001), followed by a significant 3-fold decline in 2006 (0.3%). Subsequently, *Pf* parasite prevalence remained low during 2007 (0.2%), 2008 (0.2%), 2009 (0.2%), 2010 (0.2%), and 2011 (0.7%).

**Prevalence of microscopy-positive asymptomatic malaria**

Throughout the study period, as diagnosed by microscopy and the presence or absence of fever during time of visit or two days prior, most slide-positive malaria cases were asymptomatic (n = 126/284, 47.7%) rather than symptomatic (n = 100/284, 34.8%). The remainder was indeterminate (n = 58/284, 20.4%) because of a study failure to collect temperature information. The proportion of asymptomatic microscopy-positive participants changed significantly over time (p < 0.0001), with the majority consistently asymptomatic from 2005 to 2011 (Table 3). No significant differences were observed in the clinical presentation of microscopy-positive participants by *Plasmodium* species (p = 0.2), with the majority asymptomatic for both *Pv* (45.9%) and *Pf* (40.3%), followed by symptomatic cases (36% and 31.9% respectively). The only reported mixed infection was symptomatic. The proportion of asymptomatic *Pv* microscopy-positive participants changed significantly over time (p < 0.0001), while the proportion of *Pf* microscopy-positive participants did not (p < 0.1) (Table 4).

The median age of asymptomatic cases (median = 21.8 years) was significantly higher than symptomatic cases (16.4 years, p = 0.03) (Table 5). Significant differences were observed in the clinical presentation by age group (p = 0.04), with the majority of under-fives having symptomatic malaria (52.5%), while the 5-14 and ≥15 years old groups had more asymptomatic (45.3% and 49.1%, respectively) than symptomatic
microscopy-positive individuals (36% and 30.8%, respectively). Both the 5-14 (OR = 2.8, 95% CI = 1.2 – 6.9; p = 0.02) and ≥15 years old groups (OR = 3.3, 95% CI = 1.5 – 7.5; p = 0.004) had significantly increased odds of having microscopy-positive asymptomatic individuals. When we compared parasitemia between asymptomatic and symptomatic individuals, we found significant differences in their mean log trophozoite count (p < 0.0001), which were lower in asymptomatic (mean = 5.1, SD = 0.2) than symptomatic (mean = 7.2, SD = 1.7) people (Table 5).

Over the eight years of the study, the asymptomatic malaria parasite prevalence was 1.3% and the symptomatic parasite prevalence was 1%, which changed significantly over the course of the study (p < 0.0001) with asymptomatic malaria as the most prevalent clinical presentation, except in 2004 (Table 2). As the parasite prevalence declined, the proportion of asymptomatic microscopy-positive participants changed significantly over time (p < 0.0001) (Fig. 6, Table 3). In 2004, 34.5% of all slide-positive people were symptomatic and 28.2% were asymptomatic. The following years, following the sharp decline in parasite prevalence, there were more asymptomatic than symptomatic microscopy-positive participants in 2005 (55.3% vs. 42.6%), 2006 (70% vs. 30%), 2007 (50% vs. 35.7%), 2008 (71.4% vs. 28.6%), and 2009 (75% vs. 25%), with equal proportions in 2010 (28.6%, respectively), and in 2011 there were more asymptomatic (55.6%) than symptomatic (38.9%) microscopy-positive people.

**Age-specific malaria prevalence**

When we analyzed the age-specific parasite prevalence throughout the study period (2004 to 2011), the parasite prevalence differed by age group (p = 0.05), being
lower in under-fives (2.3%), followed by the 5-14 year-old group (2.7%), and highest in the ≥15 year old population (3.3%). Similarly, the age-specific *Pv* and *Pf* parasite prevalence was lowest in the under-five group (1.9% and 0.4%, respectively), followed by the 5-14 year-old (2.1% and 0.7%, respectively), and the ≥15 year-old groups (2.4% and 0.9%, respectively). When we analyzed changes in age-specific parasite prevalence over time (Table 6), we observed that age-specific parasite prevalence declined significantly in all three age groups over time, with the ≥15 year-old groups having a more staggered decline over the study period (Fig. 7A-C).

In 2004 the age-specific parasite prevalence was the highest throughout the study period for all three age groups and was similar between age groups (p=0.7). In 2005, we observed a significant decline in parasite prevalence for all age groups (Fig. 7A-C) and found significant differences by age groups (p=0.01), being higher in the 5-14 (5.9%) and ≥15 (5.2%) year old groups and lower in the under-five group (0.5%). Since 2005, parasite prevalence in the under-five group did not change significantly over time (p=0.3). In 2006, the parasite prevalence was again significantly different by age group, being highest in the ≥15 year-old group (3.9%), followed by the under-five (1.9%) and 5-14 (0.3%) year old groups (p=0.002). Since 2006, parasite prevalence in the 5-14 year old group did not change significantly over time (p=0.07). From 2007 to 2010, the age-specific parasite prevalence remained low and similar between all age groups, until 2011 when the age-specific parasite prevalence was significantly different by age group (p=0.01), due to an increase in the parasite prevalence in the ≥15 year old group (2%).

Likewise, the age-specific *Pv* (Fig. 7B) and *Pf* (Fig. 7C) parasite prevalence was highest 2004 being similar between all age groups (p=0.2). In 2005 and 2006 only *Pv
parasite prevalence was significantly different by age group, being highest in the 5-14 year-old group in 2005, and in the ≥15 year old in 2006 (p=0.01 and p=0.006, respectively). From 2007 to 2010, the age-specific parasite prevalence was also similar between all age groups for both *Pv* and *Pf* malaria, until 2011 when it was significantly different for *Pv* (p=0.03) but not *Pf* malaria (p=0.02), with all *Pv* cases being in the ≥15 year-old group.

**Sex, occupation, and risk of malaria infection**

To better understand malaria risk in Zungarococha over the eight-year period, we assessed the association between the odds of being malaria parasite positive, with sex and main occupation (Table 7). In bivariate analysis, male participants had 1.5 times higher odds of having malaria (OR = 1.5, p=0.003). When we analyzed the species-specific association between sex and odds of having malaria, the significant association between male sex and malaria persisted for both *Pv* (OR = 1.4, p = 0.02), and *Pf* malaria (OR = 1.8, p = 0.02) (Table 7). Occupation was grouped into four categories: farmer, laborer, fisherman, student, and other occupations (e.g. student, homemaker). The majority of the study population reported having other occupations (67%), mostly as a student (50%) or homemaker (41%), followed by being a farmer (9%), laborer (4%) and fisherman (1%). Compared to other occupations, farmers (OR = 1.9, p < 0.0001) and laborers (OR = 1.9, p = 0.02) had increased odds of having malaria. When we analyzed the malaria species-specific odds of being parasite positive and type of occupation, a few differences emerged. For *Pv* malaria, farmers (OR = 1.8, p = 0.01) and laborers (OR = 1.9, p = 0.02) had significantly higher odds than students, while for *Pf* malaria farmers (OR = 2.9, p =
0.002) and fisher (OR = 7.5, p = 0.04) had significantly higher odds of malaria (Table 7). After adjusting for the effect of sex, we observed that both farmers (OR = 1.8, p = 0.003) and laborers (OR = 1.7, p = 0.05) remained at higher risk of malaria infection (Table 7). For *Pv* malaria, after adjusting for sex, no occupation was significantly at higher risk of infection, while for *Pf* malaria both farmers and fisher remained at increased risk of infection.

### 2.5 Discussion

The objective of this study was to understand the changing malaria epidemiology in Zungarococha community, Loreto, during the years that Peru reported a nationwide decline in its case burden (2, 22) and assess risk factors associated with malaria. Repeated cross-sectional surveys allowed us to observe malaria trends over an eight-year span in this rural community located in the peri-Iquitos region, as well as assess the burden of asymptomatic microscopy-positive cases in the area.

From 2005 to 2010, malaria burden changed significantly in Loreto declining from 54,291 reported cases in 2005 to 11,640 cases in 2010 (2). In Zungarococha, through the present study, we observed a declining trend in malaria burden from 2004 to 2011, with the most significant decline in parasite prevalence for total and species-specific malaria from 2004 to 2005, following the implementation of different malaria control initiatives (e.g. AMI and RAVREDA, both in 2001), followed by 2005 to 2006, shortly after PAMAFRO initiated its comprehensive malaria control strategies in the region. From 2007 to 2011, during PAMAFRO control activities (e.g. mass-distribution of LLINs), malaria burden in Zungarococha was lowest with similar age-specific prevalence. This sustained low malaria burden from 2007 to 2011 could reflect the
impact that AMI and PAMAFRO’s comprehensive control activities had on the transmission of malaria. Even though we did not evaluate the impact of the different malaria intervention strategies implemented during the study period and did not include changes in climatic variables or other contextual factors such as socio-economic status and treatment seeking behaviors in this study, comprehensive malaria control activities such as AMI and PAMAFRO that include integrated vector management and community participation have been proven to successfully control malaria transmission in African countries (23-27) and effectively complement other malaria control strategies to reduce malaria burden (28, 29). Specifically for AMI, morbidity declined considerably since its inception in 2001, with a 43.5% reduction in number of confirmed cases in 2008 (n = 44,409 cases) compared to 2001 (n = 78,544 cases) (30). Regarding PAMAFRO, morbidity did also decline considerable in the areas covered by the initiative. From its inception in 2005 (n = 56,590) until 2008 (n = 21,429), they reported a 37.9% reduction in number of confirmed cases (31).

Throughout the study, including the years of sustained low malaria prevalence, we observed a considerable proportion of asymptomatic microscopy-positive participants (47.7%), mostly in older participants. People in the 5-14 and ≥15 year age groups had a significantly higher risk of being asymptomatic. A study by Alves et al. in the Brazilian Amazon also observed a similar association between age and asymptomatic malaria in two riverine communities of Portuchuelo and Ji-Parana in Rondonia, where asymptomatics were older (medians 26.5 and 21 years) than symptomatic cases (medians 14 and 8 years) (32). Other studies conducted in Brazil have also documented the
common phenomenon of asymptomatic parasite carriage (33, 34), and asymptomatic malaria presentation is not uncommon in the Peruvian Amazon Basin (35).

Although there were no significant differences in age-specific malaria parasite prevalence during 2004, in 2005 the peak prevalence of malaria shifted to people older than five years of age and in 2006 to people ≥15 years of age. This shift in age distribution was for *Pv* but not for *Pf* malaria. Our observations suggest that the multi-lateral malaria control strategies implemented since 2001 had the greatest impact in the younger age groups. This would be most likely explained by their reduced mobility to other potential at-risk areas and limited engagement in risky occupations, whereas adults were more likely to travel to and from endemic areas for economic, social or other reasons (36) and engage in occupations that could expose them to more infectious mosquito bites. After studying the association of occupation and malaria risk, our results showed that malaria risk in the area appears to be predominately occupational, with farmers and laborers at higher risk of infection. This observation is also supported by the increased malaria prevalence in people 15 years of age or older and male individuals. Other studies conducted in rural communities in Loreto have also observed greater parasite prevalence in adults and men (5, 37), likely the result of an occupational risk. Studies conducted in the Brazilian Amazon in the state of Rondonia found that malaria risk was associated with type of occupation and performing extra-domiciliary activities (38-41). Interestingly, our study observed that laborers (construction workers) were at increased risk of malaria infection. Unprotected laborers can act as potential mosquito baits, and construction sites tend to create breeding grounds for mosquito vectors (42). A study in India observed a 5% parasite positive rate in laborers, the majority being young
adults and migrants (43). Future studies should look into better characterizing the malaria risk associated with this occupation, assessing the relationship of malaria risk with working hours, working area (e.g. within or outside the community), travel history, socio-economic status, and sleeping under a bed net.

One important limitation of this study was that we did not account for the effect of climate/environmental effects, such as temperature or rainfall, or other contextual factors on the declining malaria burden, nor their potential impact on the multi-lateral malaria control strategies implemented after the mid 1990s malaria epidemic. Therefore, we must be cautious in interpreting observations of the declining burden of malaria in Zungarococha. For example, the dry season that followed the 1990s ENSO may have assisted the National Malaria Control Program efforts to control the malaria epidemic in Loreto in 1998 (5). Future studies should include climate data to assess their effect on control strategies and malaria burden. Additionally, our malaria prevalence estimates are based only on microscopy-positive cases rather than polymerase chain reaction (PCR)-positive cases, which is a more sensitive malaria diagnostic tool. When the study by Roshanravan et al. estimated the prevalence of *Plasmodium spp*. PCR-positive prevalence (17.6%) was four times greater than the slide-positive prevalence (4.2%) (35). Moreover, three-fourth of all PCR-positive people were asymptomatic, while only one third of slide-positive people were asymptomatic. This means that the present study is only observing the “tip of the iceberg” regarding malaria burden, and underestimates the presence of asymptomatic infection in the area. Other important limitations are the missing data on fever at time of visit for almost 60 malaria cases and the definition of asymptomatic disease, which could affect our asymptomatic prevalence estimates.
Nonetheless, the present study describes the existence of a considerable proportion of asymptomatic microscopy-positive people in the area following post-epidemic comprehensive control activities, thus stressing the important challenge they pose to current national malaria control. Finally, the MIGIA cohort study might have failed to enroll people who were not at home at time of visit, possibly due to work-related reasons, and thus could have introduced selection bias.

A declining trend in malaria burden in Zungarococha community was identified between 2004 and 2007 with sustained low-prevalence between 2007 and 2011 during and following PAMAFRO’s comprehensive malaria control strategy. The identification of asymptomatic cases is consistent with the development of naturally-acquired immunity to malaria in a population living in low-endemic settings. The importance of asymptomatic malaria in seasonal and low-endemic areas resides in its ability to act as parasite reservoir, allowing for the over-seasoning and persistence of the parasite and its capability to reintroduce malaria if left unaddressed. Therefore, it is important to continue investigating the epidemiology of malaria transmission in low-endemic areas, as well as to study the mechanisms behind the development and maintenance of a naturally-acquired immunity.

2.6 Reference


13. Panamerican Health Organization (PAHO). Resistance to Antimalarials. Available from:


2.7 Tables and Figures

Table 1. Peruvian malaria treatment guidelines for uncomplicated malaria in adults and children older than six months of age

<table>
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### Table 2. Study population characteristics (n = 9,688) in Zungarococha, 2004 - 2011

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<td></td>
<td>470 46.9%</td>
<td>466 45.4%</td>
<td>561 46.4%</td>
<td>571 46.9%</td>
<td>514 43.3%</td>
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<td><strong>Age group, number (%)</strong></td>
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<tr>
<td>Symptomatic</td>
<td>49 4.9%</td>
<td>20 2.0%</td>
<td>9 0.9%</td>
<td>5 0.5%</td>
<td>6 0.6%</td>
<td>2 0.2%</td>
<td>2 0.2%</td>
<td>7 0.7%</td>
<td></td>
</tr>
<tr>
<td><strong>Bed net usage, number (%)</strong></td>
<td>848 84.6%</td>
<td>1020 99.3%</td>
<td>1207 99.9%</td>
<td>1200 98.6%</td>
<td>1187 99.9%</td>
<td>1247 100.0%</td>
<td>1320 100.0%</td>
<td>1479 100.0%</td>
<td>0.002</td>
</tr>
<tr>
<td><strong>Lifetime malaria events, median [IQR]</strong></td>
<td>3 [1, 5]</td>
<td>3 [1, 6]</td>
<td>3 [0, 6]</td>
<td>3 [0, 6]</td>
<td>2 [0, 6]</td>
<td>2 [0, 6]</td>
<td>2 [0, 6]</td>
<td>1 [0, 4]</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

n: number of study visits; %: percent; IQR: interquartile range; : missing values; indeterminate: slide-positive cases without registered fever at time of visit; other occupation: student, homemaker, and other minor occupations.
### Table 3. Characteristics of malaria cases diagnosed in Zungarococha, 2004 - 2011

<table>
<thead>
<tr>
<th></th>
<th>2004 (n = 142)</th>
<th>2005 (n = 47)</th>
<th>2006 (n = 30)</th>
<th>2007 (n = 14)</th>
<th>2008 (n = 21)</th>
<th>2009 (n = 8)</th>
<th>2010 (n = 7)</th>
<th>2011 (n = 18)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, male (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.3</td>
</tr>
<tr>
<td>75 52.8%</td>
<td>29 61.7%</td>
<td>16 53.3%</td>
<td>5 50.0%</td>
<td>9 42.9%</td>
<td>4 50.0%</td>
<td>7 100.0%</td>
<td>9 50.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age group, number (%)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.002</td>
</tr>
<tr>
<td>&lt;5</td>
<td>27 19.0%</td>
<td>1 13.3%</td>
<td>4 13.3%</td>
<td>1 7.1%</td>
<td>3 14.3%</td>
<td>3 37.5%</td>
<td>1 14.3%</td>
<td>0 0.0%</td>
<td></td>
</tr>
<tr>
<td>5-14</td>
<td>41 28.9%</td>
<td>19 33.3%</td>
<td>1 3.3%</td>
<td>3 21.4%</td>
<td>7 33.3%</td>
<td>2 25.0%</td>
<td>1 14.3%</td>
<td>2 11.1%</td>
<td></td>
</tr>
<tr>
<td>≥15</td>
<td>74 52.1%</td>
<td>27 83.3%</td>
<td>25 83.3%</td>
<td>10 71.4%</td>
<td>11 52.4%</td>
<td>3 37.5%</td>
<td>5 71.4%</td>
<td>16 88.9%</td>
<td></td>
</tr>
<tr>
<td>Species-specific malaria, number (%)</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td>0.04</td>
</tr>
<tr>
<td>P. falciparum</td>
<td>44 31.0%</td>
<td>9 19.1%</td>
<td>3 10.0%</td>
<td>2 14.3%</td>
<td>2 9.5%</td>
<td>3 37.5%</td>
<td>2 28.6%</td>
<td>10 55.6%</td>
<td></td>
</tr>
<tr>
<td>P. vivax</td>
<td>96 69.0%</td>
<td>37 78.7%</td>
<td>27 90.0%</td>
<td>12 85.7%</td>
<td>19 90.5%</td>
<td>5 62.5%</td>
<td>5 71.4%</td>
<td>8 44.4%</td>
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</tr>
<tr>
<td>Mixed</td>
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<td>0 0.0%</td>
<td>0 0.0%</td>
<td>0 0.0%</td>
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</tr>
<tr>
<td>Clinical presentation, number (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Symptomatic</td>
<td>49 34.5%</td>
<td>20 42.6%</td>
<td>9 30.0%</td>
<td>5 35.7%</td>
<td>6 28.6%</td>
<td>2 25.0%</td>
<td>2 28.6%</td>
<td>7 38.9%</td>
<td></td>
</tr>
<tr>
<td>Asymptomatic</td>
<td>45 31.7%</td>
<td>27 57.5%</td>
<td>21 70.0%</td>
<td>7 50.0%</td>
<td>15 71.4%</td>
<td>6 75.0%</td>
<td>5 71.4%</td>
<td>11 61.1%</td>
<td></td>
</tr>
<tr>
<td>Lifetime malaria events, median [IQR]</td>
<td>3 [1, 5]</td>
<td>3 [2, 6]</td>
<td>5.5 [2, 9]</td>
<td>5 [3, 10]</td>
<td>5 [1, 7]</td>
<td>1 [0.5, 4]</td>
<td>3 [1, 4]</td>
<td>3.5 [2, 8]</td>
<td>0.05</td>
</tr>
<tr>
<td>Anemia, number (%)</td>
<td>40 28%</td>
<td>18 38%</td>
<td>4 13%</td>
<td>5 36%</td>
<td>11 52%</td>
<td>2 25%</td>
<td>2 29%</td>
<td>4 22%</td>
<td>0.07</td>
</tr>
<tr>
<td>Village, number (%)*</td>
<td>32 13.4%</td>
<td>14 3.5%</td>
<td>9 1.9%</td>
<td>2 0.4%</td>
<td>3 0.7%</td>
<td>0 0.0%</td>
<td>1 0.2%</td>
<td>1 0.3%</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Zungarococha</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ninarumi</td>
<td>69 22.9%</td>
<td>16 4.6%</td>
<td>17 4.3%</td>
<td>6 1.5%</td>
<td>10 2.5%</td>
<td>4 0.9%</td>
<td>5 1.1%</td>
<td>2 0.5%</td>
<td></td>
</tr>
<tr>
<td>Lianchama</td>
<td>7 4.1%</td>
<td>9 8.9%</td>
<td>1 0.7%</td>
<td>5 4.2%</td>
<td>2 1.4%</td>
<td>4 2.6%</td>
<td>1 0.7%</td>
<td>2 1.6%</td>
<td></td>
</tr>
<tr>
<td>Puerto Almendra</td>
<td>30 21.1%</td>
<td>7 5.5%</td>
<td>3 2.0%</td>
<td>1 0.6%</td>
<td>5 3.5%</td>
<td>0 0.0%</td>
<td>0 0.0%</td>
<td>4 3.4%</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>4 2.6%</td>
<td>1 2.1%</td>
<td>0 0.0%</td>
<td>1 1.5%</td>
<td>0 0.0%</td>
<td>0 0.0%</td>
<td>0 0.0%</td>
<td>9 1.9%</td>
<td></td>
</tr>
<tr>
<td>Occupation, number (%)</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
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</tr>
<tr>
<td>Farmer</td>
<td>19 13.7%</td>
<td>7 14.9%</td>
<td>5 16.7%</td>
<td>4 28.6%</td>
<td>1 4.8%</td>
<td>0 0.0%</td>
<td>2 28.6%</td>
<td>4 22.2%</td>
<td></td>
</tr>
<tr>
<td>Laborer</td>
<td>11 7.9%</td>
<td>2 2.3%</td>
<td>4 13.3%</td>
<td>0 0.0%</td>
<td>1 4.8%</td>
<td>0 0.0%</td>
<td>0 0.0%</td>
<td>0 0.0%</td>
<td></td>
</tr>
<tr>
<td>Fisher</td>
<td>1 0.7%</td>
<td>1 2.1%</td>
<td>0 0.0%</td>
<td>0 0.0%</td>
<td>0 0.0%</td>
<td>0 0.0%</td>
<td>0 0.0%</td>
<td>0 0.0%</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>67 48.2%</td>
<td>35 74.5%</td>
<td>17 56.7%</td>
<td>9 64.3%</td>
<td>16 76.2%</td>
<td>5 62.5%</td>
<td>4 57.1%</td>
<td>14 77.8%</td>
<td></td>
</tr>
<tr>
<td>.</td>
<td>41 29.5%</td>
<td>2 4.3%</td>
<td>4 13.3%</td>
<td>1 7.1%</td>
<td>3 14.3%</td>
<td>3 37.5%</td>
<td>1 14.3%</td>
<td>0 0.0%</td>
<td></td>
</tr>
</tbody>
</table>

n: number of malaria cases; %: percent; IQR: interquartile range; .: missing values; indeterminate: slide-positive cases without registered fever at time of visit; *: percentage is village-specific prevalence; other occupation: student, homemaker, and other minor occupations.
Table 4. Characteristics of *P. vivax* and *P. falciparum* malaria cases diagnosed in Zungarochocha, 2004 – 2011

<table>
<thead>
<tr>
<th></th>
<th>2004</th>
<th>2005</th>
<th>2006</th>
<th>2007</th>
<th>2008</th>
<th>2009</th>
<th>2010</th>
<th>2011</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. vivax (n)</em></td>
<td>98 100%</td>
<td>37 100%</td>
<td>27 100%</td>
<td>12 100%</td>
<td>19 100%</td>
<td>5 100%</td>
<td>5 100%</td>
<td>8 100%</td>
<td></td>
</tr>
<tr>
<td>Sex, male (%)</td>
<td>47 47.9%</td>
<td>26 70.3%</td>
<td>15 55.6%</td>
<td>6 50.0%</td>
<td>8 42.1%</td>
<td>2 40.0%</td>
<td>5 100.0%</td>
<td>4 50.0%</td>
<td>0.1</td>
</tr>
<tr>
<td>Age group, number (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.002</td>
</tr>
<tr>
<td>&lt;5</td>
<td>23 23.5%</td>
<td>1 2.7%</td>
<td>4 14.8%</td>
<td>1 8.3%</td>
<td>3 15.8%</td>
<td>1 20.0%</td>
<td>0 0.0%</td>
<td>0 0.0%</td>
<td></td>
</tr>
<tr>
<td>5 - 14</td>
<td>26 26.5%</td>
<td>18 48.7%</td>
<td>1 3.7%</td>
<td>2 16.7%</td>
<td>7 36.8%</td>
<td>2 40.0%</td>
<td>1 20.0%</td>
<td>0 0.0%</td>
<td></td>
</tr>
<tr>
<td>≥15</td>
<td>49 50.0%</td>
<td>18 48.7%</td>
<td>22 81.5%</td>
<td>9 75.0%</td>
<td>9 47.4%</td>
<td>2 40.0%</td>
<td>4 80.0%</td>
<td>8 100.0%</td>
<td></td>
</tr>
<tr>
<td>Parasitemia, mean ±SD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trophozoites</td>
<td>6.9 ±1.8</td>
<td>6.0 ±1.9</td>
<td>4.3 ±1.6</td>
<td>5.0 ±2.6</td>
<td>5.5 ±2.3</td>
<td>5.6 ±2.8</td>
<td>7.0 ±1.9</td>
<td>5.1 ±1.6</td>
<td>0.1</td>
</tr>
<tr>
<td>Gametocytes</td>
<td>0.0 ±0.0</td>
<td>4.2 ±0.7</td>
<td>3.3 ±0.8</td>
<td>5.7 ±2.4</td>
<td>3.9 ±0.9</td>
<td>3.7 ±0.5</td>
<td>5.7 ±1.4</td>
<td>0.0 ±0</td>
<td>0.03</td>
</tr>
<tr>
<td>Clinical presentation, number (%)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Symptomatic</td>
<td>37 37.8%</td>
<td>15 40.5%</td>
<td>8 29.6%</td>
<td>4 33.3%</td>
<td>5 26.3%</td>
<td>2 40.0%</td>
<td>2 40.0%</td>
<td>3 37.5%</td>
<td></td>
</tr>
<tr>
<td>Asymptomatic</td>
<td>28 28.6%</td>
<td>21 56.8%</td>
<td>19 70.4%</td>
<td>6 50.0%</td>
<td>14 73.7%</td>
<td>3 60.0%</td>
<td>1 20.0%</td>
<td>5 62.5%</td>
<td></td>
</tr>
<tr>
<td>Lifetime malaria events, median [IQR]</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gametocytes</td>
<td>25 26%</td>
<td>11 30%</td>
<td>4 15%</td>
<td>4 33%</td>
<td>9 47%</td>
<td>2 40%</td>
<td>2 40%</td>
<td>2 25%</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><em>P. falciparum (n)</em></th>
<th>44 100%</th>
<th>9 100%</th>
<th>3 100%</th>
<th>2 100%</th>
<th>2 100%</th>
<th>3 100%</th>
<th>2 100%</th>
<th>10 100%</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, male (%)</td>
<td>28 63.6%</td>
<td>3 33.3%</td>
<td>1 33.3%</td>
<td>1 50.0%</td>
<td>1 50.0%</td>
<td>2 66.7%</td>
<td>2 100.0%</td>
<td>5 50.0%</td>
<td>0.6</td>
</tr>
<tr>
<td>Age, median [IQR]</td>
<td>20.7 [9.2, 34.7]</td>
<td>27.7 [16.8, 49.6]</td>
<td>49.5 [21.6, 56.5]</td>
<td>33.6 [14.3, 52.9]</td>
<td>50.7 [50.4, 50.9]</td>
<td>2.30 [0.8, 28.5]</td>
<td>14.2 [9.9, 27.6]</td>
<td>35.6 [17.6, 38.0]</td>
<td>0.05</td>
</tr>
<tr>
<td>Age group, number (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>0.04</td>
</tr>
<tr>
<td>&lt;5</td>
<td>4 9.1%</td>
<td>0 0.0%</td>
<td>0 0.0%</td>
<td>0 0.0%</td>
<td>0 0.0%</td>
<td>2 66.7%</td>
<td>1 50.0%</td>
<td>0 0.0%</td>
<td></td>
</tr>
<tr>
<td>5 - 14</td>
<td>15 34.1%</td>
<td>1 11.1%</td>
<td>0 0.0%</td>
<td>1 50.0%</td>
<td>0 0.0%</td>
<td>0 0.0%</td>
<td>0 0.0%</td>
<td>2 20.0%</td>
<td></td>
</tr>
<tr>
<td>≥15</td>
<td>25 56.8%</td>
<td>8 88.9%</td>
<td>3 100%</td>
<td>1 50.0%</td>
<td>2 100.0%</td>
<td>1 33.3%</td>
<td>1 50.0%</td>
<td>8 80.0%</td>
<td></td>
</tr>
<tr>
<td>Parasitemia, mean ±SD</td>
<td></td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Trophozoites</td>
<td>7.2 ±1.5</td>
<td>7.2 ±0.9</td>
<td>2.8 ±0.5</td>
<td>4.2 ±0.1</td>
<td>5.7 ±0.0</td>
<td>4.5 ±0.2</td>
<td>4.1 ±0.0</td>
<td>5.7 ±1.8</td>
<td>0.04</td>
</tr>
<tr>
<td>Gametocytes</td>
<td>3.5 ±1.1</td>
<td>4 ±1.8</td>
<td>3.4 ±1.1</td>
<td>0.0 ±0.0</td>
<td>4.1 ±0.0</td>
<td>0.0 ±0.0</td>
<td>3.6 ±0.0</td>
<td>0.0 ±0.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Clinical presentation, number (%)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td>Symptomatic</td>
<td>12 27.3%</td>
<td>5 55.6%</td>
<td>2 66.7%</td>
<td>1 50.0%</td>
<td>1 50.0%</td>
<td>0 0.0%</td>
<td>0 0.0%</td>
<td>4 40.0%</td>
<td></td>
</tr>
<tr>
<td>Asymptomatic</td>
<td>12 27.3%</td>
<td>5 55.6%</td>
<td>2 66.7%</td>
<td>1 50.0%</td>
<td>1 50.0%</td>
<td>3 100.0%</td>
<td>1 50.0%</td>
<td>5 50.0%</td>
<td></td>
</tr>
<tr>
<td>Lifetime malaria events, median [IQR]</td>
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<td></td>
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</tr>
<tr>
<td>Trophozoites</td>
<td>3 [1, 6]</td>
<td>2 [1, 8]</td>
<td>3 [0, 10]</td>
<td>3.5 [2, 5]</td>
<td>9.5 [7, 12]</td>
<td>0 [0, 1]</td>
<td>2 [0, 4]</td>
<td>3.5 [2, 5]</td>
<td>0.3</td>
</tr>
<tr>
<td>Gametocytes</td>
<td>15 34%</td>
<td>6 67%</td>
<td>0 0%</td>
<td>1 50%</td>
<td>2 100%</td>
<td>0 0%</td>
<td>0 0%</td>
<td>2 20%</td>
<td>0.07</td>
</tr>
</tbody>
</table>

n: number of parasite positive malaria cases; %: percent; IQR: interquartile range; SD: standard deviation; Parasitemia: log counts/µl.
Table 5. Characteristics of asymptomatic and symptomatic malaria cases diagnosed in Zungarococha, 2004 - 2011

<table>
<thead>
<tr>
<th></th>
<th>Asymptomatic (n = 126)</th>
<th>Symptomatic (n = 100)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, male (%)</td>
<td>68 53.5%</td>
<td>56 56.0%</td>
<td>0.7</td>
</tr>
<tr>
<td>Age, median [IQR]</td>
<td>21.80 [10.55, 38.97]</td>
<td>16.39 [7.88, 32.29]</td>
<td>0.03</td>
</tr>
<tr>
<td>Age group, number (%)</td>
<td></td>
<td></td>
<td>0.007</td>
</tr>
<tr>
<td>&lt;5</td>
<td>9 7.1%</td>
<td>21 21.0%</td>
<td></td>
</tr>
<tr>
<td>5 - 14</td>
<td>34 26.8%</td>
<td>27 27.0%</td>
<td></td>
</tr>
<tr>
<td>≥15</td>
<td>84 66.1%</td>
<td>52 52.0%</td>
<td></td>
</tr>
<tr>
<td>Parasitemia, mean (±SD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trophozoites</td>
<td>5.1 ±1.78</td>
<td>7.2 ±1.77</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Gametocytes</td>
<td>3.5 ±0.86</td>
<td>4.4 ±1.09</td>
<td>0.001</td>
</tr>
<tr>
<td>Lifetime malaria events, median [IQR]</td>
<td>4 [1,17]</td>
<td>3 [1,15]</td>
<td>0.3</td>
</tr>
</tbody>
</table>

n: number of parasite positive malaria cases; %: percent; IQR: interquartile range; SD: standard deviation; Parasitemia: log counts/µl.
Table 6. Age-specific malaria prevalence in Zungarococha, 2004 - 2011

<table>
<thead>
<tr>
<th></th>
<th>2004</th>
<th>2005</th>
<th>2006</th>
<th>2007</th>
<th>2008</th>
<th>2009</th>
<th>2010</th>
<th>2011</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasmodium spp., number (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;5</td>
<td>27 15.9%</td>
<td>1 0.5%</td>
<td>4 1.9%</td>
<td>1 0.5%</td>
<td>3 1.3%</td>
<td>3 1.3%</td>
<td>1 0.4%</td>
<td>0 0.0%</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>5 - 14</td>
<td>41 13.5%</td>
<td>19 5.9%</td>
<td>1 0.3%</td>
<td>3 0.9%</td>
<td>7 2.0%</td>
<td>2 0.6%</td>
<td>1 0.3%</td>
<td>2 0.5%</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>≥15</td>
<td>74 13.90%</td>
<td>27 5.2%</td>
<td>25 3.9%</td>
<td>10 1.5%</td>
<td>11 1.8%</td>
<td>3 0.4%</td>
<td>5 0.7%</td>
<td>16 2.0%</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>P. vivax, number (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;5</td>
<td>23 13.5%</td>
<td>1 0.5%</td>
<td>4 1.9%</td>
<td>1 0.5%</td>
<td>3 1.3%</td>
<td>1 0.4%</td>
<td>0 0.0%</td>
<td>0 0.0%</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>5 - 14</td>
<td>26 8.6%</td>
<td>18 5.6%</td>
<td>1 0.3%</td>
<td>2 0.6%</td>
<td>7 2.0%</td>
<td>2 0.6%</td>
<td>1 0.3%</td>
<td>0 0.0%</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>≥15</td>
<td>49 9.3%</td>
<td>18 3.4%</td>
<td>22 3.4%</td>
<td>9 1.4%</td>
<td>9 1.5%</td>
<td>2 0.3%</td>
<td>4 0.6%</td>
<td>8 1.0%</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>P. falciparum, number (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;5</td>
<td>4 2.4%</td>
<td>0 0.0%</td>
<td>0 0.0%</td>
<td>0 0.0%</td>
<td>0 0.0%</td>
<td>2 0.9%</td>
<td>1 0.4%</td>
<td>0 0.0%</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>5 - 14</td>
<td>15 4.9%</td>
<td>1 0.3%</td>
<td>0 0.0%</td>
<td>1 0.3%</td>
<td>0 0.0%</td>
<td>0 0.0%</td>
<td>0 0.0%</td>
<td>2 0.5%</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>≥15</td>
<td>25 4.7%</td>
<td>8 1.5%</td>
<td>3 0.5%</td>
<td>1 0.2%</td>
<td>2 0.3%</td>
<td>1 0.2%</td>
<td>1 0.1%</td>
<td>8 1.0%</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

n: number; %: percentage
Table 7. Association of sex and occupation with risk of malaria infection in Zungarococha from 2004 to 2011

<table>
<thead>
<tr>
<th></th>
<th>Plasmodium spp.</th>
<th>P. vivax</th>
<th>P. falciparum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bivariate</td>
<td>Multivariate</td>
<td>Bivariate</td>
</tr>
<tr>
<td></td>
<td>OR  [95% C.I.]</td>
<td>P-value</td>
<td>AOR  [95% C.I.]</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>1.00 Ref.</td>
<td></td>
<td>1.00 Ref.</td>
</tr>
<tr>
<td>Male</td>
<td>1.45 [1.14, 1.85]</td>
<td>0.003</td>
<td>1.11 [0.80, 1.55]</td>
</tr>
<tr>
<td>Occupation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>1.00 Ref.</td>
<td></td>
<td>1.00 Ref.</td>
</tr>
<tr>
<td>Farmer</td>
<td>1.94 [1.38, 2.73]</td>
<td>&lt;0.0001</td>
<td>1.82 [1.22, 2.70]</td>
</tr>
<tr>
<td>Laborer</td>
<td>1.87 [1.13, 3.08]</td>
<td>0.02</td>
<td>1.75 [1.01, 3.01]</td>
</tr>
<tr>
<td>Fisher</td>
<td>1.31 [0.34, 5.04]</td>
<td>0.7</td>
<td>1.23 [0.32, 4.80]</td>
</tr>
</tbody>
</table>

OR: Odds Ratio; AOR: Adjusted Odds Ratio; C.I.: Confidence Interval; Statistical significance: P < 0.05. Other: student, homeworker, and other reported occupations.
Figure 1. Loreto Department and Iquitos city in the Peruvian Amazon Basin

Malaria cases in Peru: 1939-2010. Cases of malaria due to *Plasmodium falciparum* and *Pv* were not differentiated and reported systematically until 1990. Source: Peruvian Ministry of Health, Lima, Peru.

Figure 3. Zungarococha community and main villages

Villages: ZG = Zungarococha, PA = Puerto Almendra, NR = Ninarumi, LC = Llanchama

Selection of houses of the MIGIA cohort study for weekly active case detection within ‘Pf at-risk’ zones. Weekly sampling was focused in zones with defined Pf infection risk. Based upon the detection (passive or active) of a Pf case the previous month (solid red circles), a 100-meter radius was defined (open blue circle) to detect malaria-infected individuals in selected households (open black circles).

Source: Branch O, R01 AI064831-01
Figure 5. Trend of malaria parasite prevalence in Zungarococha, 2004 – 2011

Information on the changing age-specific malaria parasite prevalence with lines representing changes between CSS years, *p-value<0.05 for significant differences of total malaria, †p-value<0.05 for significant differences of *Pv* malaria, ‡p-value<0.05 for significant differences of *Pf* malaria.
Figure 6. Proportion of asymptomatic and symptomatic malaria cases in Zungarococha, 2004 – 2011

Proportion of asymptomatic and symptomatic malaria cases at each study year. Symptomatic malaria cases were defined as microscopy-positive individuals with either a documented fever at time of visit or self-reported fever during the two previous days. Asymptomatic malaria cases were defined as microscopy-positive individuals with no documented or self-reported fever at time of visit or within the two previous days. Indeterminate malaria cases were microscopy-positive individuals who lacked information on fever at the time of visit.
Figure 7. Trend of age-specific parasite prevalence in Zungarococha, 2004 – 2011

Age-specific malaria parasite prevalence trend with lines representing changes between survey years for A: under-fives, B: 5-14 year olds, C: ≥15 year olds, *p-value<0.05 for significant differences of total malaria, †p-value<0.05 for significant differences of P. vivax malaria, ‡p-value<0.05 for significant differences of P. falciparum malaria.
Chapter Three:  Paper II – Naturally-acquired humoral immune response against PvMSP1-19 in a hypoendemic area in the Peruvian Amazon Basin

3.1 Abstract

**Background:** In high-transmission settings, development of clinical immunity to malaria appears to require high levels of parasitemia and frequent infections that often result in short-lived immune responses. Conversely, studies in low-transmission settings have found that infrequent malaria infections are capable of eliciting protective immune responses that are maintained over time. Immuno-epidemiological studies on *P. vivax* (*Pv*) malaria in a mesoendemic setting found that long-term-exposed subjects had lower anti-MSP1<sub>19</sub> antibody levels than persons who were only sporadically or less-exposed to parasites, and found no statistically significant differences between clinical groups (symptomatic vs. asymptomatic). Studies on *Pv* immunity in hypoendemic settings, where frequent infection is less likely, will be key to studying humoral immune responses to PvMSP1<sub>19</sub>, shedding light on the immuno-epidemiology and possibly informing current *Pv* vaccine development efforts.

**Methods:** A retrospective immuno-epidemiological study was conducted in a low-transmission community in Iquitos, Peru using 177 samples and data collected between 2004-2011 through active and passive case detection. Plasma samples from *Pv* mono-infected subjects before and during current infection were analyzed by enzyme-linked immunosorbent assay (ELISA) to detect PvMSP1<sub>19</sub>-specific antibodies. Differences in
antibody levels between symptomatic and asymptomatic individuals within and between pre- and current infection time points, as well as the association of pre-infection IgG antibodies with asymptomatic infection, were assessed.

**Results:** During the current documented infection, one third of *Pv* mono-infected subjects were asymptomatic. These individuals were older, had more lifetime malaria events, a longer period of residence in the area, and lower trophozoite and gametocyte counts than symptomatic individuals. Pre-infection IgG mean antibody levels against *PvMSP1* was indistinguishable between symptomatic and asymptomatic participants (p=0.7), but was correlated with time since last infection and number of lifetime malaria events. However, higher pre-infection IgG antibody quartiles were associated with increased odds of having an asymptomatic *Pv* infection. During *Pv* infection, asymptomatic individuals had significantly lower antibody levels than symptomatic individuals (p<0.001), but there was no significant correlation between pre- and current infection antibodies. Between the pre- and current infection time points, only symptomatic subjects had a significant increase in IgG antibody levels (p<0.001). Despite significant changes in antibody levels between the pre- and current infection time points (p<0.001) in symptomatic and asymptomatic individuals, no significant difference in seropositive status was observed between the groups (p=0.3).

**Conclusions:** Pre-infection anti-*PvMSP1* IgG antibodies and the infection anti-*PvMSP1* IgG antibody response appear to reflect different risks associated with clinical disease. Higher pre-infection IgG antibody quintiles were associated with increased odds of having asymptomatic *Pv* malaria during active infection, while increasing IgG antibodies during active infection were associated with clinical disease. These findings
suggest that anti-PvMSP1$_{19}$ IgG antibody responses might not be a suitable epidemiological tool to distinguish symptomatic and asymptomatic infected persons in low-endemic communities. Nonetheless, because anti-PvMSP1$_{19}$ IgG antibody quartiles were associated with increased odds of asymptomatic malaria, serology could be of use to study naturally-acquired clinical immunity in low-endemic settings.

3.2 **Background**

Despite recent efforts to control malaria, the looming widespread resistance of the parasite to existing drugs (1, 2), vector resistance to insecticides (3), population movement, and administrative failures (4) highlight the need for new efforts to contain this disease, such as the development of an efficient vaccine. Knowledge of the human immune responses to malaria infection and factors associated with the development and maintenance of naturally-acquired immunity will help current malaria vaccine efforts. Understanding how antibodies could serve as a tool for epidemiological studies to screen populations at risk of malaria and potentially diagnose infected individuals retrospectively will also be important to enhance malaria control and elimination efforts in low endemic settings where community-wide surveys are critical to actively identify malaria cases, as well as asymptomatic parasite reservoirs that contribute to the persistence of the disease.

Current public health interventions, such as indoor-residual spraying and insecticide-treated nets, have resulted in a significant reduction in the burden of malaria. However, this reduction has been less pronounced in *Plasmodium vivax* (*Pv*) than in *P. falciparum* (*Pf*) endemic settings (5). Additionally, we have limited understanding of *Pv*
malaria, particularly regarding the hypnozoite stage, and have been unable to culture the parasite continuously in vitro. This suggests that elimination of \( P_v \) malaria will be far more difficult to accomplish, and stresses the need to conduct more immunoepidemiology studies on this \( Plasmodium \) species.

Among human malaria parasites, \( P_v \) is the most widely distributed species and accounts for an estimated 70-80 million cases annually with more than two billion people at risk (6-8). \( P_v \) is the most frequent malaria pathogen in many parts of the world, such as Central and South America, South East Asia, India and Oceania. Infection with \( P_v \) has often been considered benign but current evidence demonstrated that infection with \( P_v \) can result in significant morbidity and mortality (9-13). This health impact takes a substantial toll on social and economic development in terms of disability-adjusted life years, job loss, and economic productivity, due to acute and relapsing infections, chronic malarial anemia, impaired early childhood growth and development (14), and complicated pregnancies (15).

Multiple studies have reported asymptomatic \( P_v \) malaria infections in areas with varying prevalence (16-20) that could serve as parasite reservoirs. These asymptomatic cases may help maintain malaria transmission in endemic areas and facilitate the persistence of this disease. The high frequency of asymptomatic cases suggests that subjects in low transmission areas such as Peru can develop a naturally-acquired immunity to clinical infection, contrary to what our current understanding of immunity to malaria suggests. Studies on the acquisition of naturally-acquired immunity, assessed in regions of high \( P_f \) transmission, describe a delayed acquisition of immunity, where resistance to disease results from years of frequent, closely-spaced infections (21, 22).
In Peru, \( P_v \) is mainly transmitted in the Amazon Region and represented 80% of over 64,000 malaria cases reported in 2014, with the majority in the Amazonic Department of Loreto (23). \( P_v \) transmission is often focal and heterogeneous, affecting particular segments of the population (e.g., loggers or others engaged in forest-related economic activities) (18, 19, 24). In the Peruvian Amazon Basin, the transmission and incidence of \( P_v \) malaria shows a low and seasonal transmission pattern (18), being hypoendemic in the Iquitos region. Despite low malaria transmission in this area, previous studies have reported remarkably high levels of \( P_v \) genetic diversity in Iquitos and surrounding villages as well as a high prevalence of asymptomatic cases (19, 24), which might contribute to the persistence of the parasite in the region.

The Peruvian Amazon Basin offers a unique environment to study naturally-acquired immunity to clinical disease, assessing the host’s response to malaria parasite antigens under low transmission intensity. Additionally, this environment is ideal to study the effect of infrequent exposure on the development and maintenance of naturally-acquired immunity due to the low probability of having more than one infection per year.

One of the most well studied malaria vaccine candidates is the merozoite surface protein I (MSP1), which is a blood stage glycoprotein located on the surface of merozoites that is highly immunogenic. During red blood cell (RBC) invasion, MSP1 is proteolytically cleaved into polypeptides with MSP1 C-terminal 19 kDa (MSP1\(_{19}\)) remaining attached to the merozoite surface during RBC invasion (25, 26). Given its involvement in this process, MSP1\(_{19}\) is considered important in erythrocyte invasion and the parasite’s life cycle (25). Studies on \( Pf \) have identified this protein as a major target of naturally-acquired antibodies, capable of interrupting RBC invasion by inhibiting the proteolytic
cleavage (27), and these antibodies are positively associated with protection from clinical infection (28-33), reduced parasitemia, and reduced risk of subsequent infection (30, 34-37). Only a few immuno-epidemiological studies have addressed the natural humoral response to \( P_v \) MSP1\(_{19} \) (PvMSP1\(_{19} \)).

In this study, we describe the natural humoral immune responses to PvMSP1\(_{19} \) in a low transmission area and assess its association with clinical disease, adjusting for other epidemiological parameters. We hypothesized that asymptomatic individuals would have higher PvMSP1\(_{19} \)-specific mean antibody levels prior to and during the documented infection than symptomatic individuals, and that their increased antibody levels would be associated with clinical protection, as previously described for \( P_f \) MSP1\(_{19} \) (38).

### 3.3 Methods

**Study area and study design:** Malaria has historically been present in the Loreto area, with epidemics and outbreaks influenced by changing support to the malaria control programs, environmental factors (e.g. El Niño Southern Oscillation, floods), as well as population movements that exposed naïve hosts to malaria for socio-economic reasons, such as migration to remote areas to engage in oil and mining exploration activities, as well as resettlement from areas occupied by narco-terrorist groups (39). Through intense vector control programs and changes in drug policy malaria cases declined since the beginning of the 20\(^{th}\) century, with a drastic reduction in the number of cases by 2005 (Fig. 1).

This retrospective study was performed with repository samples collected from the Zungarochocha community, an endemic area located in the northwestern region of the
Peruvian Amazon Basin that is approximately 5 kilometers away from Iquitos city, within the Malaria Immunology and Genetics in the Amazon (MIGIA) cohort study. Samples were collected during the hypoendemic malaria period from 2003 to 2011. The MIGIA parent study used active case detection, with cross-sectional community wide surveys at the beginning (January – March) and end (July – August) of the malaria season and reactive case detection with weekly home-based visits, as well as passive case detection at the local health clinic throughout the year.

The Zungarococha community is composed of four main villages (Fig. 2), with approximately 2000 inhabitants, connected by a dirt road (~2 km between villages) and with houses made of wood and thatch roofs with no screens. Only two villages have electricity (Zungarococha and Puerto Almendra) and the community health post is located in the most developed village (Zungarococha). The Zungarococha population is sustained by local agriculture and employment, and has a homogenous income status. Malaria transmission in the community is year-round with a prominent peak during the rainy season, from January to July, with \( P_v \) the predominant species. In 2003, a prospective cohort study conducted by Branch \textit{et al.} in this area estimated a force of infection (FOI) of 1.8 \( P_v \) infections/person/year and 0.46 \( P_f \) infections/person/year with more than 60% of malaria cases being asymptomatic and detectable only by PCR. They estimated by active and passive case detection a \( P_v \) and \( P_f \) prevalence of 0.39 and 0.13, respectively (18).

\textbf{Study Population:} Our study population was selected by: i) establishing a case definition of symptomatic and asymptomatic \( P_v \) malaria, ii) categorizing all participants from the MIGIA parent study based on the case definition, iii) checking the pregnancy
status of the participant, if female, and excluding those who were currently pregnant, and iv) checking the availability of samples in the Iquitos MIGIA repository. Any *Pv* slide-positive mono-infected subject, determined by microscopy, who did not present with fever at the time of microscopy diagnosis or during the two previous days was defined as an asymptomatic case. Any *Pv* slide-positive mono-infected subject who presented with a fever (axillary temperature >37.5°C) at the time of diagnosis or self-reported fever during the two days prior was defined as a symptomatic case. We selected all plasma samples that met our selection criteria (convenience sampling). We also categorized the different types of occupations by their risk of malaria exposure into: i) high risk (farmer, fisherman, and logger) and ii) low risk (e.g. student, stay-at-home, child, unemployed, retired, etc.).

We analyzed plasma samples from selected symptomatic (n=118) and asymptomatic (n=59) *Pv* mono-infected subjects to: i) assess differences in IgG antibody levels to *PvMSP1*<sub>19</sub> before (n=55, pre-infection time range: 0.5 – 8 months) and during infection (n=177) between clinical groups; ii) measure the change in IgG antibody levels to *PvMSP1*<sub>19</sub> across time points in both clinical groups; and iii) assess the effect of prior antibody levels to *PvMSP1*<sub>19</sub> on the clinical outcome (symptomatic vs. asymptomatic infection). Additionally, IgG subclass 1 (IgG<sub>1</sub>) and subclass 3 (IgG<sub>3</sub>) antibody levels to *PvMSP1*<sub>19</sub> were analyzed as these are the most cytophilic antibodies (highest affinity to the Fc receptor) and have been associated with reduced parasite density (30, 40-45). Finally, since IgM antibodies are the first to be produced in response to an initial exposure to *Pv*, differences IgM antibodies to *PvMSP1*<sub>19</sub> were compared between study groups.
**Parent study and data collection:** The parent cohort study (MIGIA) collected whole blood from infants, children, adults, and pregnant women for malaria diagnosis using three sampling strategies: i) passive case surveillance with a full-time clinical team stationed at the Zungarococha health post throughout the year, ii) active case detection through community-wide surveys at the start (January-March) and end (July – August) of the malaria season, and iii) reactive case surveillance with four weekly household visits throughout the malaria season (February – August) (18) (Fig. 3). The weekly household visits took place within a 100-meter radius from a “sentinel” house. Sentinel houses were defined as houses that had at least one *Pf* case (index case) that had either visited the health post in the previous month for malaria diagnosis (passive detection) or were detected through the community-wide survey or weekly household visits in the previous month (active detection). Upon the identification of the *Pf* index case, a 100-meter radius around that index case’s house was delimited and defined as the “*Pf* at-risk area” (Fig. 4). If at-risk-areas overlapped in a given month, the parent study assigned the randomly selected house to one of the areas. Generally, there were 20-50 houses within a given 100-meter radius area. Within the “*Pf* at-risk area”, houses were randomly selected using a random number generator to conduct weekly visits in order to capture the onset and dynamics of malaria infection, both *Pf* and *Pv*, in individuals living in close proximity to the *Pf* index case. The parent study team visited all randomly selected houses within each at-risk-area and recruited all household members until at least 30 individuals (~5–9 houses) were enrolled. All consenting participants, including the sentinel household with the index case, were included in the weekly visits. Overall, there were five to six at-risk-areas per month of active surveillance. Each participant was visited once per week for
four weeks, except when there was a positive blood slide, which resulted in additional visits. Branch et al. reported high recruitment and retention rates in 2003 (18). Because of the relative small size of each village, less than 600 square-meters, it was not unusual to sample the same at-risk areas in successive months of active surveillance. In 2003, Branch et al. reported sampling 70-90% of all the houses within Puerto Almendra and Ninarumi villages, and 50-70% of all houses within Zungarococha village during the active surveillance period.

During each visit, whether in the health post, community-wide survey, or weekly home-visits, clinical (e.g. temperature, malaria-related symptoms, hematocrit), epidemiological (e.g. number of past malaria infections, years lived in the area), and demographic information (e.g. age and sex) were recorded by the study team (physician-nurse team). The study physician measured axillary temperature using a digital thermometer, and collected 0.25 to 3 ml of blood by finger prick or venipuncture. Additionally, participants diagnosed with malaria had 3-6 ml of blood collected by venipuncture. Blood samples were used to prepare thin and thick blood smears, as well as capillary hematocrit tubes. All remaining blood was stored in EDTA-containing vacutainers for further processing and storage. During active case detection visits, blood slides, hematocrit tubes and vacutainers were transported to the laboratory for microscopy and processing. Vacutainers were centrifuged to separate plasma from packed blood cells, and frozen within 18 hours at -85°C. Capillary hematocrit tubes were centrifuged and the volume percentage of red blood cells (packed cell volume, PCV) was measured.
Malaria infections were documented by microscopic analysis of Giemsa-stained thick and thin blood smears, following standard procedures. Two expert microscopists with over 15 years of experience counted parasites, reading 200 microscopy fields. Both \textit{Pv} and \textit{Pf} trophozoites and gametocytes were counted separately. At least 500 white blood cells (WBCs) were counted before an individual was diagnosed as negative by microscopy. Species-specific parasite density (parasite/uL) was determined by the number of parasites divided by the total number of WBCs counted and multiplied by 6,000. The conversion factor of 6,000 RBCs per one WBC to determine parasite density per microliter is regularly used in the area (46).

During active case detection, priority was given to symptomatic subjects (axillary temperature \(>37.5^\circ{\text{C}}\) or reported fever within 2 days), pregnant women, children (<5 years), and anemic subjects (Hb < 10 g/dL measured by HemoCue, or hematocrit PCV <30%), who had their slides read within one day of the household visit and received species-specific antimalarial drug treatment (\textit{Pv}: 3 days chloroquine + 7 days primaquine; \textit{Pf}: 3 days artesunate + 2 days mefloquine) within one day of diagnosis. Asymptomatic subjects had their slides read within 6 days after the household visit. Asymptomatic participants who were positive by microscopy at the initial visit and were found to be symptomatic at the following weekly visit (day “i+7”) were given antimalarial drug treatment within one day of diagnosis. Asymptomatic participants who remained without symptoms at day “i+7” were re-visited the following day (day “i+8”) to perform a confirmation slide. All asymptomatic slide-positive participants at days “i” and “i+7” were given antimalarial drug treatment. For all malaria-positive slides detected at the last weekly visit, an additional visit was performed to collect an extra blood sample.
Additionally, the study team was in the vicinity throughout the course of the study, taking blood samples from anyone who presented with signs or symptoms of malaria, even outside their regular scheduled weekly visits. All malaria positive individuals were directed to the local health post to receive treatment from the MINSA. All referred cases, as well as individual who attended the health post for malaria diagnosis were asked to participate in the passive case detection arm of the MIGIA study.

The MINSA authorities administered all treatments following MINSA National Drug Policy Guidelines following directly observed treatment, with: i) non-pregnant *Pv* patients: 3 days chloroquine 10 mg/kg + 7 days primaquine 0.5 mg/kg for non-pregnant *Pv* cases, ii) pregnant and infant *Pv* patients: 2 days chloroquine 10 mg/kg + 1 day chloroquine 5 mg/kg, iii) non-pregnant *Pf* cases: 3 days artesunate 4 mg/kg + 2 days mefloquine 12.5 mg/kg, and iv) pregnant and infant *Pf* patients: 5 days clindamycin 10 mg/kg two times a day + 7 days quinine 10 mg/kg three times a day (47). To confirm treatment efficacy, a finger prick blood sample was collected and analyzed 7 and 14 days after the day of malaria diagnosis.

**Measurement of *PvMSP1*<sub>19</sub> antibody responses by ELISA:** Plasma antibody levels were measured by an indirect enzyme-linked immunosorbent assay (ELISA). The 19 kDa fragment of MSP1<sub>19</sub>, an important parasite antigen for parasite invasion (48-51) and a vaccine candidate molecule of both *Pv* and *Pf* malaria (52-63), was obtained from the Malaria Research and Reference Reagent Resource Center (MR4, Virginia - USA) representing amino acids 1639-1738 (Sal-I strain) with a of *Pv*, expressed in *S. cerevisiae* (MSP1<sub>19</sub>-His<sub>6</sub>). Nunc Maxisorp (Sigma-Aldrich, Missouri - USA) flat-bottom 96-well plates were coated with recombinant protein (0.5 μg/ml IgG, 1 μg/ml IgM) in phosphate-
buffered saline (PBS) buffer (pH 7.4) overnight at room temperature (RT). After washing six times with PBS-Tween (0.05%) (PBS-T), plates were blocked with 200 uL PBS-milk (5% non-fat milk powder) at RT for one hour. Plasma samples were diluted in this solution, and 50 uL of each sample was incubated in duplicate at RT for two hours. After washing with PBS-T, plates were incubated with diluted goat anti-human horseradish peroxidase conjugated antibodies (IgM: 1:7,000, IgG: 1:10,000) (IgM: KPL; IgG: Jackson ImmunoResearch Laboratories Inc., Pennsylvania - USA) at RT for one hour. Plates were washed again with PBS-T, developed with o-phenylenediamine substrate (OPD; 10 mg tablet Sigma Aldrich Product No. P8287) at RT for one hour, and stopped with 3N hydrochloric acid (HCl). The absorbance (optical density, OD) was read at 490 nm with the use of a microplate reader (Biotek ELx800 Spectrophotometer).

To detect IgG subclasses among anti-PvMSP1_{19} antibodies, an ELISA was performed as described above using isotype-specific mouse anti-human IgG antibodies at dilutions of 1:4000 (IgG₁) and 1:2000 (IgG₃) incubated at RT for one hour, and a tertiary anti-mouse antibody (Jackson ImmunoResearch Laboratories Inc., Pennsylvania - USA). The enzyme reaction was developed with OPD substrate and stopped with 3N HCl. A standard calibration curve made from serial dilutions of a standard reference pool of highly reactive \textit{Pv} positive (individuals diagnosed by microscopy and PCR with at least 10 lifetime malaria infections) plasma samples (n=10) collected in the Peruvian Amazon Basin from Iquitos (n=5) and Madre de Dios (n=5) was used to estimate antibody levels in ELISA arbitrary units (AU). To estimate individual plasma antibody levels, the reciprocal of the plasma dilutions of the reference pool were used to generate a nonlinear sigmoidal dose-response curve-fit. Test plasma OD values were interpolated to the curve.
and converted into AU. One ELISA arbitrary unit (1 AU) is equivalent to the reciprocal plasma dilution of the reference pool that gave the lowest OD. This method allowed estimation of the antibody levels based on a reference value, even if the value of the reference is unknown in conventional units (e.g. there is no reference standard for antibody concentration), and compare multiple measurements within and between ELISA plates (64-66). For internal positive and negative controls, we used a plasma sample from a highly reactive \( P.\) malaria-positive adult from Iquitos (determined by microscopy and PCR) and a pool of plasma samples from non-exposed non-endemic malaria-naïve adults from Lima Peru \( (n=25).\) The ELISA seropositive cut-offs were obtained from the average of a pool of non-exposed non-endemic malaria-naïve adults from Lima Peru \( (n=25)\) plus three standard deviations \( (SD).\) The estimated cut-offs to determine seropositivity were 1588 AU for total IgG, 3686 AU for IgG\(_1\), and 774 AU for IgG\(_3\).

**Statistical Analysis:** Antibody levels were tested for normality using the Shapiro-Wilk test and log-transformed (natural logarithm) for analysis. Differences in mean log antibody \( (\log \text{AU})\) levels were evaluated by the Student t-test or One-way ANOVA test. Differences in mean log antibody levels within time points were evaluated by a Student t-test, differences in mean log antibody levels between time points were evaluated by a paired Student t-test. Differences in medians were evaluated by the Wilcoxon or Kruskal-Wallis tests. Correlations were identified by the Spearman rank test. Differences in proportions were evaluated by a chi-square test. Comparison between study populations (pre-infection vs. infection) was evaluated by chi-square test for categorical variables and quintile regression (comparison of medians) for continuous variables. \( P\)-values \(<0.05\) were considered statistically significant. A generalized linear model (GLM) linear
regression was used to estimate the effect of covariates on the anti-PvMSP119 Ab response during infection. GLM logistic regression was used to estimate the odds of being asymptomatic during the current \textit{P}v infection at varying levels of pre-infection IgG and current IgG antibody levels. Model selection was determined stepwise by Akaike information criterion (AIC) and P-value <0.05 criteria. All analyses were undertaken using Stata (Version 13, Statacorp LP) and GraphPad Prism (Version 6.0, GraphPad Software, Inc.).

**Research Ethics:** This study was approved by the U.S. Naval Medical Research Unit No. 6 (NAMRU-6) Institutional Review Board (IRB approval code: NAMRU6.2013.0009). Ethical approval of the parent study was obtained from the Instituto Nacional de Salud (Human use approved protocol: 08-982), the University of Alabama at Birmingham, the Universidad Peruana Cayetano Heredia, and the New York University School of Medicine Institutional Review Boards. Prior to enrollment, written informed consent was obtained from all study participants eighteen years of age or older, or for participants between seven and eighteen years of age an assent from the minor and consent from the parents or guardians. The Johns Hopkins Bloomberg School of Public Health granted an IRB waiver given that the analysis reported here used de-identified data and samples. All study participants provided written informed consent for future use of their samples.
### 3.4 Results

#### Study Population

A total of 177 plasma samples from participants with confirmed *Pv* infection, from which 55 (31%) had pre-infection samples, were analyzed. Asymptomatic individuals were significantly older (median 17 years; IQR 9-34 years) than symptomatic individuals (median 12 years; IQR 6-24 years), reported significantly more lifetime malaria events (median 5 events; IQR 1-8 events) than symptomatic individuals (median 2 events; IQR 1-5 events), reported living in the area significantly longer (median 9 years; IQR 5-16 years) than symptomatic individuals (median 5 years; IQR 3-9 years), and had significantly lower trophozoite (median 36; IQR 24-119) and gametocyte counts (median 0; IQR 0-0) counts/μL than symptomatic individuals (median 6,650; IQR 3,265-12,687 and median 262; IQR 59-657, respectively) (Table 1). There were fewer male participants (44.8%) in the asymptomatic than the symptomatic group (60.5%). There were no differences in occupational risk between clinical groups (p=0.3), anemia (p=0.4), or time since last malaria event (p=0.1).

When we compared medians and proportions between the pre-infection sample (n = 55) and the study population (n = 177), we did not find significant differences in age (p=0.4), sex (p=0.8), lifetime malaria events (p=0.3), trophozoites (p=0.4), gametocytes (p=0.7), years of residence in the area (p=0.7), years since last malaria event (p=0.1), or anemia (p=0.3). Finally, within the study sub-sample with pre-infection plasma samples, no statistically significant differences were observed in the time between the pre- and current-infection time points between asymptomatic (median 1.1 months, IQR 0.7 – 4.7
months) and symptomatic (median 2.9 months, IQR 1.4 – 3.9 months) participants (p=0.07)

**Pre-infection anti-PvMSP19 IgG mean antibody levels were indistinguishable between symptomatic and asymptomatic individuals but were significantly correlated with time since last malaria event and lifetime malaria events**

No significant differences in the seropositive status between symptomatic (35%) and asymptomatic (38%) individuals were observed before infection (p=0.8), and anti-PvMSP19 total IgG mean antibody levels were similar (7.1 ±2.1 AU and 7.2 ±1.5 AU respectively, p=0.8) (Table 2). Accordingly, IgG subtypes 1 and 3 were also similar between clinical groups, with minimal differences between those who were symptomatic and asymptomatic (Table 2). However, when analyzed by quartiles, significant differences were observed between the clinical groups (p=0.02), with more asymptomatic subjects (67%) in the mid to high antibody categories (quartile 3 and 4) compared to symptomatic subjects (41%).

No correlation was observed between pre-infection IgG antibody levels and the time when pre-infection antibodies were measured (Spearman R=0.04 p=0.8) and between study groups (p=0.78). However, a significant negative correlation between pre-IgG antibodies and self-reported time since last malaria event was identified (Spearman R=−0.35 p=0.01) (Fig. 5A), suggesting that the more recent the past malaria event, the greater the pre-infection total IgG levels. Individuals who reported experienced their last malaria event within the previous six months, six to twelve months, or over twelve months of the current *Pv* infection had significantly different mean antibody levels
Compared to subjects who reported their last malaria event within 6 months of the current *Pv* infection (mean=9.2 log AU), mean pre-infection IgG antibodies were higher than subjects who reported their last malaria event 6-12 months (mean=7.0 log AU, *p*=0.06) and more than 12 months (mean=6.6 log AU, *p*<0.001) prior to the current infection (Fig. 5B). We did not find significant differences in mean pre-infection IgG antibodies between subjects who reported their last malaria event 6-12 months or more than 12 months prior to the current infection (*p*=1). Over seventy percent of individuals that experienced their last malaria event in the previous six months were anti-*PvMSP1* IgG seropositive (73%), while the proportion of seropositive subjects who reported their last malaria event in the previous 6-12 months or over 12 months was markedly reduced (40% and 26% respectively) (*p*=0.02).

A significant positive correlation was observed between levels of pre-infection anti-*PvMSP1* total IgG antibodies and self-reported lifetime *Pv* malaria events (Spearman R=0.36 *p*=0.007) as well as *Pv* lifetime malaria events (Spearman R=0.35 *p*=0.008), but not for *Pf* lifetime malaria events (Spearman R=0.11 *p*=0.43) (Fig. 5C). Compared to subjects who reported experiencing no previous malaria events (mean=5.9), mean pre-infection IgG antibodies were only significantly higher those who reported experiencing four or more previous malaria events (mean=7.7, *p*=0.04) but not those who reported having experienced 1-3 previous malaria events (mean=7.2, *p*=0.1). A similar but not significantly different relationship was observed between self-reported lifetime *Pv* event categories (data not shown).

When the relationship between pre-infection IgG antibodies and other variables were investigated, no significant differences between symptomatic and asymptomatic
subjects were observed except for self-reported months since last malaria event and self-reported lifetime \( P\nu \) malaria episodes (Table 3). In a multivariate analysis adjusting for age, these associations remained significant and indicated that pre-infection IgG antibody levels increased with increasing previous \( P\nu \) lifetime events and age, and was highest when the last malaria event took place within 6 months of the current infection.

**Distinct \( P\nu \text{MSP1}_{19} \) antibody responses in symptomatic vs. asymptomatic individuals during \( P\nu \) infection**

Significant differences were observed between the percentages of total IgG seropositive people, with the asymptomatic group having significantly less seropositive individuals than the symptomatic group (55% vs. 86%; \( p<0.001 \)). Also, asymptomatic subjects had significantly lower log AU total IgG antibodies compared to symptomatic individuals (\( p<0.001 \)) (Fig. 6, Table 4). We also investigated the relationship between total IgG antibodies (log AU) and other variables. In a multivariate analysis, differences between symptomatic and asymptomatic subjects in total IgG antibody levels were lost after adjusting for trophozoites, gametocytes, and previous number of malaria events (Table 5).

Consistent with the findings for total IgG serostatus and antibody levels, asymptomatic participants were less likely to be seropositive for IgG1 and IgG3 antibodies (28% and 19%, respectively) (\( p<0.0001 \)) than symptomatic participants (77% and 59%, respectively), and had significantly lower \( P\nu \text{MSP1}_{19} \)-specific IgG1 and IgG3 antibodies than symptomatic participants (\( P<0.0001 \)) (Table 4). Asymptomatic subjects had significantly lower log AU IgM antibodies than symptomatic individuals (\( p<0.001 \)).

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(Table 4). Overall, asymptomatic participants had significantly lower antibodies levels (log AU) than symptomatic subjects during the documented infection (Fig. 6)

**Changes in anti-PvMSP1 total IgG antibodies and serostatus in symptomatic and asymptomatic individuals from the pre- to current-infection time point**

We investigated the relationship between pre- and current-infection total IgG antibodies but did not find a significant correlation between both time points (Spearman R=0.2 p=0.1). Regarding changes in serostatus between time points, almost a quarter *Pv*-infected study participants, as diagnosed by light microscopy, remained anti-PvMSP1<sub>19</sub> IgG seronegative between time points (22%); 42% seroconverted to anti-PvMSP1<sub>19</sub> IgG seropositive during infection; 31% remained seropositive between time points; and 5% seroreverted to anti-PvMSP1<sub>19</sub> IgG seronegative during infection (Table 6). Differences in total IgG serostatus and log AU levels between the pre- and current-infection time points by clinical group were assessed (Fig. 7). Although no significant differences in serostatus between symptomatic than asymptomatic individuals were observed (p=0.3), with 47% of symptomatic individuals and 33% of asymptomatic individuals becoming seropositive during infection (Table 6), only symptomatic subjects had a significant increase in total IgG antibody levels ($\Delta_{SYM}=4.2$, $p<0.001$ vs. $\Delta_{ASYM}=0.7$, $p=0.2$) (Fig. 8).

Regarding IgG<sub>1</sub> and IgG<sub>3</sub> subtypes, changes in serostatus between time points, 42% and 56% of *Pv*-infected study participants, as diagnosed by light microscopy, remained anti-PvMSP1<sub>19</sub> IgG<sub>1</sub> and IgG<sub>3</sub> seronegative between time points; 42% and 33% seroconverted to anti-PvMSP1<sub>19</sub> IgG<sub>1</sub> and IgG<sub>3</sub> seropositive during infection; 13% and 7% remained seropositive between time points; and 4% seroreverted to anti-PvMSP1<sub>19</sub>
IgG\textsubscript{1} and IgG\textsubscript{3} seronegative during infection, respectively (Table 6). Significant differences in serostatus, for both IgG\textsubscript{1} (p=0.007) and IgG\textsubscript{3} (p=0.001), were observed, with more symptomatic (IgG\textsubscript{1}: 56%, IgG\textsubscript{3}: 50%) than asymptomatic individuals (IgG\textsubscript{1}: 19%, IgG\textsubscript{3}: 5%) becoming seropositive to anti-PvMSP\textsubscript{19} during infection (Table 6). Also, significant changes in log AU levels between the pre- and current-infection time points by clinical group were observed, with a significant increase in both IgG\textsubscript{1} and IgG\textsubscript{3} antibodies in symptomatic individuals ($\Delta_1=2.3$, p<0.001 vs. $\Delta_3=3.4$, p<0.001), but only for IgG\textsubscript{3} antibodies in asymptomatic subjects ($\Delta_3=1.1$, p=0.001) (Fig. 8).

**Association between pre-infection total IgG antibodies and risk of asymptomatic *P. vivax* malaria**

To investigate the relationship between the risk of asymptomatic and anti-PvMSP\textsubscript{19}-specific IgG antibodies, age, sex, time of residence in the area, and self-reported months since last malaria event, we first conducted a univariate logistic regression. Only total IgG antibody levels at the pre-infection time point were significantly associated with odds of being asymptomatic (Table 7). Pre-infection antibodies (defined in quartiles) displayed a u-shaped relationship with the outcome, peaking in the third quartile and subsequently decreasing in the fourth quartile. Intermediate total IgG antibody levels (tertile 2) increased the odds of being asymptomatic, but higher antibody levels (tertile 3) were a risk factor for symptomatic malaria. Individuals older than 5 years of age had higher odds of being asymptomatic during a *Pv* compared to younger children. No significant association between the odds
of asymptomatic $Pv$ malaria and sex, time of residence, or time since last reported malaria event was identified (data not shown).

Multivariate regression was used to model the effect of pre-infection total IgG antibodies on the risk of being asymptomatic, after adjusting for time since when the pre-antibodies were measured, antibody levels elicited during the current infection, and age. Pre-infection antibodies were associated with an increased risk of asymptomatic $Pv$ malaria, with the highest risk in the third tertile (Table 7). Individuals older than 5 years of age and time since pre-antibodies were measured were significantly associated with an increased risk of being asymptomatic.

3.5 Discussion

This study is the first to investigate the humoral response to PvMSP1$_{19}$ in a low endemic area in the Peruvian Amazon Basin and assess differences by symptoms. Findings presented in this study provide insight into $Pv$ immune responses to PvMSP1$_{19}$, a leading vaccine candidate antigen, in symptomatic and asymptomatic infected individuals both prior and during infection. The key findings are: (i) pre-infection anti-PvMSP1$_{19}$ IgG mean antibody levels were indistinguishable between symptomatic and asymptomatic individuals, (ii) pre-infection anti-PvMSP1$_{19}$ IgG antibodies were associated with past exposure and asymptomatic infection while infection anti-PvMSP1$_{19}$ IgG antibodies were associated with symptomatic infection, (iii) approximately a quarter of $Pv$-confirmed cases were anti-PvMSP1$_{19}$ IgG seronegative, while seropositive status during current infection was not significantly different between symptomatic and
asymptomatic individuals, and (iv) age greater than five years was associated with increased odds of having asymptomatic *Pv* malaria.

These findings did not confirm our initial hypothesis that asymptomatic subjects would have higher mean pre-infection antibody levels than symptomatic subjects, which would confer subsequent protection to symptomatic disease. We also failed to observe significant differences in seropositive status between those who were symptomatic and those who were asymptomatic. A study by Morais et al. carried out in the Brazilian Amazon observed that the proportion of anti-*PvMSP1*\textsubscript{19} IgG seropositive individuals was similar among non-infected individuals in the Brazilian Amazon with different malaria transmission intensities, emphasizing the high immunogenicity of the protein and its potential use as a malaria vaccine antigen (68). Interestingly, they did observe significant differences in mean antibody levels by transmission intensity, with apasitemic individuals living in a mesoendemic area in Apiacas having significant lower *PvMSP1*\textsubscript{19} antibodies compared to less frequently or sporadically-exposed subjects (p<0.0001). Perhaps our findings may be due to the fact that all participants resided in a hypoendemic area, where the number of lifetime malaria events is not sufficient to result in asymptomatic subjects having significantly more antibody levels. Although we did not find significant differences in mean pre-infection antibody levels between clinical groups, when we analyzed differences in antibody levels by categories (quartiles), we observed significant differences between asymptomatic and symptomatic subjects, with more asymptomatic subjects (67%) being in the mid-high antibody categories (quartile 3 and 4) compared to symptomatic subjects (41%).
Anti-PvMSP1\textsubscript{19} IgG antibodies were both a marker of exposure and risk of clinical infection. Pre-infection IgG antibodies were significantly associated with time since last reported malaria event, and was significantly higher in individuals who had their last malaria event within the previous six months, as well as increased with the number of lifetime malaria infections, indicative of the relationship between anti-PvMSP1\textsubscript{19} IgG antibodies and \textit{Pv} exposure. Symptomatic individuals displayed a significant boost in their total IgG antibody levels compared to asymptomatic individuals during \textit{Pv} infection, and was associated with both parasitemia and having experienced more than one lifetime \textit{Pv} event. In our multivariate model, after adjusting for age, higher pre-infection IgG antibodies (quartiles) were associated with increased risk of asymptomatic malaria, while increased antibodies elicited during infection (tertiles) were associated with reduced risk of asymptomatic malaria. A study in Papua New Guinea with \textit{Pf} infected children 1 to 4 years of age observed that antibodies elicited during active infection were associated with increased risk of disease, being a biomarker of malaria exposure in this population with low immunity (69). Stanisic \textit{et al.} also observed that \textit{Pf} anti-merozoite IgG antibodies were associated with protection from symptomatic malaria in older children 5 to 14 years of age (70), being a biomarker of protective immunity for older children with greater cumulative malaria exposure. Also, a study by Torres \textit{et al.} on \textit{Pf} MSP1\textsubscript{19} antibody responses conducted in the same study area as our study reported that subjects who were IgG-positive prior to their \textit{Pf} infection had reduced malaria-related symptoms, such as fever (38). These results suggest that anti-PvMSP1\textsubscript{19} IgG antibodies need to reach a certain threshold in order to be associated with clinical protection or naturally-acquired immunity and capable of mediating protection from
clinical malaria. Our findings allow us to better understand the immune response to this antigen in holoendemic areas as the Peruvian Amazon Basin, and guide future immune-epidemiology studies on the humoral response to better understand the development and features NAI to clinical disease in areas with low malaria transmission.

Even though the present study did not evaluate PvMSP1\textsubscript{19} IgG serostatus as a possible screening tool to identify \textit{Pv} infections, our results on seroconversion and seropositive status during a microscopy-confirmed \textit{Pv} mono-infection are informative to better understand the possible contribution that PvMSP1\textsubscript{19} might have on identifying \textit{Pv} cases, both symptomatic and asymptomatic. We observed that less than half of our confirmed \textit{Pv} cases seroconverted during infection. We did not observe significant differences between symptomatic or asymptomatic individuals, possibly due to the high immunogenicity of this antigen, as reported in other research studies (68, 71-73). Moreover, based only on PvMSP1\textsubscript{19} IgG seropositive status, we only detected 73% of all \textit{Pv}-confirmed cases. A study by Rodrigues \textit{et al.} evaluated PvMSP1\textsubscript{19} as a serological detection tool for malaria and concluded that the recombinant protein was a valuable serological assay to detect \textit{Pv} cases, with high sensitivity and specificity compared to microscopy (74). An important observation from the Rodrigues \textit{et al.} study is that they used a different recombinant proteins (amino acids 1616-1704 expressed in \textit{E. coli} or \textit{Pichia pastoris}, Belem strain) than the one used in the present study. A possible but unlikely explanation for our contrasting observations to the Rodrigues \textit{et al.} study could be that differences in the amino acid sequence in the recombinant protein could affect its folding and subsequent recognition by antibodies. Since over 70% of our study samples were seropositive to the recombinant PvMSP1\textsubscript{19} protein before and during infection, and
immunization studies that have used this protein have demonstrated partial protection in *Saimiri* monkeys by eliciting protective antibodies (59), suggests that the slightly different amino acid sequence does not affect the folding or immunogenic properties of the protein. Protein polymorphisms might also explain why some participants were not seropositive. The large PvMSP1 protein contains six highly polymorphic regions interspersed with seven conserved blocks, including a highly conserved C-terminal 19-kDa polypeptide that has the lowest nucleotide diversity of all blocks (75). To determine if protein polymorphisms in this population might explain our observations, we are planning to conduct gene sequencing.

This study demonstrated that pre-infection IgG antibodies were associated with a reduced risk of symptomatic malaria and that this association followed an inverse u-shape relationship. Our observation suggests that people with higher levels of pre-infection IgG antibodies can control a low-grade infection and remain asymptomatic. Our findings are similar to what Torres *et al.* observed in their *Pf* antibody response dynamics study that was carried out in the same hypoendemic community in the Peruvian Amazon Basin (38). However, we also found that age greater than 5 years was associated with an increased odds of asymptomatic disease presentation, contrary to what was observed by Torres *et al.* This observation might be related to previous reports of naturally-acquired immunity developing more rapidly for *Pv* than *Pf* malaria. Different immuno-epidemiological studies on *Pv* malaria conducted in areas of different transmission intensity such as Papua New Guinea (76-79), Brazil (80), Thailand (81, 82), and Sri Lanka (6) observed a difference in the rate of acquisition of naturally-acquired immunity, being faster for *Pv* than *Pf* malaria. Experimental infections involving neurosyphilis patients infected with
malaria parasites have also observed faster acquisition of clinical immunity in *Pv* than *Pf*
infected subjects (83, 84). All the aforementioned studies support the notion that
naturally-acquired immunity develops more quickly to *Pv* than *Pf*, both for homologous
and heterologous strains. Therefore, despite living in a hypoendemic area, older subjects
who are more likely to have had more prior exposure history might have developed
immunity to symptomatic malaria after only few infections. Furthermore, our observation
that asymptomatic subjects reported more malaria lifetime episodes and lower parasite
densities suggest that the observed clinical protective properties of pre-infection IgG
antibodies might reflect a greater prior exposure history and be a proxy of naturally-
acquired immunity to clinical malaria in a low endemic setting.

There are several limitations to this study, the most important being that we did
not assess *PvMSP1*<sub>19</sub> polymorphisms. Therefore, we cannot determine if seronegative
individuals either failed to make a humoral response to malaria or were seronegative
because they had antibodies against polymorphic epitopes not detected by the *PvMSP1*<sub>19</sub>
antigen used in this study. Nonetheless, the MSP1<sub>19</sub> antigen has been reported to be a
highly conserved protein (71, 85-87), which supports the generalizability and validity of
these results. *PvMSP1*<sub>19</sub> polymorphisms will be assessed in our study samples to confirm
these observations. Another limitation is that, because we cannot ascertain where in the
course of infection subjects were enrolled, this time point might not necessarily capture
an acute malaria response but could reflect a longer term immune response or the sample
was collected too early in the infection, resulting in the seronegative status. A prospective
study with close monitoring of subjects for the appearance of parasitemia would better
capture differences in the acute response between clinical groups after infection. Despite
not being able to accurately describe differences in clinical groups during acute infection, this study provides information on the humoral immune responses from a hypoendemic malaria setting and assesses how these antibodies relate to risk of clinical disease. Also, this study did not assess how pre-infection antibodies were associated with protection from subsequent infections but only their association with clinical presentation. A study by Crompton et al. (2010) in Mali observed that antibodies to MSP1, as well as other vaccine candidate antigens as AMA-1 and CSP, did not confer protection to subsequent infection with *P. falciparum* malaria (67). Future studies will look into assessing how pre-infection antibodies correlated with subsequent protection from *P. vivax* malaria, comparing antibody profiles between individuals who did not experience a malaria episode to those who did, both symptomatic and asymptomatic. Lastly, another limitation to this study is that our pre-infection antibodies were measured at different time points (range: 1 week – 8 months).

In conclusion, our findings highlight how both pre- and current-infection antibodies to the merozoite antigen PvMSP1_{19} are associated with naturally-acquired immunity to clinical malaria in a hypoendemic transmission area. It is important to be able to differentiate a protective antibody response from a response to ongoing exposures that are unlikely to provide clinical protection. Our data indicate that pre-infection IgG antibodies, which are correlated to time since last malaria infection and lifetime *Pv* events, reflect prior malaria exposure history of an individual, and are associated with increased odds of being asymptomatic during infection, making it a proxy for protection to clinical malaria, while current-infection antibodies reflect an increased risk to clinical (symptomatic) disease making it a proxy for *Pv* exposure in a hypoendemic setting. A
central aspect to consider in the selection of effective \( P_v \) vaccine targets, such as \( \text{PvMSP1}_{19} \), is the maintenance of the antibody response in areas with hypoendemic malaria. The majority of subjects in the highest pre-circulating antibody quartile reported experiencing their last malaria event within the last 6 months and that anti-\( \text{PvMSP1}_{19} \) IgG antibody levels decreased markedly after that timespan. Future studies will address this central question that will help guide current vaccine studies in the hopes of eliminating this debilitating disease.

### 3.6 References


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3.7 Tables and Figures

Table 1. Demographic and clinical characteristics of *P. vivax* infected subjects from Zungarococha, Loreto – Peru

<table>
<thead>
<tr>
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<th>Study population (n=177)</th>
<th>Subset (n=55)</th>
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<tbody>
<tr>
<td></td>
<td>Symptomatic</td>
<td>Asymptomatic</td>
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<tr>
<td>Total samples</td>
<td>119(67%)</td>
<td>58(33%)</td>
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<tr>
<td>Age, years</td>
<td>12(6 months)</td>
<td>17(9 months)</td>
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<tr>
<td>Male sex</td>
<td>72(60.5%)</td>
<td>26(44.8%)</td>
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<tr>
<td>Lifetime Plasmodial episodes, number</td>
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<tr>
<td><em>P. falciparum</em></td>
<td>2(1%)</td>
<td>5(1%)</td>
</tr>
<tr>
<td><em>P. vivax</em></td>
<td>2(1%)</td>
<td>4(1%)</td>
</tr>
<tr>
<td>Parasitemia, parasites/μL (**)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trophozoites</td>
<td>8.7(0.1)</td>
<td>4.2(0.2)</td>
</tr>
<tr>
<td>Gametocytes</td>
<td>4.7(0.2)</td>
<td>0.5(0.2)</td>
</tr>
<tr>
<td>Time to residence, years</td>
<td>5(3 years)</td>
<td>9(5 years)</td>
</tr>
<tr>
<td>Period of enrollment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Start of malaria attack season</td>
<td>51(42.9%)</td>
<td>28(48.3%)</td>
</tr>
<tr>
<td>End of malaria attack season</td>
<td>22(17.7%)</td>
<td>20(34.5%)</td>
</tr>
<tr>
<td>Dry season</td>
<td>35(29.4%)</td>
<td>10(17.2%)</td>
</tr>
<tr>
<td>Time since last malaria months</td>
<td>12(4 months)</td>
<td>20(10 months)</td>
</tr>
<tr>
<td>Anemia</td>
<td>44(36.9%)</td>
<td>19(32.8%)</td>
</tr>
<tr>
<td>Occupational risk level†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>11(9.2%)</td>
<td>5(8.6%)</td>
</tr>
<tr>
<td>Low</td>
<td>108(90.7%)</td>
<td>53(91.3%)</td>
</tr>
</tbody>
</table>

Data are median value (Interquartile range) or frequency (%). Subjects were compared between symptomatic and asymptomatic subjects by Mann-Whitney U test and Chi2 test, respectively. *P* value ≤ 0.05 was considered significant. (†) Reference categories: Agriculture, Businessman, Factory, Fisherman, Low-risk occupation, Student, Housemaid, Child, Unemployed, Retired, Taxi driver, Construction worker, Security guard, Craftsman, Poultry farmer, Trader, Ranger, Soldier, Independent.
Table 2. Differences in pre-infection anti-PvMSP1<sub>19</sub> antibody levels

<table>
<thead>
<tr>
<th>Antibody (Log&lt;sub&gt;AU&lt;/sub&gt;)</th>
<th>Symptomatic (n=34)</th>
<th>Asymptomatic (n=21)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>7.1±2.1</td>
<td>7.2±1.5</td>
<td>0.8</td>
</tr>
<tr>
<td>IgG1</td>
<td>8.2±0.9</td>
<td>8.0±0.6</td>
<td>0.3</td>
</tr>
<tr>
<td>IgG3</td>
<td>4.0±1.4</td>
<td>4.2±1.3</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Data are mean value±SD of immunoglobulin IgG and subtypes IgG1 and IgG3, (*) significant differences p<0.05, (†) Log<sub>AU</sub>: Log<sub>ELISA</sub> Arbitrary Units.
Table 3. Relations of demographic and epidemiological variables on pre-circulating anti-PvMSP1<sub>19</sub> total IgG antibodies

<table>
<thead>
<tr>
<th></th>
<th>Bivariate</th>
<th></th>
<th>Multivariate</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>coef. [95% C.I.]</td>
<td>P-value*</td>
<td>coef. [95% C.I.]</td>
<td>P-value*</td>
</tr>
<tr>
<td>Asymptomatics</td>
<td>0.13 [-0.90, 1.18]</td>
<td>0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lifetime Pv events, number</td>
<td>0.19 [0.02, 0.36]</td>
<td>0.03</td>
<td>0.15 [-0.002, 0.30]</td>
<td>0.05</td>
</tr>
<tr>
<td>Sex, female</td>
<td>0.16 [-0.85, 1.18]</td>
<td>0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>0.72 [-0.41, 1.84]</td>
<td>0.2</td>
<td>0.03 [0.005, 0.06]</td>
<td>0.02</td>
</tr>
<tr>
<td>Months since last malaria event</td>
<td>Ref.</td>
<td></td>
<td>Ref.</td>
<td></td>
</tr>
<tr>
<td>Months 11</td>
<td>-2.12 [-3.85, -0.38]</td>
<td>0.02</td>
<td>-2.01 [-3.61, -0.41]</td>
<td>0.01</td>
</tr>
<tr>
<td>Months 12</td>
<td>-2.61 [-3.72, -1.49]</td>
<td>&lt;0.001</td>
<td>-2.51 [-3.53, -1.48]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time of residence, years</td>
<td>-0.01 [-0.07, 0.04]</td>
<td>0.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total IgG antibody response was measured as Log ELISA Arbitrary Units (Log AU). Coef: regression coefficient, (*) statistical significance < 0.05.
Table 4. Anti-PvMSP19 antibody levels in symptomatic vs. asymptomatic subjects during *P. vivax* infection

<table>
<thead>
<tr>
<th>Antibody (LogAU†)</th>
<th>Symptomatic (n=119)</th>
<th>Asymptomatic (n=58)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM</td>
<td>9.4±1.9</td>
<td>7.8±1.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IgG</td>
<td>11.1±2.7</td>
<td>7.9±1.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IgG1</td>
<td>10.4±2.7</td>
<td>7.1±1.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IgG3</td>
<td>7.1±1.5</td>
<td>5.9±1.7</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are mean value±SD of immunoglobulin IgM, IgG, and subtypes IgG1 and IgG3, (*) significant differences, p<0.05, (†) LogAU: Log ELISA Arbitrary Units.
Table 5. Relationships between demographic and epidemiological variables and anti-PvMSP1<sub>19</sub> total IgG antibodies elicited during patent infection

<table>
<thead>
<tr>
<th></th>
<th>Bivariate</th>
<th></th>
<th>Multivariate</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>coef. [95% C.I.]</td>
<td>P-value*</td>
<td>coef. [95% C.I.]</td>
<td>P-value*</td>
</tr>
<tr>
<td>Asymptomatics</td>
<td>-3.24 [-4.01, -2.48]</td>
<td>&lt;0.001</td>
<td>-0.82 [-2.24, 0.61]</td>
<td>0.2</td>
</tr>
<tr>
<td>Parasitemia, log count</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>0.66 [0.52, 0.81]</td>
<td>&lt;0.001</td>
<td>0.35 [0.03, 0.66]</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Gametocytes</td>
<td>0.50 [7.81, 0.62]</td>
<td>&lt;0.001</td>
<td>0.21 [0.03, 0.39]</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Lifetime Pv event, No. event</td>
<td>2.04 [1.01, 3.07]</td>
<td>&lt;0.001</td>
<td>2.56 [1.67, 3.44]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sex, female</td>
<td>0.17 [-0.68, 1.02]</td>
<td>0.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>-0.02 [-0.04, 0.01]</td>
<td>0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Months since last malaria event</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>...</td>
<td>Ref.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1.99 [0.33, 3.66]</td>
<td>0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>-0.13 [-1.28, 1.03]</td>
<td>0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time since residence, years</td>
<td>-0.03 [0.08, 0.02]</td>
<td>0.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total IgG Antibody Response was measured as Log ELISA Arbitrary Units (Log AU). (*) Statistical significance < 0.05, coef: regression coefficient.
Table 6. Changes in pre- and patent-infection anti-PvMSP1<sub>19</sub> IgG serostatus

<table>
<thead>
<tr>
<th></th>
<th>Total IgG</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SYM</td>
<td>ASYM</td>
<td>Total</td>
</tr>
<tr>
<td>Seroneg.</td>
<td>6</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>Seroneg.</td>
<td>16</td>
<td>7</td>
<td>23</td>
</tr>
<tr>
<td>Seropos.</td>
<td>12</td>
<td>5</td>
<td>17</td>
</tr>
<tr>
<td>Seropos.</td>
<td>0</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

Data are number(%) of subjects. IgG: Immunoglobulin G, IgG1: IgG subtype 1, IgG3: IgG subtype 3, SYM: Symptomatic, ASYM: asymptomatic, Seroneg.: Seronegative, Seropos.: Seropositive.
Table 7. Bivariate and multivariate analysis of the odds of being symptomatic vs. asymptomatic during confirmed *P. vivax* infection

<table>
<thead>
<tr>
<th></th>
<th>Bivariate</th>
<th>Multivariate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR [95% C.I.]</td>
<td>P-value*</td>
</tr>
<tr>
<td><strong>Pre-IgG, categories</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quartile 1</td>
<td>1.00 Ref.</td>
<td></td>
</tr>
<tr>
<td>Quartile 2</td>
<td>3.06 [0.47,19.66]</td>
<td>0.2</td>
</tr>
<tr>
<td>Quartile 3</td>
<td>13.80 [2.05,92.04]</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Quartile 4</td>
<td>2.20 [0.33,14.73]</td>
<td>0.4</td>
</tr>
<tr>
<td><strong>Patent-IgG, categories</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-tertile 1</td>
<td>1.00 Ref.</td>
<td></td>
</tr>
<tr>
<td>P-tertile 2</td>
<td>2.14 [0.44,10.53]</td>
<td>0.3</td>
</tr>
<tr>
<td>P-tertile 3</td>
<td>0.34 [0.07,1.68]</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>Age group</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-14 years</td>
<td>1.00 Ref.</td>
<td></td>
</tr>
<tr>
<td>Age 5-14 years</td>
<td>3.27 [0.79,13.41]</td>
<td>0.1</td>
</tr>
</tbody>
</table>

OR: Odds Ratio, AOR: Adjusted Odds Ratio, Pre-IgG: Pre-infection IgG, Patent-IgG: IgG Antibodies measured during patent infection, *P*-statistical significance threshold: 0.05. Multivariate model was adjusted for time (months) since pre-IgG antibodies were measured.
“Malaria cases in Peru: 1939-2010, Cases of Malaria due to *Plasmodium falciparum* and *P. vivax* were not differentiated and reported systematically until 1990. Source: Peruvian Ministry of Health, Lima, Peru.”

Figure 2. Zungarococha community and main villages

Villages: ZG = Zungarococha, PA = Puerto Almendra, NR = Ninarumi, LC = Llanchama

Figure 3. Flowchart of the MIGIA study and repository sample selection

Parent study MIGIA enrollment and sampling scheme (2003 – 2011) and repository sample selection for the pre- and patent-infection immune-epidemiology study.
Selection of houses of the MIGIA cohort study for weekly active case detection within ‘Pf at-risk’ zones. Weekly sampling was focused in zones with defined Pf infection risk. Based upon the detection (passive or active) of a Pf case the previous month (solid red circles), a 100-meter radius was defined (open blue circle) to detect malaria-infected individuals in selected households (open black circles).

**Source:** Branch O, R01 AI064831-01
Figure 5. Relationship between pre-infection antibodies, time since last malaria event and lifetime malaria events

Log AU, Log ELISA Arbitrary Units; IgG, immunoglobulin G; \( P < 0.05 \) for significant correlations or differences of variables. (A) Correlation between pre-infection antibodies and self-reported months since last malaria event. (B) Relationship between pre-infection antibodies and categories of self-reported months since last malaria event. Medians (central horizontal lines) and interquartile ranges (boxes) are shown. (C) Correlation between pre-infection antibodies and self-reported lifetime malaria events.
Figure 6. Mean anti-PvMSP1_{19} antibody levels by clinical group

ELISA-determined plasma antibody levels (Log AU: Log ELISA Arbitrary Units) in symptomatic (dark grey) and asymptomatic (light grey) individuals, showing means and standard deviations. Antibody levels were significantly higher in the symptomatic than asymptomatic individuals (*, \( P < 0.05 \)). IgM: immunoglobulin M, IgG: immunoglobulin G, IgG_1: IgG subtype 1, IgG_3: IgG subtype 3.
Figure 7. Changes in antibody levels between pre-infection and the infection period

Individual ELISA-determined plasma IgG antibody levels to *P. vivax* MSP1<sub>19</sub> (log AU: log ELISA Arbitrary Units) during pre-infection and infection time points.
Figure 8. Anti-PvMSP119 mean antibody response during a pre- and confirmed \textit{P. vivax} infection time points

ELISA-determined plasma antibody levels (Log AU: Log ELISA Arbitrary Units) in symptomatic (dark grey) and asymptomatic (light grey) individuals, showing means and standard deviations. Differences in (A) total immunoglobulin G, IgG, (B) IgG subtype 1, and (C) IgG subtype 3, within and between study time points. (*) Significant differences $P < 0.05$ or (**) $P < 0.001$ specified in figure.
IV. Chapter Four: Paper III - Maintenance of anti-MSP119 antibodies in Pv mono-infected subjects from a hypoendemic area in the Peruvian Amazon Basin

4.1 Abstract

Background: Antibodies are essential for the effective control of malaria parasites and a critical component of naturally-acquired immunity, providing partial protection from clinical malaria. Some studies have reported rapid decline and impaired maintenance of antibody responses, suggesting impaired immunity to malaria parasites; however, other studies conducted in low-endemic settings have observed long-lasting antibody responses to the 19-kDa region of merozoite surface protein 1 (MSP119). Antibody response dynamics to *P. vivax* (*Pv*) MSP119 (*PvMSP119*) in low transmission settings, such as Brazil and Korea, reported long-lasting antibody responses from 8 months to several years post-infection. Similarly, studies of *P. falciparum* (*Pf*) MSP119 (*PfMSP119*) in a low endemic area in the Peruvian Amazon Basin described antibody persistence for more than 5 months, yet there are no studies on the maintenance of *PvMSP119* antibodies in this area.

Methods: To investigate the maintenance of antibodies against *PvMSP119* in a hypoendemic setting in the Peruvian Amazon Basin, and factors associated with sustained antibody levels, we conducted an immuno-epidemiology study with 31 plasma samples collected in Zungarococha, a low-transmission community in Loreto, from *Pv* mono-infected subjects during patent infection and 8 to 21 months post-infection.
Antibody levels to PvMSP1\textsubscript{19} were measured at both time points by an enzyme immunoassay. Changes in antibody levels and serostatus over time were assessed, the rate of antibody decline and median half-life were estimated, and variables associated with sustained antibody levels were evaluated.

**Results:** Only 35.5% of study participants were seropositive for 8 up to 15 months (median = 13.4 months) post-infection, representing a 2.3-fold decrease in the frequency of seropositive subjects from the time of patent infection. Among seropositive subjects, 43% maintained their anti-PvMSP1\textsubscript{19} seropositive status for 8-15 months. Antibody decline and maintenance of seropositive status were associated with age, with older subjects having a smaller absolute decrease in antibody levels and higher odds of remaining seropositive. Overall, the median antibody decay constant and median half-life from among participants with detectable antibody for 8 up to 15 months were -0.03 log AU/month (IQR -0.02, -0.04) and 27.5 months (IQR 19.5, 39.3) (>2 years), respectively.

**Conclusions:** Despite low transmission, anti-PvMSP1\textsubscript{19} IgG antibodies persisted for a median of 13.4 months after infection and treatment, with a median half-life of 27.5 months post-infection. Our observations support the hypothesis that naturally-acquired immunity to \textit{Pv} malaria develops in low transmission regions and maintained despite infrequent infections. can maintain naturally-acquired immunity for 8-15 months. These observations suggest that anti-PvMSP1\textsubscript{19} antibodies can over-season, possibly rendering protection to clinical malaria upon re-infection, which could perhaps explain the considerable proportion of asymptomatic people in this area despite low prevalence.
4.2 Background

*Plasmodium vivax* (*Pv*) is the most widely distributed human malaria species in the world, and the most prevalent malaria species in the Americas, accounting for approximately 70-80 million global cases per year (1). In various countries, *Pv* is the principal cause of all malaria cases. In Peru, *Pv* was responsible for approximately 84% of the 65,294 malaria cases reported in 2014 (2). Different control strategies are currently being implemented in endemic settings, such as insecticide treated nets (ITNs) and indoor residual spraying (IRS), but overall vaccines are considered the most cost-effective control measure for infectious diseases. The development of an effective *Pv* malaria vaccine has proven challenging due to complexities of the parasite, such as the inability to sustain long-term culture in vitro (3-5), lack of correlation between naturally-acquired immune responses and clinical immunity (6), as well as limited understanding of the longevity of the immune response that mediate this protection, such as antibodies.

Antibodies are essential for the effective control of malaria parasites in blood and a crucial component of the naturally-acquired immune response, capable of inhibiting red blood cell (RBCs) invasion by merozoites, blocking cytoadherence of infected RBCs (iRBCs), as well as increasing the phagocytic activity of monocytes and macrophages (7, 8). Different factors may influence the dynamics of antibody responses, such as the pathogen (e.g. antigenic variation, relapse) and intensity of exposure. Within the host, different parameters of the immune response participate in antibody maintenance over time, including the persistence of antigens on follicular dendritic cells, the generation of long-lived plasma cells (LLPC), apoptosis of plasma cells, and T cells that facilitate the survival of antigen-specific B cells (e.g., plasma cells, memory B cells) (9).
Because immuno-epidemiological studies on *Plasmodium falciparum* (*Pf*) conducted in high transmission regions observed that antibody responses to malaria parasites were short-lived (10-12), it was believed that several years of parasite exposure were required to generate acquired immunity to clinical malaria. The rapid decline in antibody levels did not reflect classical antibody response dynamics (13-15), suggesting that B cell memory to *Plasmodium* may be defective or suboptimal, resulting either from cell death shortly after infection, or memory B cell anergy or exhaustion (16).

The acquisition of naturally-acquired immunity, including the maintenance long-lasting antibodies, has been reported to be different between *Pf* and *Pv* malaria, where the latter is acquired more rapidly after fewer encounters with the parasite (17-19). *Pv* has unique biological features that may contribute to the development of naturally-acquired immunity and the maintenance of antibodies over time, such as the hypnozoites stage that can initiate blood-stage infections several months to years after initial infection (20), thus boosting the immune response during periods of low or absent malaria transmission and conferring a state of premunition through continued exposure to low density parasitemia. However, studies have described the development of naturally-acquired immunity to *Pv* parasites without repeated parasite exposure, such as for malaria therapy for neurosyphilis (21-23) as well as studies conducted in low transmission areas such as the Solomon Islands (24) and the Amazon Basin (25-27). These studies suggest that naturally-acquired immunity to *Pv* parasites may develop with less exposure than previously thought.

Several studies demonstrated long-lived antibody responses to both *Pv* and *Pf* parasites (6, 28-31), as well as antibody maintenance in low transmission areas, mostly to
Pf (27, 32) rather than Pv (33, 34). Therefore, we hypothesized that the antibody responses to PvMSP1-19, an important Pv vaccine candidate and the most intensively studied antigen as a potential target of protective immunity, is maintained in a low-endemic area in the Peruvian Amazon Basin similar to what has been reported in previous Pv studies and longer than what has been reported in previous Pf studies conducted in the area (27, 32). We measured changes in antibody levels elicited during patent Pv infection and antibody persistence for at least 8 months post-infection in mono-infected subjects.

4.3 Methods

Study area and study design: Malaria has historically been present in Peru, particularly in the region of Loreto in the Amazon Basin (35). In the early 1990s, malaria resurfaced in the country (Fig. 2) with major outbreaks in the periphery of Iquitos city, capital of Loreto, due to flooding caused by the El Niño Southern Oscillation (ENSO) that resulted in increased vector populations, oil and mining explorations that resulted in rural expansion, narco-terrorism activities that resulted in population movement of naïve hosts to malaria endemic areas, and deforestation (36, 37). The National Malaria Program and the Loreto Public Health Department used different control strategies (e.g. disruption of larval sites, fogging, IRS, ITNs) to address this major outbreak. Through intense vector control programs and changes in antimalarial drug policy, with the adoption of artemisinin-combination therapy (ACT) to treat uncomplicated Pf malaria by 2001, malaria cases declined since the beginning of the 21st century, with a drastic reduction in
the number of cases by 2005 (Fig. 3) until 2012, when heavy rains and floods resulted in another malaria outbreak in the area.

A retrospective study was carried out with samples collected from Zungarococha, an endemic community located in the northwestern region of the Peruvian Amazon Basin approximately five kilometers from Iquitos city. Repository samples from the Malaria Immunology and Genetics in the Amazon (MIGIA) cohort study collected by active and passive surveillance from 2006 to 2011 were used. The MIGIA parent study collected samples between 2004-2011 from residents of Zungarococha, Loreto (located close to Iquitos city) through community-wide surveys at the beginning (January – March) and end (July – August) of the malaria season, weekly home-based follow-up visits during the malaria season (January – July), and through passive surveillance at the local health post throughout the year.

The Zungarococha community is composed of four villages (Fig. 1), with approximately 2000 inhabitants, connected by a dirt road (~2 km distance between villages). Houses are made of wood with thatch roofs and no screens and only two villages have electricity. The population is sustained mostly by local agriculture and employment and has a homogenous income status. The community health post is located in Zungarococha village, the most developed village of the community. Malaria transmission in Zungarococha is seasonal with a prominent peak during the rainy season from January to June, with \( P_v \) the predominant species. In 2003, the MIGIA cohort study estimated a force of infection (FOI) of 1.8 \( P_v \) infections/person/year and 0.46 \( P_f \) infections/person/year with more than 60% of all malaria cases being asymptomatic,
detectable only by PCR, and \( P_v \) and \( P_f \) prevalence of 0.39 and 0.13 respectively by reactive case detection (26).

**Study Population:** The study population was selected from MIGIA participants enrolled through passive surveillance. We selected plasma samples from all symptomatic participants who were \( P_v \) slide-positive at enrollment, received standard antimalarial treatment with chloroquine and primaquine, who were slide-negative at least eight months after the documented infection, and with no other documented \( P_v \) or \( P_f \) infection (through passive or active surveillance) or documented fever during that time period, as determined from the MIGIA database records. We excluded pregnant women and participants with mixed infections, given that an altered immune system and \( P_f \) co-infection could affect interpretation of our findings, as well as individuals who had a documented fever between both time points, since it could be indicative of an infection. The MIGIA database records also allowed us to estimate the time since the last documented \( P_v \) patent infection. The MIGIA study asked study participants about their number of lifetime malaria infections (e.g. *Plasmodium spp.*, \( P_v \), and \( P_f \)). In this low-prevalence area the majority of infections were due to \( P_v \), thus self-reported lifetime *Plasmodium spp.* and \( P_v \) malaria were highly correlated (\( R = 0.97, p<0.0001 \)). We used \( P_v \) lifetime malaria events for our data analysis.

We analyzed two plasma samples from each study participant, the first one from the \( P_v \) patent infection time point (patent) and the second one collected at least 8 months after patent infection (post-patent). This time period was chosen because it is equal to what was observed in previous \( P_v \)MSP1\(_{19} \) studies carried out in Brazil and longer than what was reported in \( P_f \)MSP1\(_{19} \) studies conducted in our study area (5 months). We also
analyzed IgG subclass 1 (IgG1) and subclass 3 (IgG3) antibodies because they are the most cytophilic (with the highest affinity to the Fc receptor) and have therefore been associated with reduced parasite density, pathogen clearance and clinical protection (38-44). The selected patent and post-patent samples allowed us to assess: the i) change in antibody levels; ii) serostatus between patent infection at least eight months later; iii) variables associated with the antibody decline; iv) differences between serogroups (e.g. seropositives vs. seronegatives); v) differences between seropositive subjects who remained seropositive and those who seroreverted; vi) variables associated with an increased odds of remaining seropositive several months after a Pv mono-infection; and vii) the antibody decay constant and half-life.

**Parent study and data collection:** The parent cohort study (MIGIA) collected whole blood from infants, children, adults, and pregnant women for malaria diagnosis using three sampling strategies: i) passive case surveillance with a full-time clinical team stationed at the Zungarochocha health post throughout the year; ii) active case detection through community-wide surveys at the start (January-March) and end (July – August) of the malaria season; and iii) reactive case surveillance with four weekly household visits throughout the malaria season (February – August) (26) (Fig. 3). The weekly household visits took place within a 100-meter radius from a “sentinel” house. Sentinel houses were defined as houses that had at least one Pf case (index case) that had either visited the health post in the previous month for malaria diagnosis (passive detection) or were detected through the community-wide survey or weekly household visits in the previous month (active detection). Upon the identification of the Pf index case, a 100-meter radius around that index case’s house was delimited and defined as the “Pf at-risk area” (Fig. 3).
If at-risk-areas overlapped in a given month, the parent study assigned the randomly selected house to one of the areas. Generally, there were 20-50 houses within a given 100-meter radius area. Within the “Pf at-risk area”, houses were randomly selected, using a random number generator, to conduct weekly visits in order to capture the onset and dynamics of malaria infection, both Pf and Pv, in individuals living in close proximity to the Pf index case. The parent study team visited all randomly selected houses within each at-risk-area and recruited all household members until at least 30 individuals (~5–9 houses) were enrolled. All consenting participants, including the sentinel household with the index case, were included in the weekly visits. Overall, there were five to six at-risk-areas per month of active surveillance. Each participant was visited once per week for four weeks, except when there was a positive blood slide, which resulted in additional visits. All slide-positive individuals, regardless of clinical presentation, were directed to the local health post to receive antimalarial treatment from the MINSA. Finally, all referred cases, as well as individual who independently attended the health post for malaria diagnosis, were asked to participate in the passive surveillance arm of the MIGIA study.

During each visit, whether in the health post, community-wide survey, or weekly home-visits, clinical (e.g. temperature, malaria-related symptoms, hematocrit), epidemiological (e.g. self-reported number of past malaria infections, years lived in the area), and demographic information (e.g. age and sex) were recorded by the study team. The study physician measured axillary temperature using a digital thermometer, and collected 0.25 to 3 ml of blood by finger prick or venipuncture. Additionally, participants diagnosed with malaria had 3-6 ml of blood collected by venipuncture. Blood samples
were used to prepare thin and thick blood smears, as well as capillary hematocrit tubes. All remaining blood was stored EDTA-containing vacutainers for further processing and storage. In the active case detection visits, blood slides, hematocrit tubes and vacutainers were transported to the laboratory for microscopy and processing. Vacutainers were centrifuged to separate plasma from packed blood cells, and frozen within 18 hours at -85°C. Capillary hematocrit tubes were centrifuged and the volume percentage of red blood cells (packed cell volume, PCV) was measured. Malaria infections were documented by microscopic analysis of Giemsa-stained thick and thin blood smears, following standard procedures. Two expert microscopists with over 15 years of experience counted parasites, reading 200 microscopy fields. Both *Pv* and *Pf* trophozoites and gametocytes were counted separately. At least 500 white blood cells (WBCs) were counted before an individual was diagnosed as negative by microscopy. Species-specific parasite density (parasite/μL) was determined by the number of parasites divided by the total number of WBCs counted and multiplied by 6,000. The conversion factor of 6,000 RBCs per one WBC to determine parasite density per microliter is regularly used in the area (45).

**Measurement of *Pv*MSP119 specific antibody responses by ELISA:** Plasma antibody levels were measured by an indirect enzyme-linked immunosorbent assay (ELISA). The 19 kDa fragment of MSP119 was obtained from the Malaria Research and Reference Reagent Resource Center (MR4, Virginia - USA) representing amino acids Leu1639 trough Ser1738 (Sal-I strain) with a of *Pv*, expressed in *S. cerevisiae* (MSP119-His6) containing two epidermal growth factor-like domains. Nunc Maxisorp (Sigma-Aldrich, Missouri - USA) flat-bottom 96-well plates were coated with recombinant
protein (0.5 μg/ml for IgG, 1 μg/mL for IgM) in phosphate-buffered saline (PBS) buffer (pH 7.4) overnight at room temperature (RT). After washing six times with PBS-Tween (0.05%) (PBS-T), plates were blocked with 200 uL PBS-milk (5% non-fat milk powder) at RT for one hour. Plasma samples were diluted in this same solution, and 50 uL of each sample was incubated in duplicate at RT for two hours. After washing with PBS-T, plates were incubated with diluted (1:10,000) goat anti-human horseradish peroxidase conjugated IgG antibody (Jackson ImmunoResearch Laboratories Inc., Pennsylvania - USA) at RT for one hour. Plates were washed again with PBS-T, developed with o-phenylenediamine substrate (OPD; 10 mg tablet Sigma Aldrich Product No. P8287) at RT for one hour, and stopped with 3N hydrochloric acid (HCl). The absorbance (optical density, OD) was read at 490 nm with the use of a microplate reader (Biotek ELx800 Spectrophotometer).

To detect subclasses of human IgG among anti-PvMSP1_{19} antibodies, an ELISA was performed as described above using isotype-specific mouse anti-human IgG antibodies at dilutions of 1:4000 (IgG_{1}) and 1:2000 (IgG_{3}) incubated at RT for one hour, and a tertiary anti-mouse antibody (Jackson ImmunoResearch Laboratories Inc., Pennsylvania - USA). The enzyme reaction was developed with OPD substrate and stopped with 3N HCl. A standard calibration curve made from serial dilutions of a standard reference pool of highly reactive P_{v} positive (individuals diagnosed by microscopy and PCR with at least 10 lifetime malaria infections) plasma samples (n=10) collected in the Peruvian Amazon Basin from Iquitos (n=5) and Madre de Dios (n=5) was used to estimate antibody levels in ELISA arbitrary units (AU). To estimate individual plasma antibody levels, the reciprocal of the plasma dilutions of the reference pool were
used to generate a nonlinear sigmoidal dose-response curve. Test plasma OD values were interpolated to the curve and converted into AU. One ELISA arbitrary unit (1 AU) is equivalent to the reciprocal plasma dilution of the reference pool that gave the lowest OD. This method allowed estimation of the antibody levels based on a reference value, even if the value of the reference is unknown in conventional units (e.g. there is no reference standard for antibody concentration), and compare multiple measurements within and between ELISA plates (46-48). For internal positive and negative controls, we used a plasma sample from a highly reactive P. malaria-positive adult from Iquitos (determined by microscopy and PCR) and a pool of plasma samples from non-exposed non-endemic malaria-naive adults from Lima Peru (n=25). The ELISA seropositive cut-offs were obtained from the average of a pool of non-exposed non-endemic malaria-naive adults from Lima Peru (n=25) plus three standard deviations (SD). The estimated cut-offs to determine seropositivity were 1588 AU for total IgG, 3686 AU for IgG$_1$, and 774 AU for IgG$_3$.

**Statistical Analysis:** Data were tested for normality using the Shapiro-Wilk test and log-transformed (natural logarithm) for analysis. The difference in the mean log antibody levels between patent infection and the post-patent period was evaluated by a paired Student’s t-test. Differences in the means between groups were evaluated by an unpaired Student’s t-test. Differences in medians were evaluated using Kruskal-Wallis test. Correlations were identified by the Spearman rank test. Differences in proportions were evaluated by a chi-square test. P values <0.05 were considered statistically significant. To estimate the antibody decay constant and half-life, we considered a single exponential decay model for the antibody decline phase (see formula I) as done in other
antibody decay studies (6, 49). We estimated the antibody half-life (formula II) and decay constant (formula III) on samples that exhibited antibody decline between the post-patent time points.

\[
I) \quad \frac{dA}{dt} = -\lambda A \quad \rightarrow \quad A = A_0 \cdot e^{-\lambda t}
\]

\[
II) \quad A = A_0 \cdot \left(\frac{1}{2}\right)^{t/1/2} \quad \rightarrow \quad t_{1/2} = t \cdot \frac{\ln(1/2)}{\ln(A/A_0)}
\]

\[
III) \quad \lambda = \frac{\ln(2)}{t_{1/2}}
\]

A generalized linear model (GLM) linear regression was used to estimate the effect of other variables on the change in anti-PvMSP199 antibody response over time. GLM logistic regression was used to study the association of other variables with the odds of remaining seropositive over time. Model selection was determined stepwise by AIC and p-value <0.05 criteria. All analyses were undertaken using Stata (Version 13, Statacorp LP) and figures were developed with GraphPad Prism (Version 6.0, GraphPad Software, Inc.).

**Research Ethics:** This study was approved by the U.S. Naval Medical Research Unit No. 6 (NAMRU-6) Institutional Review Board (IRB approval code: NAMRU6.2013.0009). Ethical approval of the parent study was obtained from the Instituto Nacional de Salud (Human use approved protocol: 08-982), the University of Alabama at Birmingham, the Universidad Peruana Cayetano Heredia, and the New York
University School of Medicine Institutional Review Boards. Prior to enrollment, written informed consent was obtained from all study participants eighteen years of age or older. For participants between seven and eighteen years of age, assent from the minor and consent from the parents or guardians were obtained. The Johns Hopkins Bloomberg School of Public Health granted an IRB waiver given only de-identified data and samples were analyzed. All study participants provided written informed consent for future use of their samples.

4.4 Results

Study Population

To study the maintenance of the humoral response to PvMSP1\textsubscript{19}, we analyzed repository samples from 31 \textit{Pv} mono-infected subjects from the MIGIA cohort study collected during patent uncomplicated infection, as well as several months post-infection (median time: 12.2, range: 8.1 – 21.6 months) with no observed subsequent patent infection (Fig. 4). Most participants (61.3\%) were female (Table 1). The group’s median age was 15.2 years (IQR 8.7 – 28.6 years), had a median self-reported years living in the area of 7.8 years (IQR 2.4 – 15.7 years), had a median 7.7 months (IQR 0 – 12.9 months) since their last patent malaria infection, reported a median of 5 (IQR 2 – 11) lifetime malaria events and a median of 4 (IQR 2 – 10) lifetime \textit{Pv} events, and the majority (84\%) had self-reported experiencing at least one lifetime malaria event. Although not statistically significant, when age was categorized in tertiles, we observed that the median number of self-reported \textit{Pv} lifetime events increased by age group, being greatest in the highest tertile (ages $\geq$ 22.8 years, median 6 events) compared to the first (ages 2.9 – 9.5
years, median 3.5 events) and second tertiles (ages 12.5 – 18 years, median 3.5 events) (p=0.5). During the documented patent infection, the mean naturally log-transformed parasitemia was 7.0 ± 1.9 trophozoites/μL and 5.1 ± 1.3 gametocytes/μL, and over half the individuals (53%) were anemic. During the post-patent time point, all subjects had not detectable parasites by microscopy (trophozoites and gametocytes) and 14% of all study participants were anemic.

The mean IgG antibody level (log AU) during patent infection was 10.1 ± 3 AU, and the majority (74%) of subjects were seropositive. A moderate but significant correlation was observed between anti-PvMSP119 IgG antibody levels at the time of patent infection with trophozoite counts (Spearman R=0.51 p = 0.003) and gametocyte counts (Spearman R=0.54 p = 0.002). However, no significant correlation was observed between post-patent antibody IgG levels and parasitemia counts at the time of patent infection (trophozoites: Spearman R = -0.1, p = 0.4; gametocytes: Spearmen R = 0.2 p = 0.4), but only a positive significant correlation between post-infection total IgG antibody levels and age (Spearman R=0.7, p<0.001), suggesting that older individuals have greater post-patent IgG antibodies.

**Antibody decline was significantly associated with age and antibody levels elicited during patent infection**

We detected total IgG antibody titers lasting as long as we measured (21.6 months post infection). During the patent-infection and post-patent time points, a significant absolute change was observed in mean IgG levels from 10.1 ± 3 log AU during patent infection to 7.0 ± 1.3 log AU during the post-patent period (p<0.0001), corresponding to
a -3.1 ± 2.9 log AU absolute decline in antibody levels (Table 1 and Figure 5). The estimated median decay constant of antibodies was -0.03 log AU/month (IQR -0.02, -0.05) and the estimated median half-life was 21.3 months (IQR 13.6, 33.9 months), indicative that total IgG antibodies can over-season and persisting for almost two years.

Because antibody production can be influenced by host and parasite factors, we assessed correlations between the absolute decline in total IgG antibodies and other variables, such as age, level of parasitemia, self-reported number of lifetime Pf infections, time since patent infection, and time since last reported malaria infection. A moderate but significant negative correlation was observed between the absolute IgG decline and both trophozoite (Spearman R=-0.6, p<0.001) and gametocyte (Spearman R=-0.6, p=0.001) counts during patent infection, suggesting that individuals with greater parasite counts/μL have greater absolute IgG decline. No significant correlations were observed between absolute antibody decline and age, number of lifetime Pf infections, time since patent infection, or time since last reported malaria infection.

To understand how other variables were associated with the change in antibody levels (log AU) months after patent Pf mono-infection, GLM bivariate and multivariate regressions were used to study the absolute change in total IgG antibody levels, adjusting for other covariates such as age, trophozoite and gametocyte (log counts/μl), and antibody levels elicited during patent infection. Bivariate analysis showed that people with more parasites (trophozoites and gametocytes) and with increasing IgG antibody levels elicited during patent infection had a greater absolute decrease in antibody levels at follow-up (Table 2). A trend in absolute IgG antibody change over time and self-reported lifetime Pf event categories was observed, with the decline of antibodies being less
pronounced as the number of events increase. In a multivariate model, age was significantly associated with the absolute decline of IgG antibodies after adjusting for other covariates, with increasing age associated with a smaller decline in IgG antibodies over time (i.e. a smaller difference in IgG differences between patent and post-patent time points). IgG levels elicited during patent infection remained significantly associated with IgG absolute decline even after adjusting for other covariates. Increasing patent IgG antibodies were associated with a steeper decline in IgG antibodies over time, as expected. However, the initial significant association between parasitemia and IgG absolute decline was lost after adjusting for other covariates.

With respect to IgG$_1$ and IgG$_3$ antibodies, we also observed antibody titers as long as we measured (21.6 months post-infection). During the patent-infection and post-patent time points, a significant absolute change in mean IgG subtype levels were observed. For IgG$_1$, we observed a significant decline from 9.0 ± 3.3 log AU to 6.3 ± 0.7 log AU (p<0.0001), corresponding to a -2.7 ± 3.1 log AU absolute decline in antibody levels (Table 1). For IgG$_3$, we observed a significant decline and from 7.8 ± 2.2 log AU to 6.4 ± 0.6 log AU (p=0.002), corresponding to a -1.3 ± 2.1 log AU absolute decline in antibody levels (Table 1). Also, our estimated median decay constant and median half-life of IgG$_1$ antibodies were -0.05 log AU/month (IQR -0.04, -0.07) and 12.9 months (IQR 10.6, 16.5 months), and for IgG$_3$ antibodies were -0.02 log AU/month (-0.008, -0.04) and 46 months (IQR 18.4, 88.6 months). These observations are also indicative that IgG subtypes IgG$_1$ and IgG$_3$ can over-season, with IgG3 antibodies persisting for almost four years.
Antibody levels during the post-patent period was similar between seropositive and seronegative subjects

A significant difference in anti-PvMSP1\textsubscript{19} IgG serostatus was observed between patent infection and the post-infection period (p<0.01), with the majority of subjects (74\%, n=23) IgG seropositive during patent infection, while only 35.5\% (n=11) were seropositive during the post-infection time point, representing a 2.1-fold decrease in the frequency of seropositive subjects between the patent and post-patent period (Fig. 6). Although not statistically significant, trends in differences were observed in the median number of lifetime \textit{Pv} infections between seropositive and seronegative subjects (4 vs. 2.5 events), self-reported time since last infection (9 vs. 2 months), and median age (18 vs. 12 years) at time of infection (Table 3). No significant difference was observed in the median duration between the patent and post-patent time points between seropositive and seronegative subjects (12.4 vs. 11.2 months) (Table 3). When we assessed differences in anti-PvMSP1\textsubscript{19} IgG levels between seropositive and seronegative subjects during patent and post-patent time points, the mean IgG level during patent infection was significantly greater in seropositive than seronegative subjects (11.4 ± 2.4 log AU vs. 6.4 ± 0.6 log AU, respectively; p<0.001) as expected, but similar in the post-patent period (7.2 ± 1.4 log AU vs. 6.5 ± 0.8 log AU, respectively; p=0.17) (Table 3). Significant differences between both groups were observed in their mean absolute change in total IgG levels between both time points (p<0.001) and being greater in seropositive than seronegative subjects (−4.2 ± 2.4 AU vs. 0.1 ± 0.3 log AU, respectively) (Table 4).

As expected, significant differences (p<0.0001 and p<0.01, respectively) in IgG\textsubscript{1} and IgG\textsubscript{3} antibodies were observed between both time points. We observed that 48\%
(n=15) and 55% (n=17) of study subjects were seropositive to anti-PvMSP1\textsubscript{19} IgG\textsubscript{1} and IgG\textsubscript{3} antibodies during patent infection; while only 3% (n=1) and 19% (n=6) were seropositive to IgG\textsubscript{1} and IgG\textsubscript{3} during post-patent infection, representing a 16- and 2.9-fold decrease in the frequencies of seropositive subjects, respectively (Fig. 6). Consistent with our total IgG findings, significant differences were observed in the mean IgG\textsubscript{1} and IgG\textsubscript{3} antibody levels between both sero-groups during patent infection and was significantly greater in seropositive than seronegative subjects, as expected (Table 3). No significant differences were observed between sero-groups during the post-patent period. Similar to the total IgG observations, a significant difference in the mean absolute change was observed in IgG\textsubscript{1} and IgG\textsubscript{3} antibodies across time points by sero-groups, which was larger in the seropositive than seronegative subjects (Table 4). These observations of total IgG, IgG\textsubscript{1} and IgG\textsubscript{3} antibody levels by serostatus groups during patent infection and the post-patent period suggest that the observed absolute differences in antibody levels were determined by differences in antibody levels elicited during patent infection, which subsequently declined to similar values after eight or more months post-infection in both groups.

**Anti-PvMSP1\textsubscript{19} IgG antibodies persisted for 8-15 months after *P. vivax* infection, with older age significantly associated with increased odds of maintaining a seropositive status**

To study the persistence of the antibody responses to PvMSP1\textsubscript{19}, participants who were seropositive during patent *Pv* infection were analyzed. Among the 23 IgG seropositive subjects during patent infection, two groups were identified according to
their maintenance of seropositive status during the post-patent period: those who maintained their seropositive status (n=10) and those who seroreverted (n=13). More than half (57%) of all IgG seropositive subjects seroreverted, while 43% maintained their serostatus. We observed that subjects who remained seropositive had a median seropositivity time of 13.4 months (IQR 10.7, 14.4 months), lasting from 8 to 15 months after patent infection (Fig. 7). Also, participants who remained seropositive were significantly older than those who seroreverted, with a median (IQR) age of 40 (27 – 46) years vs. 13 (8 – 16) years respectively (p<0.001); had significantly lower trophozoite log counts during patent infection, with a mean ± SD log count of 6.1 ± 2.2 vs. 8.1 ± 1.3 respectively (p=0.01); and had significantly greater IgG mean ± SD antibody levels during the post-patent time point, with 8.5 ± 1.1 log AU vs. 6.2 ± 0.4 log AU respectively (p<0.0001) (Table 5). No significant differences were observed between both groups by sex, gametocyte log count, self-reported lifetime P. falciparum events, time since last malaria event, duration between patent and post-patent time points, or total IgG antibody levels elicited during patent infection.

When we quantified the absolute change in total IgG antibody levels, no significant differences were observed in mean absolute change between those who remained seropositive and those who seroreverted (Table 4). On the other hand, we did observe significant differences in their median decay constant and half-life (p=0.03, respectively). Using a single exponential antibody decay model, the median anti-PvMSP19 IgG decay constant during the patent and post-patent time period was -0.03 log AU/month (IQR -0.02, -0.04), with a median half-life of 27.5 months (IQR 19.5, 39.3 months) in those who remained seropositive. On the other hand, the median decay
constant and median half-life of those who seroreverted were -0.05 log AU/month (IQR -0.03, -0.06) and 13.8 months (IQR 10.8, 25.2 months).

To understand variables associated with increased odds of maintaining seropositive status after patent infection, bivariate and multivariate logistic regression analyses were conducted. The bivariate analysis showed that age and trophozoites (log count) during patent infection were significantly associated with the odds of remaining seropositive (Table 6). Specifically, older age was significantly associated (p=0.01) with increased odds of remaining seropositive 8-15 months after patent infection, while greater trophozoites log counts during patent infection were significantly associated (p=0.04) with reduced odds of remaining seropositive. After adjusting for other covariates, only age remained significantly associated with the odds of maintaining seropositive status.

With respect to the maintenance of IgG1 (n=15) and IgG3 (n=17) antibodies, only 7% (n=1) IgG1 seropositive subjects and 18% (n=3) IgG3 seropositive subjects maintained their serostatus at the post-patent period, while 93% (n=14) and 82% (n=14) seroreverted (Fig. 7). No significant differences were observed between those who remained seropositive and those who seroreverted in their IgG1 and IgG3 antibody levels during both patent and post-patent periods (Table 6). No significant absolute differences were observed between both groups in their IgG1 and IgG3 antibody levels, median half-life, and decay constant) (Table 4). The median IgG1 half-life was similar between those who maintained vs. reverted their seropositive status (p=0.8), with 14.1 months (IQR 11.8, 16.5 months) and 12.9 months (IQR 10.4, 16.3 months) respectively, and the median decay constant was also similar between both groups being -0.05 log AU/month (IQR -0.04, -0.06) and -0.05 log AU/month (IQR -0.04, -0.07) respectively (p=0.8).
median IgG half-life was similar between those who maintained vs. reverted their seropositive status (p=0.5), with 21.5 months (IQR 17.1, 50.3 months) and 42.4 months (IQR 19.2, 92.5 months) respectively, and the median decay constant was also similar between both groups being -0.03 log AU/month (IQR -0.01, -0.04) and -0.02 log AU/month (IQR -0.01, -0.04) respectively (p=0.5).

4.5 Discussion

This is the first study to investigate the maintenance of the antibody response to PvMSP1₉ in a low endemic setting in Peru, during a period of 8-15 months after infection, which provides insight into antibody persistence, evaluating changes over time, differences between groups (e.g. seropositive vs. seronegative subjects, persistent seropositivity vs. seroreversion), and variables associated with antibody decline and persistence (e.g. age). Our main findings are: (i) despite residing in a low transmission setting with reduced parasite exposure, anti-PvMSP1₉ IgG antibodies persisted for 8 to 15 months after infection remaining seropositive; and (ii) PvMSP1₉-specific IgG antibody decline and persistence were significantly associated with age, with older individuals having reduced antibody decline and increased odds of remaining seropositive.

In the Peruvian Amazon Basin, a low transmission area (26, 27, 32), a study by Clark et al. in 2011 described the longevity of the antibody response to Pf MSP1₉ (PfMSP1₉), reporting that antibodies persisted for at least 6 months in the absence of reinfection (27, 32). Moreover, Clark et al. detected PfMSP1-specific memory B cells during post-infection time points, supporting the hypothesis that antimalarial humoral
immunity can develop in low-transmission regions despite reduced exposure to malaria parasites. Studies of the maintenance of antibodies to \( P_v \) by Braga et al. and Morais et al. conducted in a rural non-endemic community distant from the Brazilian Amazon that experienced a \( P_v \) outbreak (50) reported long-lasting IgG and IgG subclasses 1 and 3 antibody responses to \( P_v \text{MSP1}_{19} \), persisting for at least 8 months post-infection (33, 34). Serum samples were collected from individuals both 8 months and 7 years after exposure to \( P_v \) during an outbreak in Mantena, Minas Gerais that lasted 50 days, who were symptomatic with patent infection during the outbreak and with no reported travel history to neighboring malaria endemic regions during that period of time. A study by Soares et al. investigated the longevity of antibodies to \( P_v \) in northern Brazil four months after infection, observing that the vast majority of treated cases had a significant decrease in anti-\( P_v \text{MSP1}_{19} \) antibody titers by the second month of follow-up and a significant change in serostatus (51). To date, there are no other studies of the maintenance of antibodies to \( P_v \) antigens in individuals who reside in a low transmission area that could support either the findings on antibody persistence in a non-endemic area by Braga et al. and Morais et al. or the hypothesis of Clark et al. that infrequent infections in low transmission areas can stimulate and maintain anti-malaria humoral responses.

This study demonstrated that despite low malaria transmission in the area, anti-\( P_v \text{MSP1}_{19} \) IgG antibodies persisted for 21 months after natural infection following treatment, and with no apparent sign of re-infection or relapse, with an estimated median half-life of two years. When we evaluated the change of serostatus over time, 43% of all seropositive subjects remained seropositive 8-15 months post-infection. These findings are concordant with other studies of \( P_v \text{MSP1}_{19} \) antibodies conducted in non- and low-
endemic settings that evaluated the change in seroprevalence and antibody decline after varying lengths of time (30, 34, 52). A study by Braga and Fontes in Minas Gerais, Brazil observed that 8 months after a Pv outbreak in a non-endemic malaria area, 61% of all infected individuals remained IgG seropositive to rPV200 (PvMSP1 carboxyl-terminal domain that includes amino acids 1357 – 1729), and that 7 years since the outbreak without apparent reinfection or relapse 47% remained seropositive (34). Also, a study by Lin et al. 2004 observed that Pv-infected Korean soldiers who had recently served in a malarial endemic region, had low but persistent anti-PvMSP119 IgG antibody levels for up to 10 months after receiving treatment, and that previously exposed residents who had lived in areas where Pv malaria had been endemic exhibited residual antibody responses for more than 30 years (30). Additionally, the study by Soares et al. observed that two months after receiving treatment 47% of PvMSP119 IgG responders maintained their seropositive status, exhibiting a dramatic reduction in their antibody levels more than an average of 13-fold during that short time period (51). Even though this last study observed changes in the seropositive status shortly after receiving treatment, it was not able to ascertain the persistence of the anti-PvMSP119 humoral response beyond that period of time.

The acquisition of malaria natural immunity may be modulated by the age at first exposure, with children being more susceptible to infection than adults after comparable number of exposures (53, 54). Our study found that the absolute decline of Pv anti-PvMSP119 IgG antibodies over time was associated with age, with older individuals having a reduced absolute decline of IgG antibodies compared to younger individuals, consistent with observations from other studies of PfMSP119 antibodies (10, 27).
However, the cumulative exposure to \( P_v \) antigens is a function of both the duration of exposure (i.e., age) and malaria endemicity (transmission intensity). The accumulation of \( P_v \) infections with age in Peru, a low endemic area, is different than high transmission areas where most individuals have probably experienced several infections during the first years of life (10) and have frequent exposure to malaria parasites. Even though we failed to observe a significant correlation between the absolute decline of antibodies and age, we found a significant correlation between age and post-patent antibody levels suggesting a slower decline and therefore greater longevity of antibodies in older individuals. We also found a significant association between age and antibody decline after adjusting for both patent-infection antibody levels and parasitemia counts. Therefore, our findings may reflect a difference in anti-\( P_v \text{MSP}1_{19} \) IgG maintenance by age in a low endemicity setting, where antibody responses continue to develop with increasing age. Since our post-patent samples were collected several months after infection, it could be possible that our observations are the result of differences in long-lived plasma cells (LLPC) or age differences in the activation and regulation of LLPC through survival signals (55). LLPC allow for continued production of anti-\( P_v \text{MSP}1_{19} \) IgG antibodies over time without restimulation with persistent antigens. When we assessed the effect of age on the odds of remaining seropositive several months after patent parasitemia, only ten (32\%) individuals maintained their seropositive status, all of whom where twelve years of age or older, while the other thirteen seropositive subjects who seroreverted were significantly younger. Therefore, our observation of the significant association of antibody decline and age suggests that age might reflect a difference in cumulative malaria exposure to malaria parasites (number of lifetime infections
encountered) rather than differences in immune maturation. We also observed that older individuals (third tertile) reported a greater number of lifetime \( P_v \) events, indicating that age reflects the cumulative exposure to \( P_v \) malaria infections over time.

There are several limitations to our study. We were unable to know with certainty that all study subjects were \( P_v \) free over the study period, and must therefore rely on self-report. Nonetheless, the cohort study from which our samples were obtained performed active case surveillance during the malaria season and passive case detection throughout the year, and any symptomatic infections would have been identified by MIGIA’s malaria surveillance. However, our study subjects could have had asymptomatic infection that would most likely have gone undetected. Asymptomatic infections can still boost immune responses, possibly altering their antibody decline kinetic and antibody maintenance over time. A prospective study with close monitoring of parasitemia would have been ideal to ascertain the maintenance of antibodies in a low endemic setting in the absence of any reinfections or relapse. Furthermore, a prospective study with a greater sample size and repeated measurements, including multiple data points from the same subjects, would have allowed us to study the antibody kinetics and assess the effect that treatment has on the kinetics of the antibody response. Additionally, even though we observed significant differences in antibody decline and maintenance by age, we were not able to ascertain if these differences were more prominent shortly after infection cleared or gradually over time. Despite having only two time points, this study provides valuable information on the longevity of anti-\( P_v \text{MSP1}_{19} \) antibodies and the effect of age on antibody maintenance in a low endemic area. Finally, in order to capture significant differences of antibody decline by other variables, as lifetime \( P_v \) events, a greater sample
size will be needed in order to describe antibody dynamics and malaria epidemiology more accurately.

This study evaluated the persistence of humoral responses and described the magnitude of the decline 8-15 months after \( P_v \) mono-infection in a low endemic setting. Antibodies were maintained for 8-15 months in the absence of apparent relapse or reinfection, and the antibody decline over time was associated with age. Individuals living in a low endemic setting can maintain antibody responses to \( P_v \text{MSPI}_{19} \) for more than eight months with a median half-life of over two years. Our observations as well as other studies on antibody maintenance in low-endemic settings are an encouraging sign for ongoing \( P_v \) malaria vaccine initiatives, where an effective vaccine may result in long-lasting antibody responses despite infrequent antigen exposure.

4.6 Reference


45. Lucas C. Personal communication. U.S. Naval Medical Research No. 6 (NAMRU-6). 2015.


4.7 Tables and Figures

Table 1. Characteristics of the study population

<table>
<thead>
<tr>
<th></th>
<th>Patent</th>
<th>Post-patent</th>
<th>P-value**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, female (%)</td>
<td>12 61%</td>
<td>12 61%</td>
<td>1.0</td>
</tr>
<tr>
<td>Age, median years</td>
<td>15.2 [8.7,28.6]</td>
<td>16.2 [10,29.9]</td>
<td>0.6</td>
</tr>
<tr>
<td>Parasitemia, count/μL(%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trophozoites</td>
<td>7 (1.9)</td>
<td>0.0 0.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Gametocytes</td>
<td>5.1 (1.3)</td>
<td>0.0 0.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Lifetime P. events, median</td>
<td>4.0 [2,10]</td>
<td>6.0 [2,10]</td>
<td>0.5</td>
</tr>
<tr>
<td>Months since last malaria, median</td>
<td>7.7 [0,12.9]</td>
<td>12.2 [9.2,14.1]</td>
<td>0.01</td>
</tr>
<tr>
<td>Antibodies, mean logAU</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG1</td>
<td>10.1 ±3.0</td>
<td>7.0 ±1.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IgG2</td>
<td>9.0 ±3.3</td>
<td>6.3 ±0.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IgG3</td>
<td>7.8 ±2.2</td>
<td>6.4 ±0.6</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Seropositives (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG1</td>
<td>23 74%</td>
<td>11 36%</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>IgG2</td>
<td>15 48%</td>
<td>1 3%</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IgG3</td>
<td>17 55%</td>
<td>6 19%</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Anemia (%)</td>
<td>16 52%</td>
<td>4 13%</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Data are median values [IQR], mean (SD), or frequencies (%) of individuals. (*) Mean (SD) of naturally log-transformed parasitemia. (**) Significant differences P < 0.05. Total immunoglobulin G (IgG), IgG subtype 1 (IgG1) and IgG subtype 3 (IgG3) antibodies were measured as ELISA log arbitrary units (log AU). Seropositive cut-offs were obtained from the average of a pool of non-exposed non-endemic malaria-naive adults from Lima - Peru plus three standard deviations.
Table 2. Bivariate and multivariate analyses of the absolute change in anti-PvMSP119 IgG antibodies months after patent \textit{P. vivax} infection

<table>
<thead>
<tr>
<th></th>
<th>Bivariate</th>
<th>Multivariate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>coef. [95% CI.]</td>
<td>P-value*</td>
</tr>
<tr>
<td>Sex (male)</td>
<td>1.12 [-0.94, 3.18]</td>
<td>0.29</td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.01 [-0.06, 0.07]</td>
<td>0.85</td>
</tr>
<tr>
<td>Parasitemia [log (count)]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trophozoites</td>
<td>-0.85 [-1.29, 0.39]</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Gametocytes</td>
<td>-1.06 [-1.88, 0.23]</td>
<td>0.01</td>
</tr>
<tr>
<td>Patent infection IgG</td>
<td>-0.86 [-0.99, 0.71]</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Lifetime \textit{Pv} events</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-3.83 [-6.71, 0.96]</td>
<td>0.01</td>
</tr>
<tr>
<td>2</td>
<td>-2.46 [-5.33, 0.42]</td>
<td>0.09</td>
</tr>
<tr>
<td>0</td>
<td>-1.12 [-4.06, 1.81]</td>
<td>0.45</td>
</tr>
<tr>
<td>Months since patent infection</td>
<td>-0.22 [-0.54, 0.11]</td>
<td>0.19</td>
</tr>
<tr>
<td>Months since last malaria</td>
<td>-0.05 [-0.15, 0.06]</td>
<td>0.38</td>
</tr>
</tbody>
</table>

Antibodies were measured by ELISA as arbitrary units (AU). Coef., coefficient; CI, confidence interval; (*) Statistical significance: \( P \leq 0.05 \).
Table 3. Socio-demographic characteristics and antibody differences between serostatus groups during patent *P. vivax* mono-infection

<table>
<thead>
<tr>
<th></th>
<th>Seropositive (n=23)</th>
<th>Seronegative (n=8)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex,</strong> female (%)</td>
<td>16 70%</td>
<td>3 38%</td>
<td>0.11</td>
</tr>
<tr>
<td><strong>Age,</strong> median years</td>
<td>17.9 [9.5, 37.7]</td>
<td>11.9 [4.4, 15.9]</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>Parasitemia,</strong> median count/μL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Merozoites</td>
<td>2663 [431.0, 346.0]</td>
<td>337.0 [187.5, 1233.0]</td>
<td>0.1</td>
</tr>
<tr>
<td>Gametocytes</td>
<td>116 [0.0, 346.0]</td>
<td>0.0 [0.0, 31.5]</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>Lifetime</strong> P events, median</td>
<td>4.0 [3.1]</td>
<td>2.5 [0.5, 5]</td>
<td>0.1</td>
</tr>
<tr>
<td>Months since patent infection, median</td>
<td>12.4 [9.2, 14.7]</td>
<td>11.3 [9.0, 12.7]</td>
<td>0.4</td>
</tr>
<tr>
<td>Months since last malaria, median</td>
<td>8.6 [0, 16.5]</td>
<td>1.7 [0, 8.8]</td>
<td>0.08</td>
</tr>
<tr>
<td><strong>Patent</strong> antibodies, mean log AU</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgGt</td>
<td>11.4 ±2.4</td>
<td>6.4 ±0.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IgG1</td>
<td>10.0 ±3.3</td>
<td>6.2 ±0.1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>IgG3</td>
<td>8.3 ±2.2</td>
<td>6.2 ±0.7</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>Post-patent</strong> antibodies, mean log AU</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgGt</td>
<td>7.2 ±1.4</td>
<td>6.5 ±0.8</td>
<td>0.2</td>
</tr>
<tr>
<td>IgG1</td>
<td>6.4 ±0.8</td>
<td>6.2 ±0.01</td>
<td>0.6</td>
</tr>
<tr>
<td>IgG3</td>
<td>6.5 ±0.7</td>
<td>6.2 ±0.2</td>
<td>0.3</td>
</tr>
<tr>
<td><strong>Post-patent</strong> seropositive (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgGt</td>
<td>10 43.5%</td>
<td>1 12.5%</td>
<td>0.1</td>
</tr>
<tr>
<td>IgG1</td>
<td>1 6.7%</td>
<td>0 0.0%</td>
<td>0.3</td>
</tr>
<tr>
<td>IgG3</td>
<td>3 17.7%</td>
<td>3 21.4%</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Data are median values (IQR), mean (SD), or frequencies (%) of individuals. (*) Significant differences *P* < 0.05. Total immunoglobulin G (IgG), IgG subtype 1 (IgG1) and IgG subtype 3 (IgG3) antibodies were measured as ELISA log arbitrary units (log AU). Seropositive cut-offs were obtained from the average of a pool of non-exposed non-endemic malaria-naïve adults from Lima, Peru plus three standard deviations.
**Table 4. Change in antibody levels between patent infection and the post-patent period by serostatus and maintenance groups**

<table>
<thead>
<tr>
<th>Change in Ab titers</th>
<th>Serostatus (n=31)</th>
<th>P-value*</th>
<th>Maintenance (n=23)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td></td>
<td>Mantained</td>
</tr>
<tr>
<td>IgGt</td>
<td>-4.15(±2.44)</td>
<td>0.10(±0.96)</td>
<td>&lt;0.0001</td>
<td>-3.27(±2.74)</td>
</tr>
<tr>
<td>IgG1</td>
<td>-3.65(±3.14)</td>
<td>0.05(±1.12)</td>
<td>&lt;0.01</td>
<td>-3.66(±3.68)</td>
</tr>
<tr>
<td>IgG3</td>
<td>-1.82(±2.27)</td>
<td>0.05(±0.72)</td>
<td>&lt;0.01</td>
<td>-2.36(±2.35)</td>
</tr>
</tbody>
</table>

Total immunoglobulin G (IgGt), IgG subtype 1 (IgG1) and IgG subtype 3 (IgG3) antibodies (Ab) were measured as ELISA log Arbitrary Units (log AU). Values reported are means (SD). Maintenance: seropositive people at time of infection who either maintained their seropositive serostatus [Mantained] or seroreverted [Reverted] by the post-infection time point. *P-value: Significant differences (P<0.05).
Table 5. Differences between maintenance groups

<table>
<thead>
<tr>
<th></th>
<th>Maintained (n=10)</th>
<th>Reverted (n=13)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, female (%)</td>
<td>7 70%</td>
<td>9 69%</td>
<td>0.90</td>
</tr>
<tr>
<td>Age, median years</td>
<td>40.0 [27.2,51.6]</td>
<td>12.7 [8.3,15.5]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Parasitemia, median count/µL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trophozoites</td>
<td>630.0 [47,8118]</td>
<td>2970.0 [1218,6720]</td>
<td>0.05</td>
</tr>
<tr>
<td>Gametocytes</td>
<td>43.5 [0,346]</td>
<td>149.0 [36,263]</td>
<td>0.3</td>
</tr>
<tr>
<td>Lifetime events, median</td>
<td>5 [3,11]</td>
<td>4.0 [3,9]</td>
<td>0.9</td>
</tr>
<tr>
<td>Months since last malaria, median</td>
<td>10.5 [0,23.7]</td>
<td>8.1 [4.8,13.7]</td>
<td>0.9</td>
</tr>
<tr>
<td>Patent antibodies, mean log AU</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>11.7 ±2.7</td>
<td>11.1 ±2.2</td>
<td>0.5</td>
</tr>
<tr>
<td>IgG1</td>
<td>10.3 ±3.9</td>
<td>9.9 ±2.8</td>
<td>0.8</td>
</tr>
<tr>
<td>IgG3</td>
<td>8.9 ±2.7</td>
<td>7.8 ±1.8</td>
<td>0.2</td>
</tr>
<tr>
<td>Post-patent antibodies, mean log AU</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>8.5 ±1.1</td>
<td>6.2 ±0.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IgG1</td>
<td>6.6 ±1.2</td>
<td>6.2 ±0.005</td>
<td>0.3</td>
</tr>
<tr>
<td>IgG3</td>
<td>6.6 ±0.6</td>
<td>6.4 ±0.8</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Data are median values (IQR), mean (SD), or frequencies (%) of individuals. (*) Significant differences P < 0.05. Total immunoglobulin G (IgG), IgG subtype 1 (IgG1) and IgG subtype 3 (IgG3) antibodies were measured as ELISA log arbitrary units (log AU). Antibody maintenance groups were categorized as seropositive people at time of infection who either maintained their seropositive serostatus (Maintained) or seroconverted (Reverted) by the post-infection time point.
Table 6. Bivariate and multivariate analyses of the odds of remaining IgG seropositive months after patent *P. vivax* infection

<table>
<thead>
<tr>
<th></th>
<th>Bivariate</th>
<th></th>
<th></th>
<th>Multivariate</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR [95%CI]</td>
<td>P-value*</td>
<td>AOR [95%CI]</td>
<td>P-value*</td>
<td></td>
</tr>
<tr>
<td>Sex (male)</td>
<td>0.96 [0.16,5.79]</td>
<td>0.9</td>
<td></td>
<td>1.19 [1.01,1.42]</td>
<td>0.04</td>
</tr>
<tr>
<td>Age (years)</td>
<td>1.29 [1.04,1.38]</td>
<td>0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parasitemia (log count)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trophozoites</td>
<td>0.50 [0.26,0.96]</td>
<td>0.04</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gametocytes</td>
<td>0.95 [0.42,2.12]</td>
<td>0.9</td>
<td></td>
<td>1.30 [0.34,3.93]</td>
<td>0.7</td>
</tr>
<tr>
<td>Patent infection IgG</td>
<td>1.13 [0.79,1.61]</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lifetime Plasmodium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.00 [0.11,5.81]</td>
<td>0.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.80 [0.04,14.64]</td>
<td>0.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.67 [0.16,15.14]</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Months since patent infection</td>
<td>1.02 [0.79,1.32]</td>
<td>0.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Months since last malaria</td>
<td>1.02 [0.95,1.10]</td>
<td>0.6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Antibodies were measured by ELISA in Arbitrary Units (log AU). OR: Odds Ratio; AOR: Adjusted Odds Ratio; Patent infection IgG: Antibodies measured during patent *P. vivax* mono-infection. (*) Statistical significant P < 0.05.
Villages: ZG = Zungarococha, PA = Puerto Almendra, NR = Ninarumi, LC = Llanchama

Fig. 1. Malaria cases in Peru: 1939-2010, Cases of Malaria due to *Plasmodium falciparum* and *P. vivax* were not differentiated and reported systematically until 1990.

Source: Peruvian Ministry of Health, Lima, Peru

Selection of houses within the MIGIA cohort study for weekly active case detection within ‘\(Pf\) at-risk’ zones. Weekly sampling occurred on zones with defined \(Pf\) infection risk. Based upon the detection (passive or active) of a \(Pf\) case the previous month (solid red circles), a 100-meter radius was defined (open blue circle) to detect malaria-infected individuals in selected households (open black circles).

Source: Branch O, R01 AI064831-01
Figure 4. IgG antibody responses to PvMSP1\textsubscript{19} antigen over time in plasma samples from \textit{P. vivax} mono-infected subjects

ELISA-determined plasma IgG antibody levels to \textit{P. vivax} MSP1\textsubscript{19} (log AU: log ELISA Arbitrary Units) over time in months, showing means, standard errors, and number of subjects in parenthesis.
Figure 5. Changes in individual antibody levels between infection and the post-infection period

Individual ELISA-determined plasma IgG antibody levels to *P. vivax* MSP1<sub>19</sub> (log AU: log ELISA Arbitrary Units) during infection and post-infection time points.
Figure 6. Changes in seropositive status between infection and the post-infection period

Percentage of seropositive plasma samples during patent infection (dark grey) and several months after patent infection (light grey). Total immunoglobulin G, IgG<sub>T</sub>; IgG subtype 1, IgG<sub>1</sub>; IgG subtype 3, IgG<sub>3</sub>. Seropositivity was determined using an estimated cut-off value. (*) $P$-value < 0.01; (**) $P$-value < 0.001.
Figure 7. Changes in serostatus within subjects who were seropositive during patent *P. vivax* mono-infection

Percentage of seropositive individuals who either maintained their serostatus (dark grey) or seroreverted (light grey) several months after patent infection. Total immunoglobulin G, IgG; IgG subtype 1, IgG₁; IgG subtype 3, IgG₃. Seropositivity was determined using an estimated cut-off value.
V. Chapter Five: Conclusions and Recommendations

5.1 Summary of Major Findings

This research examined the declining burden of *Pv* malaria in a community in the Peruvian Amazon Basin and studied humoral responses to *PvMSP1*$_{19}$ during a low-malaria transmission period in symptomatic and asymptomatic individuals residing in the area. Findings of this dissertation on the presence of asymptomatic microscopy-positive people in a rural community in the Peruvian Amazon Basin during a low-malaria transmission period, who are capable to serving as parasite reservoirs, points to the existence of naturally-acquired immunity (NAI) in the population, underlines their relevance for the sustainable elimination of malaria, and stresses the need to further our knowledge on malaria ecology and NAI in low endemic areas. Our findings on the anti-*PvMSP1*$_{19}$ humoral response highlight differences in antibody responses between symptomatic and asymptomatic individuals, its association with clinical presentation, and antibody maintenance over time, as well as the potential usefulness of anti-*PvMSP1*$_{19}$ IgG antibodies to assess parasite exposure in community-wide surveys in low endemic settings.

There is limited understanding of the humoral response to *Pv* malaria infections, particularly in areas with low malaria transmission, such as the Peruvian Amazon Basin. Most studies on the humoral response to malaria infections have either assessed *Pf* malaria or *Pv* malaria in experimental infections or following acute and chronic exposure. The few studies that assessed the natural humoral response to *Pv* malaria in low transmission settings have either assessed differences in the response by exposure,
comparing subjects residing either in meso-endemic areas to less frequently or sporadically exposed subjects residing in low-endemic areas (1), or by transmission intensity by comparing antibody responses from subjects residing in areas of different endemicity and length of residence (1-3).

Based on the intrinsic biological differences between *Pv* and *Pf* malaria, such as the hypnozoite stage responsible for disease relapse, preference for reticulocytes over mature RBCs with less-redundant invasion pathways, and earlier differentiation into gametocytes facilitating parasite transmission to mosquito vectors even before clinical infection is evident, highlight the need to conduct *Pv*-specific immuno-epidemiological studies rather than making extrapolations from *Pf* studies, even when performed in the same geographical area. Only a few studies assessed the association between antibody levels (4) and clinical presentation of disease, and/or assess differences between symptomatic and asymptomatic individuals, none of which have taken place in the Peruvian Amazon Basin and none for *Pv* malaria. A study by Braga *et. al* observed that PfMSP1_{19} IgG antibodies were higher among subjects with long-term exposure, who resided in areas where malaria prevalence was more intense, and among asymptomatic patients (4).

The lack of knowledge of the immune response to *Pv* malaria, due to the limited number of studies on the subjects as well as the intrinsic biological challenges that *Pv* malaria has, hinders the advancement of our understanding of the development and maintenance of NAI to *Pv*, information that could then be exploited to guide current malaria vaccines strategies on for example vaccine boosting. Finally, the importance of asymptomatic malaria in seasonal and low-endemic areas resides in its ability to act as
parasite reservoir, allowing for the over-seasoning and persistence of the parasite. Therefore, to support current public health control and elimination strategies implemented in low-transmission settings, such as guiding different approaches to active case detection, it will important to continue investigating the ecology and sero-epidemiology of malaria transmission in low-endemic areas.

5.1.1 *Paper I*

Paper I characterizes the changing malaria epidemiology in the study area, assessing the burden of *Pv* and *Pf* malaria, as well as the prevalence of asymptomatic individuals, as diagnosed by light microscopy. It describes both *Pv* and *Pf* malaria trends over an eight-year time span from 2004 to 2011 during the post-epidemic period when multilateral interventions and initiatives were launched with the goal to reduce disease burden in the region. Even though we cannot directly associate specific control activities to the declining burden of malaria, there is an evident decline in malaria burden in the community, most significantly during the earlier years of the survey not too long after the launch of intensified control activities. Following the sharp decline in malaria burden and the implementation of a comprehensive malaria control strategy that included the first mass distribution of LLINs in the region, malaria prevalence achieved sustained low levels for several years. The presence of a higher proportion of asymptomatic microscopy-positive than symptomatic residents in this seasonal and low-endemic area suggests the existence of NAI. Finally, our results on the risk of malaria throughout the study period suggest that it is occupationally-related. We conclude that despite the evident decline in malaria burden, asymptomatic individuals infected with *Pv* pose an
important challenge in the sustained control of malaria in the area, and future studies on malaria ecology, immunology and epidemiology in low endemic settings should provide with valuable information to help current public health control and elimination strategies, such as help determine the use of active case detection strategies, either reactive or proactive case detection, to most effectively control and reduce malaria burden in the area.

5.1.2 Paper II

Paper 2 presents the first study to assess differences in anti-PvMSP1<sub>19</sub> antibody levels between symptomatic and asymptomatic individuals in a community with low malaria transmission in the Peruvian Amazon Basin both prior to and during <i>Pv</i> infection. Symptomatic and asymptomatic <i>Pv</i>-infected individuals had no significant differences in their mean antibody levels months prior a documented patent infection. However, during patent infection, only symptomatic individuals had a significant boost in their anti-PvMSP1<sub>19</sub> IgG response. Despite no significant differences in mean pre-infection antibody levels, increasing pre-infection antibody concentrations were positively correlated with the number of self-reported lifetime infections and inversely correlated with the self-reported time since last infection. Individuals in the mid- to high-category of pre-infection IgG antibody had increased odds of asymptomatic malaria infection, while individuals with current infection in the mid- to high-category IgG antibody levels had increased odds of symptomatic infection, indicative that anti-PvMSP1<sub>19</sub> IgG antibodies are both a marker of exposure and protection in this population. Finally, based on changes in seropositive status between pre- and current-infection time points, anti-
PvMSP1$_{19}$ IgG antibodies might not be useful to detect malaria infections, particularly asymptomatic infections.

5.1.3 Paper III

Paper 3 expands these studies of IgG responses to PvMSP1$_{19}$ antigen, specifically its maintenance over time in individuals residing in a low-transmission area. It successfully demonstrated that seropositive anti-PvMSP1$_{19}$ IgG antibodies can be maintained for eight to fifteen months in a low endemic community in the Peruvian Amazon Basin, with an estimated half-life of over two years with no apparent re-infection or relapse, and with a third of the study participants remaining seropositive over the study period. In addition to documenting sustained antibody levels and seropositive status, older individuals had smaller absolute declines in IgG antibodies and increased odds of remaining seropositive. These observations, combined with observations from other $Pv$ immuno-epidemiological studies conducted in low endemic settings and experimental $Pv$ infections, suggest that infrequent malaria infections as experienced in the study community are capable of eliciting an immune response that can over-season, which could explain the considerable prevalence of asymptomatics in low transmission settings.

5.2 Study Limitations

For Paper 1, while the MIGIA dataset is a rich source of information, the lack of information regarding PCR malaria diagnosis, distance to a water source, and site of occupation, does not allow us to more precisely estimate the burden of malaria and better
characterize risk factors. Although the MIGIA dataset had information on malaria cases diagnosed by microscopy, a more sensitive diagnostic molecular method would have identified a substantial number of asymptomatic submicroscopic infections. Therefore, our results are most likely an underestimate of the real malaria burden in the area. Previous studies conducted in other peri-Iquitos areas described higher malaria prevalence when diagnosed by PCR (14.2%) compared to light microscopy (2.9%) (5). To better understand malaria burden and risk in the area and inform local antimalarial strategies for control and elimination, it is crucial to capture the full extent of malaria infections, particularly asymptomatic parasite carriers who pose a public health risk by becoming a parasite reservoir, contributing to the ongoing transmission and persistence of malaria.

Also, another important limitation of this thesis was the definition of asymptomatic malaria. Even though the absence of fever is an objective observation that can be confirmed by measuring the body temperature during the study visit, the narrow window of time used to categorize clinical groups and the subjective self-reporting of fever within two days prior the study visit might not accurately classify individuals as symptomatic or asymptomatic in Papers 1 and 2, leading to potential misclassification. Furthermore, asymptomatic subjects without confirmed or reported fever at time of visit or during the prior two days may subsequently develop fever. Even though the parent study collected information on individuals who were asymptomatic at time of survey visit and who remained parasitemic and symptom-free one week later, their plasma samples were not available. Therefore, for Paper 2, we selected plasma samples from individuals who were asymptomatic not only at time of the survey visit (cross-sectional), as
performed in other studies (4, 6-10), but also included a two day pre-visit window for self-reporting of fever, similar to the approach of Afrane et. al on Pf malaria in highland areas with varying endemicity in western Kenya (11). Even though we cannot ascertain if our asymptomatic individuals are truly asymptomatic, since Pv malaria fevers typically recur every 48 hours (tertian malaria), our three-day window (survey/visit day and two days prior) should have captured febrile individuals. Still, this study cannot determine when asymptomatic individuals were first infected; therefore, we cannot ascertain if their infection was acute or chronic.

Other important limitations of this study are that we could not exclude mixed sub-microscopic infections nor assess for PvMSP1$_{19}$ polymorphisms. Because the parent study database did not include PCR analysis, sample selection for Papers 2 and 3, and our case definition in Papers 1 and 2 could only be made based on microscopy results. Even though mixed Pv-Pf mixed infections could have been missed by microscopy, we believe mixed infections with patent Pv and sub-microscopic Pf malaria are uncommon and therefore might not have had a significant impact on our observations. An unpublished study performed by the Naval Medical Research Unit No. 6 (NAMRU-6) in riverine remote communities along two rivers in the Peruvian Amazon basin (Corrientes and Yavari rivers) found that only 5.5% of 108 Pv cases diagnosed by microscopy were mixed Pv-Pf infections by PCR. Other studies on malaria epidemiology in Peruvian Amazon Region of Loreto have also reported infrequent mixed infections (12).

Another important limitation for Papers 2 and 3 is that this study did not assess PvMSP1$_{19}$ polymorphisms; therefore, we were unable to determine if the seronegative subjects either failed to generate a strong humoral response, had antibodies against
polymorphic epitopes not recognized by the recombinant antigen used in our study, or if developed short-lived memory responses. Even though a previous study by Kosek et al described a high degree of Pv diversity in the Peruvian Amazon (13), the PvMSP119 gene is considered to be highly conserved (14).

The results on antibody changes over time in Paper 3 are also subject to our inability to ascertain if our participants experienced exposure to malaria parasites between study time points (range: 8-21 months). Possible undetected infections could have boosted and altered the natural antibody decline. Any changes due to immune boosting would have gone unnoticed because our analysis included only two time points. Nonetheless, our selected samples were collected between 2006-2011, when malaria prevalence was below 2% and after all participants received directly observed treatment including primaquine for radical cure, therefore making relapse or reinfection unlikely, and from individuals without documented microscopy-positive malaria infections and fever between both time points.

5.3 Recommendations for Future Research

Future studies in the Peruvian Amazon Basin should focus on better characterizing malaria burden, including climate data to investigate its relationship with the changing malaria epidemiology over time, as well as include spatial and network data to assess for spatial (‘hot-spots’) and/or group clustering (‘hot-pops’) of malaria risk. It would also be interesting to work on a mathematical model to try predicting the occurrence of asymptomatic malaria using self-reported history of malaria, socio-demographic and epidemiological variables, as well as serology data, to potential guide
future targeted malaria control strategies. Also, it would be interesting to continue studying the persistence of anti-\textit{Pv} antibodies over longer periods of time in people who continue to reside in this low-transmission area with infrequent boosting of their immune system. Other studies have assessed antibody responses after 7 years (15) or even decades (3, 16) after being exposed to malaria, but had either left the endemic area or were only briefly exposed after a single outbreak with no further exposure during their lifetime. Such a study would help us understand the maintenance of the naturally acquired humoral response to \textit{Pv} malaria in a ‘low dose’ scenario with infrequent boosting. Additionally, it would be interesting to assess for the quality of the immune response elicited to \textit{Pv} malaria, performing agglutination and avidity assays on antibodies that are maintained over time. Also, in order to describe and assess changes in the breadth of the humoral response to \textit{Pv} malaria, as well as search for additional malaria antigens possibly associated with clinical protection in low-endemic settings following a limited number of exposures, it would be interesting to perform a protein microarray antibody profile to \textit{Pv} malaria, as performed for \textit{Pf} malaria. It would also be interesting to explore antibody maintenance to other \textit{Pv} antigens and obtain defined kinetic antibody profiles, which would allow us to assess for possible serological biomarkers of exposure that could help quantify their exposure rather than just describe it, as well as potentially translating it to low-cost field-based surveillance tools. A low transmission setting as the Peruvian Amazon Basin, where malaria infections are infrequent throughout time would be an ideal setting to assess for these biomarkers. Finally, studying the immune memory to this and other antigens will help us understand the development and maintenance of NAI to \textit{Pv} malaria in low-transmission settings. Examining peripheral blood mononuclear cells
(PBMCs) samples from *Pv*-infected individuals by ELISPOT and flow cytometry would allow us to study the memory B cell (MBCs) response to *Pv* malaria, as well as to characterize different populations of MBCs including naïve, activated, and atypical populations.

5.4 Policy implications

This dissertation focused on the declining burden of malaria in a rural community in the Peruvian Amazon Basin, assessing the presence of asymptomatic microscopy-positive cases as well as characterizing naturally-acquired humoral responses to *Pv* infection. Despite several limitations, this study has policy implications for the control and elimination of malaria, principally in areas of low *Pv* malaria transmission and pre-elimination settings. As malaria burden declines, its epidemiology will become more complex, with the identification of asymptomatic parasite reservoirs being critical for achieving sustainable malaria elimination. Although not addressed in this thesis, we found within MIGIA’s nine-year survey (2003-2011) using active and passive case detection, only 11% of 1133 asymptomatic cases (absence of fever) reported in the study were detected by passive case surveillance alone. Our findings from Paper 1, using MIGIA’s active case surveillance data from 2004-2011, support the WHO’s recommendation of using nucleic acid amplification methods in low transmission settings to increase the sensitivity of surveys and to map the prevalence of malaria (17). Even though our definition of asymptomatic might be oversimplified, knowing that microscopy sensitivity is significantly lower than PCR or other nucleic acid amplification methods, different important question arise, such as: i) who are we missing?, ii) what is the
contribution of asymptomatic malaria to transmission?, iii) what factors are associated with the epidemiology of the asymptomatic reservoir?, and iv) when and how should we target them? In order to address these questions and perform malaria epidemiological surveys we will need to incorporate molecular diagnostic techniques in the surveillance of malaria in countries/areas with low malaria transmission.

Pertaining to the diagnosis of malaria in low transmission and pre-elimination settings, Paper 2 and 3 touch indirectly on the usefulness of serological techniques for malaria diagnosis. Based on our observations on anti PvMSP119 humoral response, in which several microscopy-confirmed cases were not identified by serology (Paper 2), IgG antibodies reflected both exposure and current infection (Paper 2 and 3), and the long duration of antibodies with an estimated half-life of 2 years (Paper 3), a serological test to this conserved antigen might not be useful in differentiating recent from old infections, particularly in asymptomatic cases. Nonetheless, the use of serology to assess past exposure to the parasite at a population level could help identify at-risk populations in low-transmission settings in geographically restricted areas (‘hot spots’), populations at increased risk (‘hot pops’), as well as to monitor changes in malaria transmission over time. It will be important to standardize serological assays to ensure the generalizability of results, a task that should be addressed in future studies.

5.5 References


CURRICULUM VITAE

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EDUCATION AND TRAINING

Aug 2010 - Present  Doctor of Philosophy Candidate, International Health, Global Disease
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Dissertation title: Asymptomatic P. vivax malaria burden and naturally-
acquired humoral response to PvMSP19 in a low endemic malaria area
in the Peruvian Amazon Basin.

Aug 2008 – May 2009  Master of Health Science, Molecular Microbiology and Immunology,
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Master’s thesis title: Estrogen and Systemic Lupus Erithematosus

Mar 2002 – Dec 2007  Bachelor of Science, Biology, Universidad Peruana Cayetano
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PROFESSIONAL EXPERIENCE

Oct 2015 – Present  Research Scientist II, Department of Parasitology, U.S. Naval
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Aug 2012 – Present  Research Scientist I, Department of Parasitology, U.S. Naval Medical
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Jun 2009 – May 2010  Project Coordinator, Universidad Peruana Cayetano Heredia,
“Prevalence and transmission of Trypanosoma cruzi in rural
communities of the high jungle of Northern Peru”, Lima, Peru
Mar 2008 – Jul 2008  **Research Assistant**, Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, MD, USA


Jan 2007 – Jan 2008  **Research Assistant**, Instituto de Medicina Alexander von Humboldt (IMTAvH), Universidad Peruana Cayetano Heredia, Lima, Peru

Jan 2004 – Mar 2004  **Research Assistant**, McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University School of Medicine, Baltimore MD, USA

**PROFESSIONAL QUALIFICATIONS**

**PUBLICATIONS**

2015  
**Prevalence and Transmission of Trypanosoma cruzi in People of Rural Communities of the High Jungle of Northern Peru.**  

2012  
**Epidemiology of tobacco use and dependence in adults in a poor peri-urban community in Lima, Peru.**  
Weygandt PL, Vidal-Cardenas E, Gilman RH, Avila-Tang E, Cabrera L, Checkley W.  

2010  
**Rhizomelic chondrodysplasia punctata type 1: report of mutations in 3 children from India.**  

**POSTER PRESENTATIONS**

2014  
**Antibody response against PvMSP1-19 in symptomatic and asymptomatic *P. vivax* mono-infected subjects from a low endemicity area in the Peruvian Amazon Basin.**  
American Society of Tropical Medicine and Hygiene, 63rd Annual Meeting, New Orleans, USA, 2014.
2013

**Exploration of malaria and other vector-borne illnesses incidence in entomological workers.**
American Society of Tropical Medicine and Hygiene, 61st Annual Meeting, Atlanta, USA, 2013.

**RESEARCH GRANT PARTICIPATION**

**2014**

**Title of Grant and Sponsoring Agency:** “Validation of a Magneto Optical Diagnostic (MOD) RDT” – Fondo Nacional del Desarrollo Científico (FONDECYT) Ministerio de Salud del Peru: 2015-2018

**Principal Investigator and Funding Level:** Elisa Vidal-Cardenas - $235,000 over 3 years

**Main Grant Objective:** To evaluate the performance of a novel RDT in the Peruvian Amazon Basin.

**Principal Responsibilities of Individual:** Lead Investigator to evaluate the novel mobile MODS RDT; Management of field and laboratory activities; Data compilation and analysis; Manuscript preparation.

**2012**

**Title of Grant and Sponsoring Agency:** “Malaria infections in human-baited mosquito collectors in Peru” – Global Emerging Infections Surveillance and Response System (GEIS): 2012-2013

**Principal Investigator and Funding Level:** Andres G. Lescano - $34,000 over 1 year

**Main Grant Objective:** To estimate the risk of malaria and other mosquito-transmitted diseases in individuals who perform human-baited mosquito collections.

**Principal Responsibilities of Individual:** Associate Investigator; Data analysis; Manuscript preparation

**PROFESSIONAL DEVELOPMENT**

**Nov 2014**


**Oct 2011**


**Jul 2010**

*Certificate in Tropical Medicine,* Institute in Tropical Medicine and Public Health, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA

**Nov 2009**

* Advances in malaria research: In the lab and the field,* Johns Hopkins Malaria Research Institute, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA
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<td>Metaxenic diseases and their vectors</td>
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<td>2012 - 2015</td>
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<td>2003 - 2004</td>
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### MEMBERSHIP

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<td>ASTMH, American Society of Tropical Medicine and Hygiene</td>
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<td>2006 - Present</td>
<td>APEB, Asociación Peruana de Estudiantes de Biología</td>
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### ADDITIONAL INFORMATION

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