Clinical impact of submicroscopic malaria on delivery outcomes and the role of VAR2CSA antibodies in pregnant women from Colombia

by

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Abstract

Malaria caused by the genus *Plasmodium* (*P.*) is one of the oldest and most important parasitic infections globally. Pregnant women are at an increased risk for malaria, where the disease poses a significant risk to both maternal and child health. Better diagnosis of malaria can have an immediate impact on patient care, and provide a valuable tool to describe the prevalence of disease and host-parasite interactions. Although microscopy is the gold standard for malaria diagnosis, more sensitive tests are needed to identify submicroscopic infections (SMIs). In this thesis, I developed an RT-qPCR assay to identify *P. falciparum* and *P. vivax* infections at the submicroscopic level, validated it using panel of clinical samples from Colombia, and demonstrated the superior sensitivity directly to qPCR for screening SMIs at the species level.

The impact of malaria in pregnancy (MiP) has been well described. However, less is known about the effects of SMIs in pregnancy, particularly in areas of lower transmission where *P. falciparum* and *P. vivax* co-exist. To investigate the impact of SMIs in pregnancy, we performed a longitudinal study to follow pregnant women from an endemic area of Colombia with co-circulating *P. falciparum* and *P. vivax*. A cohort of 180 pregnant women from Colombia were followed through antenatal visits from recruitment until delivery. Malaria was diagnosed by light microscopy, SMIs by RT-qPCR, and placental malaria by histopathology. We evaluated the impact of SMI on maternal anemia, low birth weight (LBW), pre-mature birth (PTB), and small for gestational age (SGA) babies. We observed that SMIs occurred frequently in our pregnant population. Twenty-five percent (45/180) of the women were diagnosed with at least one SMI during follow-up. While SMIs were not generally associated with adverse outcomes at delivery, we showed a novel finding that women who had a mixed *P. falciparum* and *P. vivax* SMI during pregnancy, were significantly associated with risk for PTB compared to women without infection.

VAR2CSA, a protein belonging to the *P. falciparum* erythrocyte membrane protein-1 family, is expressed on the surface of infected red blood cells (iRBCs) and mediates binding of the iRBC to chondroitin sulfate A (CSA) found in the placenta. Antibodies specific to VAR2CSA have been shown to confer protection against MiP based on studies conducted in Africa. To test this in our population, we measured serum VAR2CSA-specific antibody levels by ELISA, tested the function of these antibodies by the ability to inhibit adhesion of iRBCs to CSA *in vitro*, and evaluated their role in protection from adverse clinical outcomes at delivery. We showed that over 60% of women analyzed in our study had antibodies to the full-length VAR2CSA protein at inclusion, regardless of parity. Antibody levels decreased during pregnancy and boosting was not observed following subsequent *Plasmodium* exposure. We observed that the function of antibodies, defined as the ability to inhibit CSA adhesion *in vitro*, and not antibody levels, were significantly associated with protection from SMI-related maternal anemia at delivery.

The work in this thesis involved the validation of a highly sensitive screening diagnostic for SMIs at the species level and also laid a framework for development of a direct-from-blood RNA-based diagnostic to be used in the field or at the point-of-care. Results from the longitudinal study revealed a high frequency of SMIs in pregnancy in this region and enhance our understanding of both the impact of SMI in pregnancy as well as the protective role of VAR2CSA-specific antibodies.

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List of Abbreviations

AMA-1 – apical membrane antigen 1

ANC – antenatal care

ARDS – acute respiratory distress syndrome

AU – arbitrary unit

bp – base pair

BSA – bovine serum albumin

cDNA – complementary DNA

CI – confidence interval

CQ – chloroquine

CSA – chondroitin sulfate A

C_T – cycle threshold

DAPI – 4',6-diamidino-2-phenylindole

DNA – deoxyribonucleic acid

dNTP – deoxyribonucleotide triphosphate

EBL – erythrocyte binding ligand

EDTA – ethylenediaminetetraacetic acid

ELISA – enzyme-linked immunosorbent assay

GP6D – glucose-6-phospghate dehydrogenase

Hb – hemoglobin

HEPES – 4-(2-hydroxyethyl)-1-perazineethanesulfonic acid

Hz – hemozoin

IPTp – intermittent preventative treatment in pregnancy

IBA – inhibition of binding assay

IE – infected erythrocyte

iRBC – infected red blood cell

IRS – indoor residual spraying

ITN – insecticide treated net

LB – Luria-Bertani

LBW – low birth weight

LOD – limit of detection

MiP – malaria in pregnancy

MIQE – minimum information necessary for publication of qPCR experiments

MJ – movement junction

MSP – merozoite surface protein

NaCl – sodium chloride

NAT- nucleic acid-based test

NTC – no template control

OD – optical density

PBS – phosphate-buffered saline

PCR – polymerase chain reaction

POC – point of care

PQ – primaquine phosphate

PTB – pre-term birth

qPCR – real-time polymerase chain reaction or quantitative polymerase chain reaction

RBC – red blood cell

RNA – ribonucleic acid

RNase – ribonuclease

RP-C – RPMI-complete

RP-I – RPMI-incomplete

rpm – revolutions per minute

RPMI – Roswell Park Memorial Institute buffer

RT – room temperature

RT-qPCR – reverse transcription qPCR

SD – standard deviation

SGA – small for gestational age

SMI – submicroscopic infection

SP – sulfadoxine-pyrimethamine

TAE – Tris, sodium acetate trihydrate, EDTA

T_M – Melting temperature

TNA – total nucleic acid

VSA – variant surface antigen

Chapter 1 - Introduction

1.1. Malaria

1.1.1. Brief history

As one of the oldest diseases known to mankind, malaria or malaria-like symptoms has been described for more than 4,000 years [1]. The term malaria originates from the 18th century Italian "*mala aria"*, which directly translates to "bad air" [2]. Malaria-like symptoms, more often described as intermittent fever, have been documented as far back as 2700 BC in the Nei Ching, a historical Chinese medical writing [1], to the Greek doctrine of fevers by Hippocrates in the fourth century BC [3]. However, the mechanism of infection and disease transmission would remain unclear for several thousand years. It would not be until the end of the 19th century when French army surgeon, Charles Louis Alphonse Laveran, would discover the causative parasites in blood from a patient suffering from malaria [4]. Shortly after, Ronald Ross, a British medical officer in the Indian Medical Service, would discover that the malaria parasite was transmitted by the mosquito [5].

1.1.2. Life cycle

Malaria is caused by protozoan parasites of the *Plasmodium* genus. While more than 200 *Plasmodium* species have been described, there are five species of *Plasmodium* capable of infecting humans: *P. falciparum*, *P. vivax*, *P. ovale, P. malariae*, and *P. knowlesi*. *Plasmodium* spp. that infect humans have a complex life cycle and are dependent on two hosts: the female *Anopheles* mosquito, which is the definitive host, and an intermediate host, the human host. A detailed outline of the parasite's life cycle is described below and is depicted in Figure 1.1.

1.1.2.1. Pre-erythrocytic stage

Sporozoites are inoculated into the human host during a blood meal from an *Anopheles* mosquito infected with *Plasmodium* parasites. The mosquito probes the skin and either feeds directly from a pool of blood generated from a damaged surface or by directly cannulating from a subcutaneous blood vessel [6]. To prevent the blood from coagulating, saliva from the mosquito, mixed with a few hundred sporozoites, are deposited [6]. Once injected, the sporozoites begin moving in a random gliding pattern, leaving the injection site and entering the blood stream by breaking through the endothelial barrier [7]. Not all sporozoites that enter the human host make it to the blood stream; some are destroyed in the skin (0.5-5%) [8], while some enter the lymphatic vessel and migrate to a draining lymph node (~20%), where most are internalized by dendritic cells [7].

Sporozoites that enter the blood vessels rapidly travel through the endothelium and migrate to the liver, where they settle and infect a hepatocyte in a process called productive invasion [9]. Within the hepatocyte, the sporozoite undergoes nuclear replication and develops via a trophozoite stage into the mature liver schizont. In the case of *P. falciparum*, this can take a period of five to seven days and results in a liver stage schizont containing several thousand merozoites [10]. After inducing cell death of the hepatocyte, merozoites bud off in the form of a merosomes, membrane bound vesicles each containing 100-200 merozoites [10]. These merosomes exit the liver through the endothelial cell layer [11] and ultimately break off, releasing the merozoites into the blood stream where they invade red blood cells (RBCs) signalling the erythrocytic stage of development.

Not all sporozoites that enter the hepatic cell will immediately mature into the liver stage schizont. In the case of the human parasites *P. vivax* and *P. ovale*, an alternative pathway following hepatocyte invasion is the development into a dormant form known as the hypnozoite. Hypnozoites

may remain dormant for weeks, or even months or years, and the mechanism behind the development into dormancy or the activation of hypnozoites is not known [12].

1.1.2.2. Erythrocytic stage

The erythrocytic stage of infection marks the onset of malaria pathology in the human host. Following merosome disintegration, released merozoites efficiently invade RBCs through a mechanism involving erythrocyte surface glycophorins A/B/C and sialic acid as receptors [13-15]. Erythrocyte binding ligands (EBLs) are a family of *Plasmodium* type I transmembrane proteins that interact with reticulocyte binding proteins [16]. These EBLs have been shown to play a central role in invasion. While *P. falciparum* parasites have the affinity to invade both immature erythrocytes (reticulocytes) and mature erythrocytes, the sole invasion target for *P. vivax* is reticulocytes. Merozoite attachment to RBCs is mediated by low affinity interactions between merozoite surface proteins (MSP) and host cell receptors on the RBC. Initial contact between the merozoite and erythrocyte can occur at any point on the merzoite surface, but is rapidly followed by reorientation of the merozoite to allow for invasion [17]. The reorientation process is mediated by apical membrane antigen 1 (AMA-1), which orients the merozoite so that the apical end is directly opposing the surface of the erythrocyte membrane [18]. A moving junction (MJ) is formed between the merozoite and erythrocyte through which the parasite can enter the host cell [19].

Upon penetration of the RBC, a parasitophorous vacuole is formed in which the parasite remains and continues to feed off the hemoglobin inside the cell [20]. This stage of the parasite is also known as the ring stage, a name derived from the morphology of the parasite as seen under a microscope [21]. As the parasite continues to feed and grow (~12 hours from point of erythrocyte invasion), hemozoin crystals, a product of active digestion of hemoglobin, and Maurer's clefts begin to

form, signalling the onset of the trophozoite stage [21, 22]. The parasite continues to undergo asexual reproduction leading to schizogony. Multiple nuclear divisions result in a multinucleated cell that eventually form anywhere between 8-32 merozoite buds [23]. Lysis of the infected RBC (iRBC) leads to the release of these merozoites back into the blood stream, allowing for merozoite infection of new RBCs and the erythrocytic cycle continues.

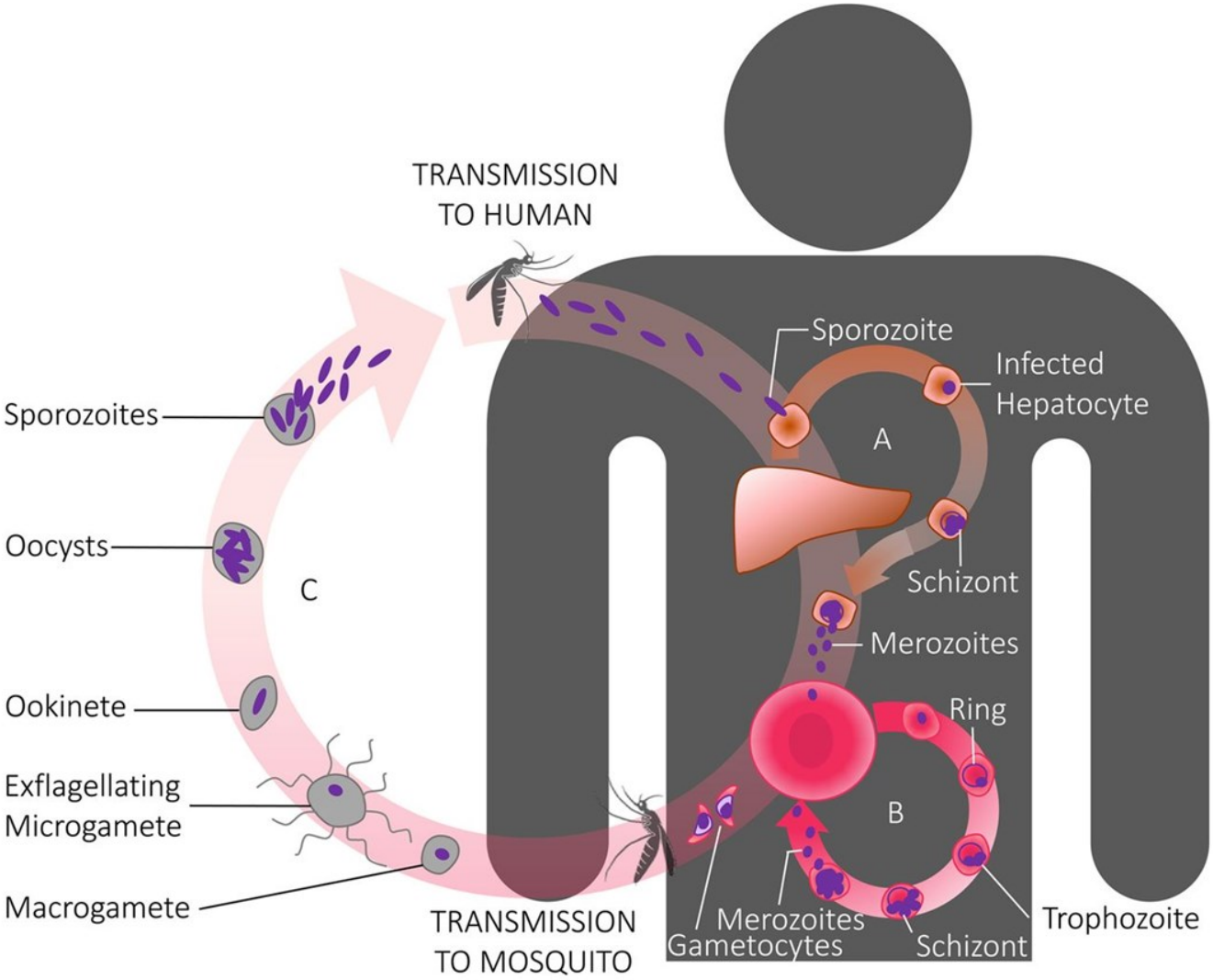


Figure 1.1. The malaria life cycle (A) The hepatic or pre-erythrocytic cycle, (B) erythrocytic cycle, and (C) sexual cycle. Infection begins in the intermediate human host when an infectious mosquito injects sporozoites during a blood meal and ends in the intermediate host when the mosquito uptakes gametocytes to continue the life cycle in the definitive host – a female *Anopholes* mosquito. Source: Anti-malarial strategies among vulnerable populations: exploring bed net underutilization among internally displaced persons and novel adjunctive therapies for cerebral malaria, *MSc Thesis, University of Alberta* 2017 - reproduced with permission from Brooks HM.

1.1.2.3. Sexual stage in Anopheles spp.

A subset of parasites will cease asexual reproduction and differentiate into the sexual cells (gametocytes). Haploid macrogametocytes (female) and microgametocytes (male) are the precursors of female and male gametes, respectively. Mature gametocytes may be ingested when a female *Anopheles* mosquito feeds on an infected host. The gametocytes undergo further development in the midgut of the mosquito, differentiating into their respective female and male gametes. The gametes fertilize in the stomach leading to the formation of a zygote. This zygote transforms into a motile ookinete, traverses the midgut and penetrates the epithelial cells to encyst between the midgut epithelia and basal lamina, and continues to develop as an oocyst [24]. These oocysts continue to grow, generating several hundreds to thousands of sporozoites, which migrate to the salivary gland of the mosquito, re-initiating the cycle and leading to opportunities to infect new intermediate hosts [25].

1.1.3. Global burden of malaria

Malaria is the leading parasitic cause of disease in the world. In 2016, there were an estimated 216 million cases of malaria globally, an increase of approximately five million from 2015 [26]. Deaths attributable to malaria in 2016 were estimated at 445,000 [26]. A vast majority of these malaria mortalities occurred in sub-Saharan Africa, in children under the age of five (Figure 1.2). *P. falciparum* is the most prevalent malaria species and is responsible for almost all cases of malaria in Africa (99%) [26]. While *P. vivax* is the second most prevalent malaria species, it is the most globally distributed species of human-causing malaria and accounts for approximately 50% of the global malaria burden outside of Africa [27, 28]. For countries with active cases of malaria, the burden of malaria can be classified as follows: hypoendemic (parasite rate in children ages 2-10 years: 0-10%), mesoendemic

(parasite rate in children ages 2-10 years: 10-50%), hyperendemic (parasite rate in children ages 2-10 years: 50-75%), or holoendemic (parasite rate in children ages 2-10 years: >75%) [29].

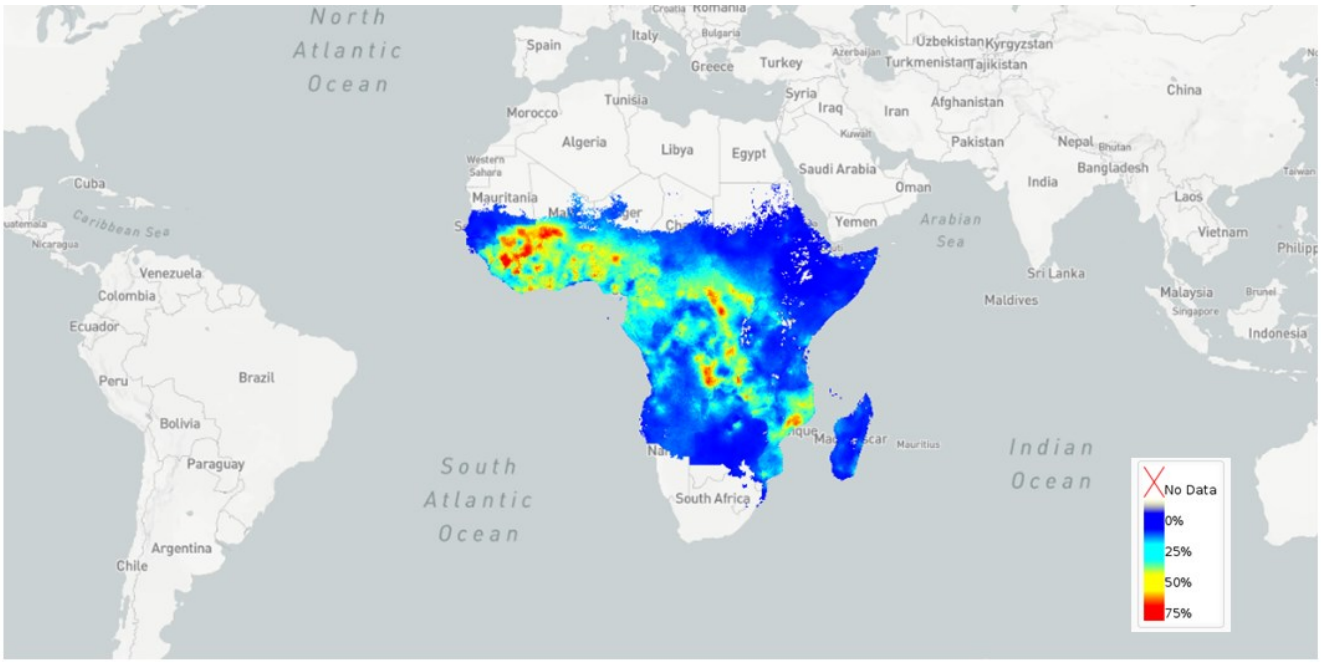


Figure 1.2. *P. falciparum* endemicity in Africa. Color grade describes the estimated proportion of children ages 2-10 years old in the population that are infected with *P. falciparum* parasite at any given time, averaged over the year for 2015. Adapted from The Malaria Atlas Project – data generated from Bhatt *et al.*, *Nature* 2015.

The true burden of malaria is hard to estimate, particularly in low resource countries where data collection and quality are poor. In these countries, particularly in regions of hyperendemicity, the unavailability of malaria diagnostic tools can lead to the under-diagnosis of malaria cases [30]. However, the same can be true in regards to the over-diagnosis of malaria cases, in which malaria infections are diagnosed on the basis of fever and not confirmed by some form of diagnostic test [31].

The burden of malaria is traditionally assessed by microscopy. However, individuals harbouring low-level parasites in the absence of clinical disease are often missed. These infections are often below the limit of detection of current field diagnostics [32]. Low-level infections that are undetectable by microscopy alone can be defined as a submicroscopic infection (SMI). The increased use of molecular diagnostics for the detection of SMIs in surveys have continuously demonstrated higher numbers of infected individuals compared to when detecting infections with microscopy alone [33], questioning our understanding of the true burden of malaria. A systematic review identified that microscopy misses on average half of all *Plasmodium* infections in endemic areas compared with diagnosis by molecular diagnostics [32].

1.1.4. Transmission

The transmission of malaria is a dynamic process that involves several interlinked factors. There are currently 91 countries with ongoing malaria transmission [26]. Malaria in these countries can be defined as either stable or unstable, a classification that was determined by the average number of feeds that a mosquito takes on a human being during its life [34]. Areas with stable malaria can be described as having stable transmission conditions with malaria infections occurring for multiple months in a year, often over many years. Unstable malaria refers to more seasonal transmission, as seen

in areas of low endemicity. Factors that affect the rate of malaria transmission can include both climatic factors (e.g. such as temperature and rainfall) and non-climatic factors (e.g. human population dynamics or migration, mosquito vectors) [35]. The adoption of malaria control measures, such as the use of insecticide treated nets (ITNs) and indoor residual spraying (IRS) will also affect the transmission of malaria in a particular region.

1.1.5. Clinical presentation of malaria

While *P. falciparum* is the most virulent species of human malaria, the manifestation of disease and symptoms is broad; ranging from an asymptomatic infection to severe malaria, which can include multiple organ failure and death [36]. Malaria is primarily associated with the presence of fever. In individuals without immunity, initial symptoms can include fever, headache, and chills, which appear 10-15 days after the initial infection from a mosquito bite [24]. If untreated within the first 24-48 hours, *P. falciparum* can progress to severe malaria, often leading to death. The manifestations of severe malaria include cerebral malaria, severe anemia, acute respiratory distress syndrome (ARDS), hyperparasitemia (>5%), metabolic acidosis, or multiple organ failure [37]. In areas of high transmission, individuals may also develop partial immunity, resulting in asymptomatic cases of malaria [38].While there is no standard definition for asymptomatic malaria, it is generally accepted as an infection of any level of parasitemia in the absence of fever or other acute symptoms [39]. *P. vivax* infections, while generally considered more benign, still contributes to significant morbidity [40]. Additionally, *P. vivax* poses the threat of relapsing infections, caused by the activation of latent live-stage hypnozoites.

1.1.6. Treatment of disease

The first effective described treatment for malaria dates back to the second century BC in China, with the use of the *Qinghao* plant (*Artemisia annua*) [1]. It would not be until 1971 when the active ingredient of *Qinghao* would be isolated and identified as artemisinin by Chinese scientists. In the 1600s, bark from the Peruvian cinchona tree was used by Jesuit missionaries in South America to treat malaria [41]. Cinchona bark would later be referred to as "Jesuits' bark" or "sacred bark" for its effectiveness in treating the disease. Prior to 1820, treatment with cinchona bark was administered by first drying the bark, grinding it into a fine powder, and mixing it into a drinkable liquid to be consumed. However 1820, Pierre Joseph Pelletier and Joseph Caventou successfully isolated and extracted quinine (the active ingredient of cinchona bark) from the bark, replacing whole cinchona bark as the standard treatment for malaria [42].

In 1934 chloroquine (CQ), initially referred to as resochin, was developed by German scientists as an alternative treatment for quinine and would be used heavily by the WHO for their global malaria eradication campaign following World War II [43]. With a similar mechanism of action to quinine, CQ actively inhibits heme polymerase activity, resulting in the accumulation of free heme, which is toxic to the parasite [44]. Emerging resistance to CQ was followed by the development of sulfadoxine-pyrimethamine (SP), which was introduced as a treatment in Thailand in 1967 [43]. However, resistance to SP would appear in the same year and would spread quickly throughout Asia.

While CQ and SP are still being used to treat malaria today, the best available treatment, specifically for *P. falciparum* infection, is artemisinin-based combination therapy (ACT), a combination of an artemisinin derivative paired with a partner drug that targets both the sexual and asexual blood stage parasites [26]. The mechanism of action appears to involve the heme-mediated decomposition of the endoperoxide bridge, producing carbon-centred free radicals [45]. The WHO

recommends that all cases of suspected malaria be confirmed by diagnostic testing prior to administering treatment [10]. Treatment based on presumptive diagnosis, cases in which a malaria diagnostic test is not performed and the presence of fever is the main indicator of disease, should only be considered when diagnostic tools are unavailable (e.g. in the field, under-resourced clinics) [46]. In cases of infection with *P. vivax* or *P. ovale*, patients must also be treated with primaquine phosphate (PQ) following blood stage treatment. PQ targets dormant liver stage hypnozoites that can cause relapse infections. While the mechanism of PQ is not well understood, proposed mechanisms involve disruption of parasite DNA structure and reactive oxygen species that may interfere with the electron transport chain in the parasite [47]. A critical challenge to treating *P. vivax* infections is that PQ may not be used to treat patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency or pregnant patients; there is currently no alternative treatment for *P. vivax* infections in pregnancy. Primaquine is not safe in pregnancy; therefore radical cure is not indicated until after delivery.

1.1.7. Emerging antimalarial drug resistance

Drug resistance to antimalarials has been a recurring problem for decades. Resistance of *P. falciparum* to early generation drugs, such as CQ and SP, has been documented as early as the 1950s [10]. ACT, the optimal choice for malaria treatment, is the combination of the drug artemisinin and a partner drug. In recent years, *Plasmodium* resistance to artemisinin derivatives has been reported in five countries within the Greater Mekong sub-region (Cambodia, Lao People's Democratic Republic, Myanmar, Thailand, and Vietnam) [26]. In 2014, the discovery of a molecular marker of artemisinin resistance, a mutant kelch-13 propeller domain protein (K13-propeller; PF3D7_1343700), was associated with both *in vivo* and *in vitro* artemisinin resistance [48]. Early identification of emerging or low-level resistance to artemisinin will be integral to controlling the spread of drug resistance. In 2015, the WHO launched the Strategy for Malaria Elimination in the Greater Mekong Sub-region (2015-

2030) and developed national level malaria control programs and elimination plans in cooperation with the countries within the Greater Mekong Sub-region [49].

1.2. Diagnostics for malaria

1.2.1. Microscopy

To this day, microscopy remains the gold standard method for malaria diagnosis [26]. Patient blood samples are collected and spread out on a microscope slide, also referred to as a "blood smear". The specimen is fixed with methanol and stained, most often with Giemsa stain, prior to examination. A skilled microscopist can differentiate *Plasmodium* species, which is important for following appropriate treatment guidelines. However, there are several challenges in the use of microscopy as a primary diagnostic tool. These include: availability of equipment and electricity, time consumption and labour intensity, and the lack of skilled technologists in affected areas, which often lead to poor interpretation of the peripheral smear [50]. In the hands of a skilled microscopist, microscopy has a detection limit of $5x10^3$ – $5x10^4$ parasites/mL (p/mL) [51, 52] however, in field conditions a detection limit of about $5x10^4$ – $1x10^5$ p/mL is more realistic [53], limiting its use at low density parasitaemia. Several national malaria control programs train local microscopists with variable degrees of success [53]. WHO training materials are available and still commonly used [36, 54]. Improving diagnostic accuracy in malaria control programs are both technically and financially challenging [26]. Continued support is essential to ensure accurate diagnosis and appropriate treatment.

1.2.2. Rapid diagnostic tests (RDTs)

Though microscopy has been in use for over 100 years, the past few decades gave rise to alternative methods for malaria diagnosis, such as the detection of malaria antigens by indirect immunofluorescence antibody assays (IFA) and enzyme-linked immunosorbent assays (ELISA) [55, 56]. These methods to detect malaria antigens form the basis of several of the commercial malaria rapid diagnostic tests (RDTs) available today [52]. A RDT is a single-use device that allows for the diagnosis of malaria at the point of care (POC). Most commercially available RDTs use immunochromatography, require a small amount of blood (5-15μL), and provide results in approximately 20 minutes [53]. According to the WHO [57], RDTs must be capable of reliably detecting 10^5 p/mL, the equivalent of 0.002% parasitaemia, must have a minimum sensitivity of 95% compared to microscopy, and a minimum specificity of 90% for *Plasmodium* species. Most commonly used RDTs only detect *P. falciparum*; however, some RDTs on the market capable of detecting and/or differentiating between *P. falciparum* and non-*falciparum* infections (*P. vivax*, *P. malariae*, and *P. ovale*). RDT usage has expanded significantly all over the world in recent years. From 2010 – 2016, 1.66 billion RDTs were sold globally by manufacturers eligible for the Malaria RDT product testing program [26].

1.2.3. Molecular diagnostics

The rise in asymptomatic malaria and SMIs have created a demand for more sensitive diagnostic tests. Nucleic acid based molecular diagnostics (or NATs, Nucleic acid-based tests) provide a highly sensitive and specific means of detecting malaria parasites. Amplification of parasite DNA using polymerase chain reaction (PCR), or loop-mediated isothermal amplification (LAMP), are two methods that can detect submicroscopic low-level parasitaemias. However, both methods require specialized equipment and laboratory settings, and are not suitable in most field settings [50, 58]. Although most molecular assays are not POC, they have become increasingly implemented for

surveillance and surveying because they have higher sensitivities than microscopy or RDTs, and also provide definitive species identification [59].

1.2.3.1. Polymerase chain reaction (PCR) and Loop-mediated isothermal amplification (LAMP)

In the context of malaria elimination, one of the major challenges that must be addressed is the demand for more sensitive diagnostics to identify the submicroscopic reservoir of infection. The superiority of PCR to microscopy as a diagnostic tool for malaria has been extensively described [32, 33, 58]. The increased sensitivity and specificity are highly desirable in areas of low malaria transmission intensity, where a great proportion of asymptomatic cases are submicroscopic [32, 60]. To maximize sensitivity, the majority of tests for malaria have been developed to target multi-copy genes, including the *Plasmodium* 18S ribosomal RNA (rRNA) gene [61, 62], subtelomeric targets [63], and mitochondrial genes such as cytochrome *b* [64] and *Plasmodium* mitochondrial cytochrome C oxidase III [65].

LAMP is a molecular technique that can also detect specific genes of a target microorganism. LAMP and conventional PCR are similar in that both methods amplify DNA; however, in contrast to PCR, LAMP can be substantially more cost-effective in that it does not require the sophisticated equipment required to run PCR [66]. LAMP uses simple equipment, such as a water-bath and ultra-violet lamp, and does not require a thermocycler for amplification nor a gel imaging system for analyzing results [67]. A study in Colombia using a LAMP kit to amplify *Plasmodium* mitochondrial DNA (mLAMP) displayed sensitivities in the range of 89.5-93.3% and a specificity in the range of 95.9-100% compared to PCR [59] . One limitation to this specific method is its inability to differentiate non-*falciparum Plasmodium* species. Further, this particular kit was unable to differentiate mixed *Plasmodium* infections from *P. falciparum* mono-infections.

1.2.3.2. Real-time PCR (qPCR)

In terms of molecular diagnostics, PCR remains the most commonly used method as it allows for explicit identification of malarial species and can be adapted for high-throughput applications [68]. Real-time PCR (qPCR) has further improved the application of PCR in that it is highly sensitive for single *Plasmodium* species infections and mixed infections, automated, quantitative, and has a very low risk of contamination that was a limiting factor in conventional PCR [69]. Several qPCR methods for diagnosing malaria have been published displaying very good sensitivity and specificity for not just *P. falciparum*, but other *Plasmodium* species as well [62, 70-72], with a limit of detection (LOD) ranging from 127 to 4000 p/mL.

1.2.3.3. *Reverse transcription qPCR (RT-qPCR)*

Another malaria diagnostic tool that has been increasingly used in the past few years is reverse transcriptase quantitative PCR (RT-qPCR). RT-qPCR is an adaptation of qPCR technology that allows for the detection of RNA. RT-qPCR may be performed as either a one-step or two-step reaction. In a two-step reaction, reverse transcription and PCR occur in separate reactions; RNA is first reverse transcribed into complementary DNA (cDNA) and is then transferred to a separate reaction tube in which the cDNA is amplified by a DNA polymerase. In a one-step reaction, reverse transcription and amplification is performed in a single reaction in one tube. Utilizing the high copy numbers of RNA targets can greatly increase the sensitivity of the test. It is estimated that that there are several thousand RNA copies of 18S rRNA per parasite [73]. One published RT-qPCR method targeting a conserved region on the 18S rRNA gene describes a limit of detection of 2 p/mL at the genus level [74]. More work and research are required in order to attain this same sensitivity at the species-specific level.

1.2.4. Minimum information for publication of Quantitative Real-Time PCR Experiments (MIQE)

There is currently no standard molecular diagnostic test for malaria and the reported methods use several different approaches for sample processing, amplification, and detection [75]. A multitude of papers have been published over the last few decades describing methods for the molecular diagnosis of malaria. With no standard, comparing and evaluating the methods to one another can be challenging. The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) are a set of guidelines that describe the minimum information necessary for evaluating any form of diagnostic qPCR experiment [76]. The establishment of these guidelines was to ensure the integrity, transparency, and full disclosure of methods to allow other investigators to assess and reproduce the results. A full list of these items can be found in Table 1.1. These guidelines provide structure for the development of qPCR-based diagnostic methods and was the framework for the validation of the PCR work described in the later chapters of this thesis.

Table 1.1. Checklist of minimum information for publication of qPCR experiments for authors, reviewers and editors. [a] Adapted from [76]

Item to check	Importance	Item to check	Importance
Experimental design		**qPCR oligonucleotides**	
Definition of experimental and control groups	E	Primer sequences	E
Number within each group	E	RTPrimerDB identification number	D
Assay carried out by the core or investigator's laboratory?	D	Probe sequences	D
Acknowledgment of authors' contributions	D	Location and identity of any modifications	E
Sample		Manufacturer of oligonucleotides	D
Description	E	Purification method	D
Volume/mass of sample processed	D	**qPCR protocol**	
Microdissection or macrodissection	E	Complete reaction conditions	E
Processing procedure	E	Reaction volume and amount of cDNA/DNA	E
If frozen, how and how quickly?	E	Primer, (probe), Mg^{2+}, and dNTP concentrations	E
If fixed, with what and how quickly?	E	Polymerase identity and concentration	E
Sample storage conditions and duration (especially for FFPE[2]samples)	E	Buffer/kit identity and manufacturer	E
Nucleic acid extraction		Exact chemical composition of the buffer	D
Procedure and/or instrumentation	E	Additives (SYBR Green I, DMSO, and so forth)	E
Name of kit and details of any modifications	E	Manufacturer of plates/tubes and catalog number	D
Source of additional reagents used	D	Complete thermocycling parameters	E
Details of DNase or RNase treatment	E	Reaction setup (manual/robotic)	D
Contamination assessment (DNA or RNA)	E	Manufacturer of qPCR instrument	E
Nucleic acid quantification	E	**qPCR validation**	
Instrument and method	E	Evidence of optimization (from gradients)	D
Purity (A_{260}/A_{280})	D	Specificity (gel, sequence, melt, or digest)	E
Yield	D	For SYBR Green I, C_q of the NTC	E
RNA integrity: method/instrument	E	Calibration curves with slope and *y* intercept	E
RIN/RQI or C_q of 3′ and 5′ transcripts	E	PCR efficiency calculated from slope	E
Electrophoresis traces	D	CIs for PCR efficiency or SE	D

Inhibition testing (C_q dilutions, spike, or other)	E	r^2 of calibration curve	E
Reverse transcription		Linear dynamic range	E
Complete reaction conditions	E	C_q variation at LOD	E
Amount of RNA and reaction volume	E	CIs throughout range	D
Priming oligonucleotide (if using GSP) and concentration	E	Evidence for LOD	E
Reverse transcriptase and concentration	E	If multiplex, efficiency and LOD of each assay	E
Temperature and time	E	**Data analysis**	
Manufacturer of reagents and catalogue numbers	D	qPCR analysis program (source, version)	E
C_qs with and without reverse transcription	D[3]	Method of C_q determination	E
Storage conditions of cDNA	D	Outlier identification and disposition	E
qPCR target information		Results for NTCs	E
Gene symbol	E	Justification of number and choice of reference genes	E
Sequence accession number	E	Description of normalization method	E
Location of amplicon	D	Number and concordance of biological replicates	D
Amplicon length	E	Number and stage (reverse transcription or qPCR) of technical replicates	E
In silico specificity screen (BLAST, and so on)	E	Repeatability (intraassay variation)	E
Pseudogenes, retropseudogenes, or other homologs?	D	Reproducibility (interassay variation, CV)	D
Sequence alignment	D	Power analysis	D
Secondary structure analysis of amplicon	D	Statistical methods for results significance	E
Location of each primer by exon or intron (if applicable)	E	Software (source, version)	E
What splice variants are targeted?	E	C_q or raw data submission with RDML	D

[a] (E) Essential information must be included and submitted with the manuscript. (D) Desirable information should be submitted if available or if requested.

[b] FFPE, formalin-fixed, paraffin-embedded; RIN, RNA integrity number; RQI, RNA quality indicator; GSP, gene-specific priming; dNTP, deoxynucleoside triphosphate.

[c] When assessing a reverse-transcription assay, absence of DNA is essential during RNA extraction step. Sample must be validated as DNA free and a no-reverse transcription control is recommended.

1.2.5. Target populations for malaria diagnosis

Pregnant women and young children are the most vulnerable populations to adverse clinical outcomes from malaria, contributing a large proportion to the global malaria morbidity and mortality [26, 77]. As such, these two demographics are regarded with high priority for clinical diagnosis and are sometimes referred to as a "hot population", defined as an at-risk group (population) that can be targeted for enhanced malaria control [78]. It has become increasingly accepted that these two populations contribute significantly to the submicroscopic reservoirs of infection, harbouring low-level parasites in the absence of clinical disease [32]. With the use of sensitive diagnostics, it is important to detect these infections, but to also identify the species in order to guide appropriate control measures and treatment.

1.3. Malaria in pregnancy

1.3.1. Burden of MiP

Malaria in pregnancy (MiP) poses a significant risk to both the mother and the fetus and can lead to adverse pregnancy outcomes [79]. It is estimated that over 50 million pregnant women are exposed to malaria each year [80]. The impact of the disease causes substantial maternal and fetal morbidity and mortality. In Africa, it is estimated that 10,000 maternal and over 100,000 infant deaths occur annually as a result of malaria infection during pregnancy [77]. Microscopy, RDTs, and PCR are the major options for the detection of MiP. Additionally, histopathology of placental specimens can also be used to identify parasites and assess inflammatory responses or damage. The use of PCR and other molecular diagnostics for SMIs have demonstrated how diagnosis by microscopy alone underestimates the total burden of MiP; in nine studies investigating the frequency of submicroscopic MiP, the mean ratio of microscopy to PCR prevalence was 0.46 (95% CI 0.35-0.57) [80]. Both *P.*

falciparum and *P. vivax* can cause MiP and have been associated with adverse pregnancy outcomes, which will be described below.

1.3.2. MiP control and prevention

Prevention and control of MiP are heavily reliant on multiple control measures; two previously mentioned measures include the use of ITNs and IRS. In African regions of high endemicity, intermittent preventative treatment in pregnancy (IPTp) with sulfadoxine-pyrimethamine is implemented through antenatal follow-up [10]. However, implementations of these control measures have been suboptimal; in 2014, only 39% of pregnant women reported sleeping under an ITN [81], and in 2016, only 31.5% of eligible pregnant women received three or more doses of IPTp [26]. In addition to these control measures, effective case management of malaria and anemia are also imperative [82].

1.3.3. Pathology and outcomes of MiP

Pregnant women are particularly vulnerable to infection with *P. falciparum,* which can sequester in the placenta and cause placental malaria, maternal anemia (<11 g/dL), pre-term birth (PTB, <37 weeks gestational age at delivery), and low birth weight (LBW, <2500 g) infants [79]. MiP has been significantly associated with an increased risk of congenital malaria [83], infant malaria [84-86], infant anemia [81], and other febrile illnesses [87, 88]. Most of our knowledge regarding MiP comes from Africa, in areas of high *P. falciparum* transmission. Disease pathogenesis in low transmission areas is very similar; however, acquisition of immunity is delayed (discussed in further detail in section 1.3.4.) [89]. The increased vulnerability to malaria of pregnant women over the non-pregnant population can be attributed to the sequestration of iRBCs in the placenta, a common occurrence with *P. falciparum* infections [80]. Although some studies showed that *P. vivax* parasites

can cytoadhere to placental tissue *ex-vivo* [90, 91], this is not considered a pathogenic mechanism of *P. vivax* infection [89, 92]. *P. vivax* infection during pregnancy was associated with adverse outcomes in studies from Thailand and Indonesia [93, 94]; however, studies in Latin America reported varying results. One study in Colombia demonstrated that *P. vivax* infection during pregnancy was associated with lower birth weight [60], while another study, also in Colombia, demonstrated no change in mean birth weight, gestational age (GA), or hemoglobin levels at delivery [95]. In studies from Brazil, Bolivia, Peru, and Venezuela, *P. vivax* was associated with anemia, reduced birth weight, and histological changes in the placenta [96-99].

1.3.3.1. Placental sequestration

In *P. falciparum* malaria, cytoadherence is mediated by the interactions between the parasite protein VAR2CSA, a member of the *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) family, which is expressed on the surface of iRBCs, to chondroitin sulphate A (CSA) found on the surface of synctiotrophoblasts and in the intervillous space [100]. A study conducted by Doritchamou *et al.* [101], has shown that *var2csa* transcripts are among the highest in malaria isolates from pregnant women and that the expression of VAR2CSA on the surface of iRBCs are generally restricted to only placental parasites and occurs during pregnancy. The structure of the VAR2CSA protein consists of multiple Duffy binding-like (DBL) domains and inter-domain (ID) regions, of which DBL 2, 3, 5, and 6 have been associated with binding to CSA [102]. Placental sequestration often leads to inflammatory responses, most notable is the accumulation of monocytes in the placenta, which is often associated with maternal anemia, fetal growth restriction, and LBW [79, 103].

1.3.3.2. Immunity

Women in their first pregnancy are more susceptible to placental malaria as this is their first exposure to placental parasites expressing VAR2CSA and they have not developed immunity against this antigen. Pregnant women acquire antibodies to VAR2CSA following exposure to MiP in a parity-dependent manner [79]. These antibodies can block adhesion of iRBCs to CSA *in vitro* [104, 105], and are associated with protection from placental malaria and other adverse birth outcomes [106, 107]. Additionally, antibodies can opsonize iRBCs for phagocytosis have been associated with protection from maternal anemia [108]. Recent studies question whether VAR2CSA-specific immunity is parity-dependent. One study conducted in Colombia demonstrated that primigravid women, men, and children had high levels of antibodies against VAR2CSA [109]. Another study conducted in Cameroon observed VAR2CSA-specific antibodies in children and non-pregnant teenagers [110]. These findings challenge our current understanding of VAR2CSA and demonstrate a need into further study.

1.3.4. Submicroscopic MiP

The application of molecular diagnostics has revealed that SMIs are frequently detected in pregnant women. Submicroscopic MiP in women from sub-Saharan Africa, as detected by nested PCR and/or qPCR, were associated with adverse outcomes in several cross-sectional [111-114] and longitudinal studies [115, 116]. Few studies examined the effects of SMIs in regions where *P. falciparum* and *P. vivax* co-circulate. *P. falciparum* and *P. vivax* SMIs detected at delivery by qPCR were associated with poor outcomes in one study from Papua New Guinea (PNG) [103] but not in studies conducted in Colombia and India [60, 117]. In a multi-centre study of pregnant women in Colombia, Guatemala, Brazil, India, and Papua New Guinea (PNG), submicroscopic *P. falciparum* and *P. vivax* infections detected by qPCR were not associated with either maternal anemia or low birth weight [118]. These findings warrant further investigation to determine whether pregnant women

infected with a SMI from a co-endemic region of *P. falciparum* and *P. vivax* are at risk for adverse clinical outcomes.

1.3.5. MiP in Latin America

Only until recently, MiP in Latin America has received very little recognition as a public health problem, largely due to the low prevalence of malaria and high proportion of supposedly benign *P. vivax* infection [119]. Recent studies demonstrating the high burden of SMIs [60, 120] and the shift in focus to target pregnant asymptomatic women for malaria elimination has caused MiP in Latin America to garner increased attention.

There are 21 countries endemic for malaria in the region with an estimated 120 million people living in areas at risk for malaria [26, 121]. An estimated 4.3 million pregnancies are at risk for malaria infection each year [122]. Over 90% of the burden of disease is concentrated within the Amazon basin, a region that borders the countries of Brazil, Colombia, Guyana, Peru, and Venezuela [123]. *P. falciparum* and *P. vivax* co-circulate within this region and are characterized by a lower malaria transmission with clusters of high endemicity in some areas [119]. The prevalence of MiP as measured by microscopy is generally low in Latin America, less than 10% in Peru, Bolivia, Honduras, and Brazil [96, 98, 124-128], under 15% in Colombia [60, 129, 130], and under 30% in Venezuela [131]. However, the application of molecular diagnostics for malaria revealed an alarming proportion of SMIs in this region. Five studies that compared microscopy to PCR to investigate the frequency of MiP can be summarized in Table 1.2. While low rates of SMIs were observed in areas of Peru and Brazil [127, 128], high rates of SMIs were observed in Northwest Colombia [60, 129, 130].

Table 1.2. Studies of submicroscopic infections in pregnancy in Latin America. Adapted from [119]

Study Site	Time and Year of Collection	Microscopic Prevalence	Submicroscopic Prevalence	Species by PCR	Clinical Outcomes	Reference
Peruvian Amazon	Delivery 2004	(M) 1.0% (P) 0.5	(M) 5.6% (P) 4.7%	(M) 64% Pv, 18% Pf, 9% Pm, 9% Pf/Pm (P) 44% Pv, 44% Pf, 11% Pm	None examined based on PCR; no correlation of hemozoin positive placentas with birthweight or gestational age	[128]
Northwest Colombia	Delivery 2005-2009	(M) 13% (P) 9%	(M) 19% (P) 17%	(M) 74% Pv, 26% Pf (P) 65% Pv, 31% Pf, 4% Pf/Pv	None	[130]
Northwest Colombia	Delivery 2008-2011	(M) 9.1% (P) 3.3%	(M) 4.9% (P) 13.2%	(M) 65% Pv, 35% Pf (P) 65% Pf, 35% Pv	One stillbirth reported	[129]
Northwest Colombia	Delivery 2005-2011	(M) 14% (P) 9%	(M) 35% (P) 48%	(M) 49% Pf, 47% Pv, 4% Pf/Pv (P) 51% Pf, 36% Pv, 13% Pf/Pv	No association with birthweight, gestational age, or anemia; inflammation detected in the placenta	[60]
Atlantic Forrest, Brazil	ANC 2012-2013	(M) 1.6% (P) N/A	(M) 4%	M) 43% Pv, 57% Pm (P) N/A	None	[127]

[a] M, maternal peripheral blood; P, placental blood.
[b] Percent PCR positive women minus the percentage of women with a microscopic infection.
[c] N/A, not available; *Pf, P. falciparum, Pm, P. malariae; Pv, P. viva*x

There are currently seven countries (Argentina, Belize, Costa Rica, Ecuador, El Salvador, Mexico, and Paraguay) in Latin America that are in the pre-elimination or elimination phases of malaria control [26, 121]. As the total number of malaria cases continue to decline, this will result in a greater proportion of undetected asymptomatic infections and SMIs [26]. In the context of elimination, it will be important to target this population, and the pregnant women represent an integral target population [132]. A major challenge will be the ability to detect SMIs near or at POC. While retrospective molecular diagnosis may be useful for surveillance and epidemiological studies, it does not support active case detection [119]. In the context of Latin America, it will be important that these POC diagnostics are not only sensitive enough to detect low-level SMIs, but also species-specific in order to facilitate appropriate malaria treatment.

Another challenge for MiP control in Latin America is access to antenatal care (ANC), particularly to those living in remote and inaccessible locations. A 2008-2009 national survey in Brazil, looking at indigenous people's health and nutrition [133], reported that only 46.1% of women nationwide had at least one prenatal consultation during their first trimester of pregnancy. An observational cross-sectional study conducted in Colombia [134] compared the frequency of ANC (how often pregnant women would visit), quality of ANC (assessment of variables received at ANC; e.g. physical examination, maternal education), and timing of ANC (how early pregnant women would seek ANC) on the impact of LBW. Results revealed that 97.59% of women who gave birth from November 2006 – November 2010 had received some form of ANC and that both the quality and number of ANC visits were associated with LBW. Access to a healthcare facility and skilled professional also varies from country to country in Latin America [135]. High rates of ANC and delivery by health care personnel are not only affected by location, but pregnant women also need to believe that such services must be worthwhile for the women to use them. Several factors, including

cultural beliefs or socioeconomic status, may affect the results of deliveries and accessibility to health care facilities.

The impact of SMIs on delivery outcomes has not been systematically assessed. As described in section 1.3.4, studies have shown conflicting results on the impact of submicroscopic *P. falciparum* and *P. vivax* infections on delivery outcomes when compared to studies based in Africa. A few key things must be taken into consideration when to comparing submicroscopic MiP in Latin America to submicroscopic MiP in Africa. Firstly, IPTp is not administered during antenatal follow-up in Latin America, as the WHO only recommends IPTp for regions with moderate to high transmission. Secondly, unlike most high transmission regions in Africa, these low transmission regions in Latin America have the co-circulation of both *P. falciparum* and *P. vivax*. Additionally, a study has shown that in this region antibodies against VAR2CSA, which has been correlated with protection, appear to be acquired independent of parity [109]. MiP immunity in this region is likely complicated by the low endemicity of the region and predominance of *P. vivax* infections [119], resulting in a broad range of severity of delivery outcomes, and warranting further investigation.

1.4. Thesis Objectives

Our lab group's initial investigation into the prevalence of submicroscopic MiP in Colombia revealed a high frequency of infection in pregnant women at delivery [60]. However, contrary to the many studies in Africa [111-116] that demonstrated associations of SMIs in pregnancy with adverse birth outcomes, delivery outcomes in Colombia were mostly normal. Given the substantial endemicity of malaria in this region, these results prompted further investigation to the clinical relevance of submicroscopic MiP, as well as defining host immunity of MiP in this region. My thesis, as part of a larger collaborative project, aims to explore the impact of MiP in women from Colombia and to ask

whether SMIs in pregnancy are associated with adverse outcomes at delivery. This research was divided into three main objectives:

i) To determine the prevalence of submicroscopic malaria and *Plasmodium* species distribution in pregnancy in an endemic region of Colombia;

ii) To characterize the host immune response to SMI, specifically in regards to the function of anti-VAR2CSA antibodies; and

iii) To evaluate clinical outcomes at delivery for both the mother and the newborn.

To accomplish these objectives, the work described in this thesis can be grouped into three sections.

Section 1. Validation of an RT-qPCR method for the detection of submicroscopic *P. falciparum* and *P. vivax* infections.

In this section, I describe the validation of a malaria screening method that I applied to our longitudinal study (described in Chapter two) to identify SMIs. This method was validated with 519 blood samples, negative by thick-smear, from febrile and asymptomatic cohorts from Colombia. Chapter three describes the development of a sensitive species-specific RT-qPCR method to differentiate between *P. falciparum* and *P. vivax* infections at the submicroscopic level.

Section 2. Evaluating the impact of submicroscopic malaria infection on pregnant women from Colombia

Chapter four describes the longitudinal study conducted in Colombia characterizing the impact of SMIs in pregnancy and evaluating the role of VAR2CSA antibodies in protection from adverse clinical outcomes. Samples from Colombian women collected throughout pregnancy were screened retrospectively using the method validated in chapter three. We measured anti-VAR2CSA antibody

levels from serum and assessed their function to inhibit binding of iRBCs to CSA *in vitro* and correlate these results to delivery outcomes.

Section 3. Development of a direct-from-blood species-specific malaria molecular diagnostic to be used at point-of-care

In this section, I explored the potential of RNA-based diagnostics for use at POC. By adapting previously described direct-from-blood methods, I demonstrated that species-specific RNA detection can be performed directly from clinical blood samples. The work in chapter five describes the validation of a direct-from-blood RT-qPCR method to differentiate SMIs at the species level between *P. falciparum* and *P. vivax*.

Chapter 2. Materials and Methods

2.1. Study design

The experiments presented in this thesis form part of a prospective, longitudinal study designed by our lab group and collaborators to monitor women during the course of pregnancy and at delivery. A cross-sectional study of two cohorts were run in parallel to examine malaria infections in non-pregnant women and men: 1) patients who presented at the local health clinic with fever; and 2) participants who were asymptomatic. Data from these three groups were used to evaluate the prevalence of submicroscopic malaria and compare species distributions.

2.1.1. Malaria in pregnancy cohort

To investigate the impact of SMIs in pregnancy, we followed a cohort of 200 women longitudinally through pregnancy until delivery. A total of 402 women were recruited into the study, of which 200 women completed the full follow up. One hundred and eighty women were selected for studying the impact of SMIs (13 women were excluded due to abortion or still birth and 7 women were excluded due to a smear-positive malaria infection). Women were recruited at the antenatal care clinic and followed until delivery at the local hospital. Women were encouraged to attend the antenatal clinic on a monthly basis where they were provided with free medical examinations, antenatal vitamins, and were tested for *Plasmodium* spp. infection by thick smear. Women who tested positive by microscopy were treated according to Colombian health guidelines (artermether-lumefantrine or quinine-clindamycin for *P. falciparum* infections and chloroquine for *P. vivax* infections).

2.1.2. Non-pregnant cohort

Samples were collected from two separate non-pregnant cohorts: 1) febrile patients residing in a malaria-endemic district of Colombia (n=347); and 2) samples from asymptomatic subjects residing in

the same malaria-endemic district in Colombia (n=351). Samples were collected between September 2013 and May 2016. The febrile cohort consisted of patients (aged 7-87 years) who presented to the malaria clinic in Puerto Libertador with suspected malaria and consented to participate in the study. The asymptomatic cohort included participants (aged 9-82 years) living in the villages who were enrolled as part of a community-based survey and consented to provide venous blood samples to test for malaria. Study participants who were positive by microscopy were treated in accordance with Colombian health guidelines. For the method validation presented in chapter three, febrile patient samples (n=274) and asymptomatic subject samples (n=245) were selected based on the availability of samples and a negative diagnosis by microscopy.

2.1.3. Study site

The study was performed in the municipality of Puerto Libertador in the Department of Córdoba (7°53′17″N 75°40′18″W). The population of Puerto Libertador was 44,694 in 2013 and 49,179 inhabitants in 2016; 63% of the population resided in rural areas. The municipality is located within the Urabá-Altos Sinú-San Jorge-Bajo Cauca region, which accounts for 60% of all malaria cases in Colombia [136, 137]. Epidemiologic characteristics of this region were described previously [120, 136, 138]. Briefly, the intensity of transmission in this region is low and stable (two rainy seasons, two dry seasons) [136], with co-circulation of both *P. falciparum* and *P. vivax* in a ratio of 2:1 [137]. The mean parasite index of Puerto Libertador, measured as the number of malaria cases/1,000 inhabitants, was 13.29 in 2000-2016 [137]. The entomological inoculation rate in this municipality ranges from 3.5 to 4.8 infective bites per person per year [139]. The most widely distributed *Anopheles* species are *A. nuneztovari, A. albimanus,* and *A. darlingi [140]*.

2.1.4. Sample size calculation

The following sample size calculation pertains to the prospective observational study of submicroscopic malaria in pregnancy described in chapter four. Our primary statistical analysis was a comparison by T-test of the birth weight of infants born of pregnancies complicated by SMI versus those without evidence of infection. Using a standard sample size calculation in R (R Core Team, version 3.1.2, 2014; R Package 'pwr' Version 1.2-1, https://github.com/heliosdrm/pwr) with 80% power, α=0.05 level of significance, assuming a prevalence of SMI of 45% [60], and a normal birth weight of mean (standard deviation [SD]) 3230 (473) g among Colombian newborns [100], a sample size of 115 mother-infant pairs was needed to detect a clinically significant difference of 250 g in birth weight between groups.

2.1.5. Sample collection

Venous blood samples (2 mL) were obtained by venipuncture and collected into EDTA tubes from each participant in the study (pregnant and non-pregnant). In the pregnancy cohort, venous blood samples were collected at each antenatal visit. In addition to peripheral blood, placental blood (2 mL) was collected at delivery by cutting a 2 mm deep incision on the maternal side of the placenta and collecting blood with a blunt syringe. Placental tissue was collected by excision immediately after delivery and processed at the Laboratorio de Dermatopatologia, Universidad de Antioquia. Whole blood was separated into serum and packed RBCs by centrifugation and stored at -20°C for downstream analyses.

2.1.6. Data collection

All participants in the study completed a physical examination and a questionnaire to collect the following data: age, weight, height, demographic information, history of malaria, bed net usage, drug/medication history, symptoms (e.g. presence of fever, cough, chills, diarrhea, bleeding, or other

symptoms). Hemoglobin (Hb) levels were measured using a HemoCue Hb 201+ System (HemoCue) following the manufacturer's protocol. In addition, women enrolled in the pregnancy cohort underwent further examinations throughout antenatal follow-up. Additional data collected from the pregnancy cohort include: history of pregnancies and gestational age at enrolment. Gestational age was determined by ultrasound, which was performed at the hospital by an obstetrician on 181/187 women. Twenty-six (14%) women had an ultrasound during the first trimester, 128 (71%) women during the second trimester, and 27 (15%) women during the third trimester. Birth weight and APGAR scores (measured one minute and five minutes post-partum) were measured at delivery.

2.1.7. Inclusion and exclusion criteria

For the pregnancy cohort, consenting women were included in the study regardless of age or parity. Exclusion criteria were: residence outside the rural area of Puerto Libertador; renal, heart or respiratory disease, hepatic cirrhosis, sexually-transmitted infections (including HIV-1), behavioural disorder, seizures in the last 24 hours prior to enrolment, jaundice, generalized edema, or any other chronic disease; antimalarial treatment within the two weeks prior to the time of enrolment, high-risk pregnancy by pathologies other than gestational malaria; missing samples from enrolment or delivery; miscarriage or stillborn infant.

For the non-pregnant cohorts, all consenting participants were included in the study regardless of age or sex, where the only exclusion criteria was residence outside the rural area of Puerto Libertador or women who were pregnant.

2.1.8. Reference samples

Clinical blood samples independent of the cohorts described in sections 2.1.1 and 2.1.2 were used for method validation. Three groups of patient samples were obtained from the Alberta Provincial Laboratory for Public Health (ProvLab): 1) a negative control group (n=25, negative by microscopy and qPCR); 2) a blinded panel of samples from patients who travelled to malaria-endemic regions (n=77, 17 negative by microscopy and qPCR); and 3) a sample from a patient diagnosed with *P. vivax* used for serial dilutions (confirmed by microscopy and qPCR).

2.1.9. Ethics

All participants provided voluntary consent and were free to withdraw from the study at any time. If the participant was under 18 years of age, a parent or legal guardian provided informed consent. Ethical approval was obtained by the Health Research Ethics Board at the University of Alberta in Canada (Pro00041720) and the Comité de Ética of Instituto de Investigaciones Médicas of Universidad de Antioquia in Colombia (009-2013, 002-2015, 009-2016). Pools of control sera from primigravid and multigravid women were obtained from Benin (protocol N°21/CER/ISBA/13) and Uganda (protocol HDREC368).

2.2. Nucleic acid methods and techniques

2.2.1. DNA extraction

DNA was extracted from packed RBCs using a QIAamp DNA Blood Mini Kit (QIAGEN) following the manufacturer's protocol with the following modifications: 150 µL of blood was combined with 50 µL of 1X PBS for initial sample mixture and eluted in a final volume of 150 µL.

2.2.2. Total nucleic acid extraction

Total nucleic acid (TNA) was extracted from packed RBCs using a MagMAX 96 DNA Multi-Sample Kit (Applied Biosystem, Foster City, CA, USA) following the manufacturer's protocol with the following modifications: 150 µL of blood was extracted and eluted in the same volume, and a manual bench-top vortex was used in place of a plate shaker to minimize aerosol contamination. Additional precautions were followed to prevent cross-contamination during extraction including unidirectional workflow, minimal production of aerosols, and use of deep well plates. To this end, multiple positive and negative controls (minimum of six each) were included during extraction and carried throughout the workflow. In cases where controls failed, all samples on the plate were re-extracted from the original blood samples.

2.2.3. RNA extraction from TRIzol

RNA was isolated from RBC samples preserved in TRIzol (Thermofisher). Briefly, TRIzol samples (stored at -80°C) were thawed on ice and 500 µL of the RBC-TRIzol suspension was transferred to a 1.5 mL Eppendorf tube with 0.2x volume of chloroform. The tube was mixed by multiple tube inversions and followed by a 15 minutes centrifuge step at 13,000 rpm at 4°C to separate phases. The supernatant (containing the RNA) was removed and transferred to a new Eppendorf tube and combined with an equal volume of cold isopropanol. The mixture was allowed to precipitate at -20°C overnight. Following precipitation, the solution was pelleted by centrifuging at 13,000 rpm at 4°C for 10 minutes and washed with 2x volume of 75% ethanol. The supernatant was removed and after allowing the pellet to air dry for 10 minutes, was re-suspended in 100 µL of RNAse/DNAse free water.

2.2.4. Saponin lysis

Saponin lysis of RBCs was performed on a subset of clinical samples to enrich for parasite DNA prior to DNA extraction. Packed RBC pellets were combined with five pellet volumes of PBS with 0.05% saponin (Sigma S-4521) and incubated on ice for ten minutes. Samples were then washed twice with PBS and carried through to column DNA extraction (as in section 2.2.1).

2.2.5. Analysis of low parasite DNA yields

For downstream analysis by qPCR, DNA extracted from samples with low parasite DNA yields were enriched using the REPLI-g kit (QIAGEN, Maryland USA) for whole genome amplification following the manufacturer's protocol or by ethanol precipitation. Briefly, the DNA sample was combined with sodium acetate (pH = 5.2) to a final concentration of 0.3 M. Two volumes of 100% ethanol were added and allowed to incubate overnight at -20°C. Following centrifugation, the supernatant was decanted, washed and spun in 70% ethanol, and re-suspended in nuclease free water.

2.2.6. Polymerase chain reaction

Standard polymerase chain reactions (PCRs) were performed for amplification of target DNA to be used for later downstream analyses. PCR was performed on a T100 Thermal Cycler (BioRad) using Hot Star Taq DNA Polymerase (QIAGEN) under the following conditions: 1X PCR buffer (QIAGEN), 0.2 mM each of deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), and deoxythymidine triphosphate (dTTP), 2.5 mM $MgCl_2$, 0.2 µM of each primer, 2.5 units (U) Hot Star Taq DNA polymerase, 5 µL DNA template, with nuclease-free water to adjust to a final volume of 50 µL. Following an initial denaturation step at 94°C for up to

5 minutes, three-step cycling (95°C for 15 seconds (denaturation), 60°C for 30 seconds (annealing), 72°C for one minute (extension)) was performed for a total of 35-40 cycles, with a final extension at 72°C for ten minutes, followed by incubation at 4°C.

2.2.7. Gel electrophoresis

PCR products were analyzed by agarose gel electrophoresis. Agarose gels were prepared by dissolving 0.5-2% (w/v) agarose (ThermoFisher, Massachusetts, USA) in 1X TAE buffer [40 nM Tris (Invitrogen, California, USA), 20 mM sodium acetate trihydrate (Fisher Scientific), 1 mM ethylenediaminetetraacetic acid (EDTA) dehydrate (Sigma Aldrich)] and casted with 1X SYBR Safe (Invitrogen) (diluted from a 10,000X stock). Agarose gels were submerged in 1X TAE buffer and DNA samples combined with 1X DNA loading dye (QIAGEN) (diluted from a 5X stock) were loaded into wells. A 100 bp DNA ladder (Invitrogen) was run in duplicate on each gel for product size approximation. Agarose gels were run between 80-120V for up to two hours. Gels were visualized using a transilluminator on a FLA-5100 imager (Fujifilm).

2.2.8. Gel extraction

Following agarose gel electrophoresis, PCR products that were needed for further downstream analyses (Sanger sequencing or plasmid preparation) were extracted and purified. PCR products were purified using a QIAquick PCR Purification kit (QIAGEN) following the manufacturer's protocol. If multiple bands were observed on the gel, the appropriate DNA was excised and purified using a Gel Extraction Kit (QIAGEN) following the manufacturer's protocol.

2.2.9. DNA ligation

DNA ligation of PCR products was performed using a pGEM-T Easy Vector Kit (Promega, Wisconsin, USA) following the manufacturer's protocol. Insert DNA was quantified using a spectrophotometer prior to ligation reactions and all ligations were performed using a T4 DNA ligase. Vector DNA (50 ng) was combined with the insert DNA in a 1:3 or 1:4 vector to insert ratio. The amount of insert DNA required was calculated using the online calculator at http://www.promega.com/biomath/. Reactions were incubated overnight at 4°C. Ligations were transformed into *Escherichia coli* DH5α, as described below in section 2.2.10.

2.2.10. Bacterial transformations

Bacterial transformations were carried out using Subcloning Efficiency DH5α Competent Cells (Invitrogen) following the manufacturer's protocol. Competent cells and DNA ligations were incubated at 42°C for 20 seconds to induce heat shock. Fifty μL of bacterial culture was spread on Luria-Bertani (LB) plates (LB base (Difco) and 15 g/L agarose) containing 100 μg/mL carbenicillin (Sigma). Plates were incubated overnight at 37°C.

2.2.11. Plasmid isolation

Colony isolation was performed by transferring one colony from selective media (outlined in section 2.2.10) to 2 mL of LB broth containing 100 μg/mL carbenicillin. The culture was transferred to fresh media containing carbenicillin and incubated overnight at 37°C in 50 mL conical tubes on a plate shaker. Plasmid DNA was isolated using a QIAprep Miniprep Kit (QIAGEN) following the manufacturer's protocol.

2.2.12. Controls

For *P. falciparum*, positive extraction controls were prepared from synchronized 3D7 ring-stage parasites cultured in human blood (See section 2.4). In addition to cultured parasites, positive controls for *P. falciparum* and *P. vivax* were prepared by cloning the respective 18S rRNA genes into a pGEM-T vector plasmid (Promega) and spiking the plasmid into uninfected blood and reported as copies/µL. DNA copy number was calculated using the online calculator at https://www.thermofisher.com/ca/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/dna-copy-number-calculator.html using the plasmid size and molar mass per base pair. Negative human blood was used as a no template control during extraction and carried through the workflow and PCR.

2.3. Malaria diagnosis

Malaria was diagnosed at the time of sample collection by thick smear. Slides were read by a trained microscopist. All microscopists underwent proficiency testing at the beginning of the study for external quality assurance. Diagnosis of submicroscopic infections was performed retrospectively as detailed below.

2.3.1. Microscopy

Blood smears from peripheral or placental blood were stained with Giemsa or Field stain. Parasitemia was determined by thick smear and calculated by counting the number of parasites per 200

leukocytes, based on a mean count of 8,000 leukocytes per microlitre of blood. Samples were considered negative if no parasites were detected in at least 200 high power (1,000x) fields.

2.3.2. qPCR

Submicroscopic malaria infections were detected by qPCR at the genus level using a previously published assay [62] and a modified assay developed in our laboratory to detect *P. falciparum* and *P. vivax* [70]. These assays were previously used to measure parasitemia by quantifying samples against a standard curve. Briefly, the assay was performed on an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, California, USA) with the following thermal profile: 15 minutes activation step at 95°C, 45 cycles of 15 seconds denaturation at 95°C and one minute annealing/extension at 60°C. For the reaction, 5 μL of template was added to 20 μL reaction mix containing TaqMan Universal PCR Master Mix (Thermofisher), 200 nM of primers, and 50 nM probe. The genus-level reaction uses primers and probes that target a region of the 18S rRNA gene that is conserved across all six species of *Plasmodium* [62] while the species-specific reaction targets an internal sequence that is variable across species [70]. Species-specific primers were designed to target type A (asexual) rRNA genes on chromosomes five and seven for *P. falciparum* and chromosomes three and ten for *P. vivax.* Primers are listed in Table 2.1 below. Samples with a cycle threshold (C_T) $\leq$ 45 were considered positive. This C_T cut-off was determined based on successful sequencing of *Plasmodium*-specific amplicons confirming positive samples with C_Ts of 40-45.

Table 2.1. List of primers and probes used in this study

Species	Primer or probe	Conc. (nM)	Sequence (5' – 3')	Reference
***Plasmodium* spp.**	Plasmo1 – F primer	200	GTT AAG GGA GTG AAG ACG ATC AGA	[62]
***Plasmodium* spp.**	Plasmo2 – R primer	200	AAC CCA AAG ACT TTG ATT TCT CAT AA	[62]
***Plasmodium* spp.**	Plasprobe	50	FAM-ACC GTC GTA ATC TTA ACC ATA AAC TAT GCC GAC TAG-TAMRA	[62]
***Plasmodium* spp.**	Kamau – F primer	200	GCT CTT TCT TGA TTT CTT GGA TG	[74]
***Plasmodium* spp.**	Kamau – R primer	200	AGC AGG TTA AGA TCT CGT TCG	[74]
P. falciparum	Pf2 – F primer	200	CCG ACT AGC TGT TGG ATG AAA GTG TTA A	[70]
P. falciparum	Pf2 – R primer	200	CCG ACT AGG TGT TGG ATG AAA GTG	[60]
P. falciparum	Falcprobe	200	FAM-AGC AAT CTA AAA GTC ACC TCG AAA GAT GAC T-BHQ-2	[70]
P. vivax	Viv – F primer	800	CCG ACT AGG CTT TGG ATG AAA GAT TTT A	[70]

P. vivax	Pv1 – F primer	200	CGC TTC TAG CTT AAT CCA CAT AAC TGA TAC	[141]
P. vivax	Pv2-6 – R primer	200	TCC AAG CCG AAG CAA AGA AAG TCC TTA AAA	-
P. vivax	Vivprobe	200	FAM-AGC AAT CTA AGA ATA AAC TCC GAA GAG AAA ATT CT-BHQ-2	[70]
P. malariae	Mal – F primer	200	CCG ACT AGG TGT TGG ATG ATA GAG TAA A	[70]
P. malariae	Malaprobe	50	FAM-CTA TCT AAA AGA AAC ACT CAT-MGBNFQ	[62]

2.3.3. RT-qPCR

For *Plasmodium* species determination, RT-qPCR was performed on an ABI 7500 Fast Real-Time PCR System using the same species-specific *P. falciparum* and *P. vivax* primers and probes used for qPCR [70] (Section 2.3.2) and the following thermal profile: 20 seconds reverse transcription (RT) step at 50°C, 20 seconds at 95°C, 40 cycles of 3 second denaturation at 95°C and 30 seconds annealing/extension at 60°C. For the reaction, 5 µL of template was added to a 5 µL reaction mixture containing TaqMan Fast Virus 1-Step Master Mix (Thermofisher), 800 nM of primers, and 200 nM probe (Table 2.1). The cut-off for the RT-qPCR reaction was set to $C_T \leq 40$. Samples with a C_T between 38 and 40 were re-run in triplicate and called positive if two out of three replicates had C_T values ≤ 40. Only samples that were positive in both the genus assay and species assay were considered a positive result for infection. Gametocytes are unable to be detected by this method as the primers in this assay target A-type rRNA transcripts expressed only in asexual blood stage *P. falciparum* and *P. vivax* parasites, restricting detection to only asexual stage parasites.

2.3.4. Direct from blood qPCR

For reactions performed directly from blood, qPCR was performed using either a CFX Connect Real-time PCR (Bio-Rad, California, USA) or Open qPCR (CHAI Biotechnologies, California USA) to detect malaria infections. Reactions were performed either in a liquid mastermix or in desiccated hydrogel [142] (Aquila Diagnostics Systems Incorporated, Alberta, Canada). Each reaction contained 40X SYBR green (Life Technologies), 0.5 µL Omni Klentaq (OKT) enzyme (VitaNavi Technology, Missouri, USA), 1X OKT Buffer (VitaNavi Technology), 1X PCR-enhancement cocktail (PEC-1) (VitaNavi Technology), 200 µM dNTPs (Sigma Aldrich, Missouri, USA), with a final blood concentration of 10%. Primer concentrations ranged from 100-400 nM per reaction depending on the

assay (Table 2.1). Thermocycling conditions for the CFX were as follows: 10 minute initial denaturation step at 95°C, followed by 40 amplification cycles (20 seconds denaturation at 95°C, 30 seconds annealing at 58°C, and 30 seconds extension at 72°C), final extension for 2 minutes at 72°C, followed by standard melt curve analysis (10 second hold at 95°C, followed by a data gathering step from 65°C-95°C at a ramp speed of 0.5°C/second). Thermocycling conditions for the Open qPCR were similar to those above with the following modifications: melt curve analysis was performed with a ramp speed of 0.09°C/second.

2.3.5. Direct from blood RT-qPCR

RT-qPCR directly from blood was performed to detect infections at the species level for *P. falciparum* and *P. vivax*. Reactions were performed using either a CFX Connect Real-time PCR (Bio-Rad) or Open qPCR (CHAI). The protocol for the species-specific detection is similar to the genus detection assay that was described previously [143]. Briefly, each reaction contained the following: 40X SYBR green (Life Technologies), 1X Buffer (VitaNavi Technology), 0.5 µL enzyme (VitaNavi Technology), 3.2 units/µL Ribolock RNase Inhibitor (Thermofisher), and primers at a final concentration of 400 nM (Table 2.1). Blood samples were added in a 2.5 µL volume to a final concentration of 10%. Thermocycling conditions for the CFX were performed as follows: 30 minute reverse transcription step at 60°C, 3 minute initial denaturation step at 95°C, followed by 45 amplification cycles (20 seconds denaturation at 95°C, 30 seconds annealing at 60°C, and 30 seconds extension at 70°C), final extension for 2 minutes at 70°C, followed by standard melt curve analysis (10 second hold at 95°C, followed by a data gathering step from 65°C-95°C at a ramp speed of 0.5°C/second). Thermocycling conditions for the Open qPCR were similar to those above with the following modifications: melt curve analysis was performed with a ramp speed of 0.09°C/second.

2.3.6. Diagnosis by placental histopathology

Placental tissue was collected immediately after delivery and processed at the Laboratorio de Dermatopatologia, Universidad de Antioquia. Methods for scoring were described elsewhere [19]. Briefly, active infections were diagnosed by the presence of *Plasmodium* parasites, past infections by the presence of hemozoin only, and chronic infections by the presence of both hemozoin and *Plasmodium* parasites.

2.4. Parasite cultures

P. falciparum 3D7 parasites and CS2 parasites were cultured *in vitro*, as described previously [144]. All culture protocols are described briefly in the sub-sections below.

2.4.1. Establishing parasite cultures from frozen ampoules

Parasites obtained through MR4 and stored in liquid nitrogen were used to establish cultures *in vitro*. Frozen parasites in cryogenic tubes were thawed in a 37°C water bath and transferred to a 15 mL conical tube. Additionally, RP-Incomplete (RP-I) buffer (RPMI 1640 supplemented with L-glutamine (Thermofisher) and 25 mM HEPES (Invitrogen)) and RP-Complete (RP-C) buffer (RP-I buffer supplemented with 0.225% Sodium bicarbonate (Invitrogen), 40 µg/mL gentamicin (Sigma), 11 mM glucose (Sigma), 200 µM hypoxanthine, 0.5% ALBUMax II (Invitrogen)) was warmed to 37°C in a water bath. A volume of 0.1x of 12% sodium chloride (NaCl) was added drop-wise while gently shaking the tube and allowed to incubate at room temperature for five minutes. 10x volume of 1.8% NaCl was added drop-wise while swirling and followed by centrifugation at 500 g for five minutes at

room temperature. Supernatant was removed and the pellet was washed with 10x volume of RP-I, followed by a five minute centrifuge at 1500 rpm. Supernatant was removed and packed RBCs was added to a 0.3 mL total pellet volume with a starting parasitemia between 0.1-1.0%. RP-C buffer was added to a total volume of 10 mL (approximately 3-5% hematocrit) and transferred to a petri dish. Petri dishes were gassed (5% CO_2, 5% O_2, 90% nitrogen) for one minute at 20 L/min and placed in a 37°C incubator.

2.4.2. Changing culture media

Media for cultures were changed daily with the exception of the day after sub-cultivation (section 2.4.3.). RP-C buffer was warmed to 37°C in a water bath. Tilting the petri dish, old media was aspirated through a sterile pipet. Cells were re-suspended with 10 mL of RP-C buffer and gassed for one minute at 20 L/min and placed in a 37°C incubator.

2.4.3. Sub-cultivation of parasites

Parasites were sub-cultured twice weekly. RP-I and RP-C buffer was brought to 37°C in a water bath. Parasitemia was determined by microscopy of Giemsa stained thin smears. The culture was transferred to a 15 mL conical tube and centrifuged for five minutes at 1500 rpm at room temperature. The supernatant was removed and the pellet was washed with 10 mL of RP-I buffer, followed by a five minute centrifugation at 1500 rpm. Cells were diluted to a parasitemia of 1% in multiple tubes with packed RBCs and brought to a 3% hematocrit in RP-C buffer. Cultures were transferred to petri dishes and gassed for one minute at 20 L/min and placed in a 37°C incubator.

2.4.4. Sorbitol synchronization

Parasite cultures were synchronized at the ring stage by sorbitol. Briefly, cultures from petri dishes were transferred to a 15 mL conical tube and spun down at 1500 rpm for five minutes. The supernatant was removed and 5x volumes of sorbitol was added and allowed to incubate at 37°C for five minutes. Following a five minute centrifugation at 1500 rpm, the supernatant was removed and the culture was set up to a 3% hematocrit as previously described (section 2.4.1.-2.4.3) and placed in a 37°C incubator.

2.4.5. Cryopreservation of parasitized RBCs

Cultures with high parasitemia (>5%) and a high proportion of ring-stage parasites were selected for freezing. RP-I buffer was brought up to 37°C in a water bath. Cultures were transferred to a 15 mL conical tube and spun down at 2000 rpm for five minutes. The supernatant was removed and the pellet was washed with 10 mL of RP-I buffer. The buffer was removed after a five minute centrifugation at 1500 rpm. An equal volume of deep-freeze solution buffer (3% sorbitol, 0.65% NaCl, and 28% glycerol) was added drop-wise. A volume of 0.5 mL were aliquoted into each ampoule and stored in liquid nitrogen.

2.4.6. Purification of CS2 schizonts and late trophozoites

CS2 parasites were selected on CSA (Sigma-Aldrich) to enrich for the expression of VAR2CSA. Mature-stage parasites were purified using a VarioMACS according to the manufacturer's protocol (Miltenyl Biotec). Expression of VAR2CSA on the surface of iRBCs infected with *P. falciparum* CS2 was confirmed by flow cytometry using a rabbit anti-VAR2CSA polyclonal antibody (1:40 dilution) and detected using a goat anti-rabbit secondary conjugated to AlexaFluor 647 (1:500

dilution, Life Technologies). Parasite DNA was stained with 5 µg/mL of DAPI. Surface labeling of DAPI-positive iRBCs was quantified by flow cytometry (Fortessa X20) and analyzed using FlowJo v.7.6 (TreeStar).

2.5. Analysis of functional antibodies

2.5.1. Quantifying VAR2CSA antibodies

The levels of specific IgG antibodies against VAR2CSA were measured in serum samples by enzyme-linked immunosorbent assay (ELISA) using a method described previously [109]. Briefly using BD Falcon petri dishes, full-length VAR2CSA recombinant protein kindly provided by Dr. Ali Salanti (0.5 µg/mL) was incubated with 100 µL of human serum (1:1000 dilution) and detected with a horseradish peroxidase-conjugated anti-human IgG (1:6000 dilution, A0170 Sigma-Aldrich). A pool of serum samples from multigravid women from Uganda served as a positive control. Twenty sera samples from Canadian residents with no history of travel to malaria-endemic areas served as negative controls. Optical densities were calculated as arbitrary units (AU) using the following formula: AU = $(OD_{sample} - OD_{background}) / (OD_{referenece\ pool} - OD_{background})$ x 100% [145]. Seropositivity was defined as AU >2 SD above the mean absorbance of the negative controls (cut-off = 19.7 AU).

2.5.2. Preabsorption of human serum

Serum samples were pre-incubated with uninfected RBCs to avoid non-specific interaction with CSA during inhibition of binding assays (See section 2.5.3.). Sera were incubated with an equal volume of washed AB+ RBCs for one hour at 4°C. Serum was separated by pelleting RBCs through

centrifugation at 2000 g for ten minutes. An additional centrifugation step was performed at 800 g for ten minutes to remove any residual RBCs.

2.5.3. Inhibition of binding assays

An *in vitro* inhibition of binding assay (IBA) was used to assess the functionality of antibodies from patient serum. Petri dishes (Falcon) were coated overnight at 4°C with 10 individual spots of 20 µL of phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and soluble CSA (sCSA, 50 µg/mL; Sigma-Aldrich). Spots were blocked with 3% BSA in 1X PBS for 1 h at 37°C. CSA-selected mature-stage CS2 iRBCs were adjusted to 20% parasitemia in suspension and blocked in a 3% BSA/RPMI buffer for 30 min at room temperature (RT). iRBCs were incubated with sCSA (500 µg/mL) or serum (1:3 dilution) for 30 min at RT, added to the plate, and incubated for 15 min at RT. Unbound cells were washed with 24 mL of 1X PBS on a plate rocker for 10 min, fixed with 1.5% glutaraldehyde in 1X PBS and stained with 5% filtered Giemsa. The entire area of each spot was imaged using an EVOS FL Auto (Life Technologies) with a 4X objective and the number of bound iRBCs was quantified using Image J (NIH).

Sera samples were run in triplicate across three separate plates within the same experiment. A pool of sera from fifty unexposed Colombians from Medellín was used as a negative control and was included on two spots on each plate to control for variable binding of iRBCs across the plate. Five replicates of sCSA were run in parallel as a positive inhibition control for every experiment. The threshold for functional inhibition was based on the mean count of bound parasites from all replicates of the Colombian unexposed pool (across all plates and experiments, n=29 plates) minus 2 SD, divided by the mean from the same pool (% Inhibition = 1 – [Mean-2SD/Mean] X 100). Based on these criteria, samples that inhibited binding of iRBCs to CSA by $\geq$ 39.1% were considered positive for

functional inhibition. Functional inhibition per sample was calculated using the following: % Inhibition = ([Binding control – Test sample]/[Binding control – sCSA control]) X 100. The pool of sera from the unexposed population served as the binding control on each plate.

2.6. Statistical analysis

Graphs and figures were generated using Prism v7 (GraphPad). Categorical variables were compared using the Chi-square test or Fisher's exact test, as appropriate. For comparisons between continuous variables, a D'Agostino-Pearson omnibus normality test was used to determine whether data followed a Gaussian distribution, and comparisons were made using parametric tests (Student's *t*-test or one-way ANOVA) or nonparametric tests (Mann-Whitney or Kruskal-Wallis for unpaired comparisons and Wilcoxon matched-pairs test for paired comparisons), as appropriate.

For method validation experiments, the limit of detection (LOD) for molecular diagnostic assays was determined by probit analysis using MedCalc 16.4 (MedCalc Software). Repeated measures of serial dilutions for qPCR and RT-qPCR assays were used to calculate the LOD with 95% confidence intervals.

Associations between risk factors and adverse birth outcomes were quantified using relative risk with 95% binomial confidence intervals. Classification of infants as small for gestational age (SGA) followed published methodology [146] and was specifically adapted to our Colombian population based on the birth weight of term infants in Colombia (40 weeks of gestation). Infants below the 10^{th} percentile were defined as SGA (Z-score <-1.28).

A linear mixed-effects model was used to study the effect of SMIs during pregnancy on the levels of anti-VAR2CSA antibodies. R (R Core Team, version 3.1.2, 2014) and *lme4* was used to perform a linear mixed-effects analysis of antibody levels as a function of gestational age and exposure to malaria. As fixed effects, gestational age was entered, and SMI as a time-dependent covariate into the model. Intercepts were modelled for each individual as random effects. Visual inspection of residual plots did not reveal any obvious deviations from homoscedasticity or normality. The p-value for the hypothesis (effect of SMI on antibody levels) was obtained by likelihood ratio test of the full model (including SMI) against the model without SMI. Linear regression was used to test the association between functional antibodies and birth outcomes. Multivariable linear regression models were used to verify associations between key predictor variables (maternal age, parity, maternal hemoglobin concentration at enrolment, malaria infection, timing (trimester) of infection, antibody levels (AU), and inhibitory antibodies (% inhibition)) and key outcomes, coded as continuous variables (gestational age at delivery, weight-for-gestational age Z-score, birth weight, and maternal hemoglobin concentration at delivery). The R package *MASS* [147] and the function *step*, was used to perform backward stepwise selection of variables for inclusion in multivariable linear regression models.

Chapter 3 – Validation of an RT-qPCR method for the detection of submicroscopic *P. falciparum* and *P. vivax* infections

Portions of this chapter have been published:

Gavina K, Arango E, Larrotta CA, Maestre A, Yanow SK. A sensitive species-specific reverse transcription real-time PCR method for detection of *Plasmodium falciparum* and *Plasmodium vivax*. *Parasite Epidemiol Control*. 2017;**2**(2):70-6.

Catalina Larrotta performed the real-time PCR screening of asymptomatic Colombian participants (section 3.5). I performed all other experiments in this chapter and wrote the original manuscript under the supervision of Dr. Stephanie Yanow.

3.1. Introduction

While microscopy remains the gold standard for diagnosis of malaria in the field, molecular diagnostics such as PCR and qPCR provide superior sensitivity and specificity and are used for epidemiological analyses within laboratory settings [32, 148]. As countries strive towards disease elimination, there is a growing demand for sensitive molecular diagnostics to target the submicroscopic reservoir of infection. While a number of methods demonstrating excellent sensitivity for the detection of *Plasmodium* spp. at the genus level have been published [68, 73, 74, 149-151], sensitivities of tests to determine the species of *Plasmodium* are typically much poorer [62, 70-72]. The work in this chapter describes a sensitive species-specific RT-qPCR method to differentiate between *P. falciparum* and *P. vivax* infections at the submicroscopic level. This assay was validated with blood samples, negative by microscopy, from febrile and asymptomatic cohorts from Colombia, and compared directly to a qPCR-based method as the gold standard. The data from this chapter demonstrate that the RT-based qPCR method provides superior sensitivity for *Plasmodium* species identification compared to DNA-based qPCR methods.

3.2. Results

3.2.1. Reaction efficiencies for *P. falciparum* and *P. vivax* RT-qPCR assays

We performed multiple replicates of serial dilutions prepared from *P. falciparum* 3D7 parasites (10^5 p/mL to 10^0 p/mL) and a *P. vivax* plasmid surrogate (10^6 copies/µL to 10^1 copies/µL) to test the efficiency of the RT-qPCR species-specific assays (Figure 3.1). The RT-qPCR reaction efficiency for the *P. falciparum* assay was 90.2%, based on serial dilutions of cultured parasites (R^2= 0.9975, slope of -3.583). For the *P. vivax* assay, the reaction efficiency using the plasmid standard curve was 106.8% (R^2=0.9984, slope of -3.17). Non-specific amplification was not observed based on gel electrophoresis

of amplified products.

3.2.2. Analytical sensitivity and specificity

Analytical sensitivity was determined from the dilution series described above (10^5 to 10^0 p/mL for *P. falciparum* cultured parasites and 10^6 to 10^0 copies/µL for *P. falciparum* and *P. vivax* plasmid surrogates, respectively), run in replicates of six in three independent experiments. Six additional replicates performed in three additional experiments were run as samples approached the LOD (10^1 p/mL and 10^0 p/mL for the *P. falciparum* assay and 10^1 copies/µL and 10^0 copies/µL for the *P. vivax* assay). The LOD with 95% confidence intervals derived from the *P. falciparum* standard was 10 p/mL (Figure 3.2a), 19 copies/µL for the *P. falciparum* plasmid surrogate (Figure 3.2b), and 18 copies/µL for the *P. vivax* plasmid surrogate (Figure 3.2c). It should be noted that the plasmid surrogates used in this study served merely as positive controls for the lowest amount of nucleic acid template that can be detected for this target DNA.

We expected that the high sensitivity observed with the RT-qPCR assays was attributed to the increased copies of 18S rRNA transcript over genomic targets within the parasite [73]. To test this, we compared the C_T values for RT-qPCR performed on *P. falciparum* parasites (10^4 p/mL) extracted with and without the addition of RNase A. Samples extracted without RNase A had a mean C_T value of 22.81 ± 0.21, while samples extracted with the addition of RNase A had a mean C_T value of 31.12 ± 0.16. We observed a significant increase in C_T for samples treated with RNase A (8.3 cycles greater; $p<0.0001$, two-tailed paired *t*-test), consistent with just under ~1000 times more RNA target than gDNA template extracted from parasites.

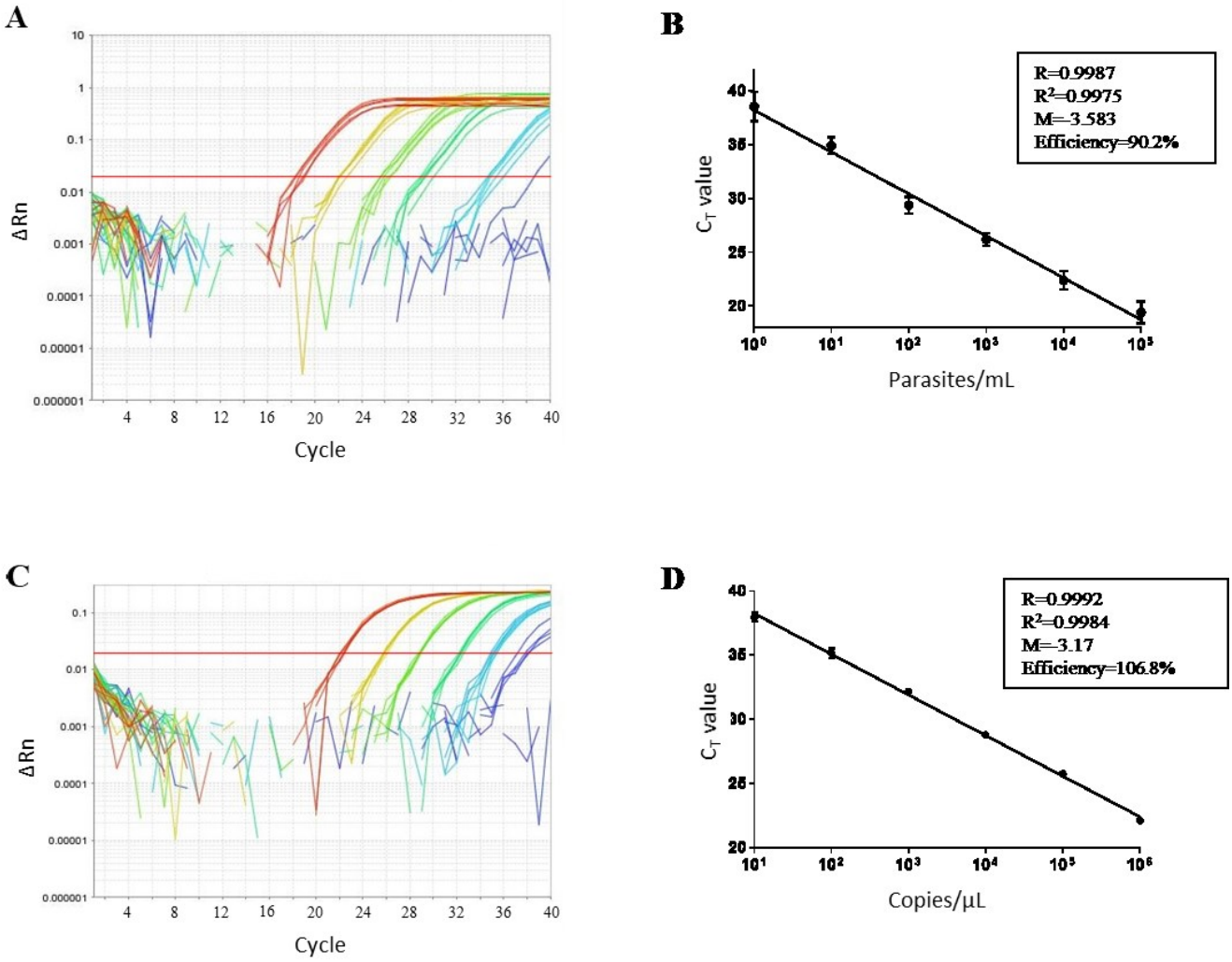


Fig. 3.1: Dilution series and reaction efficiencies for species-specific RT-qPCR assays. (A) Real-time amplification and **(B)** regression curve from serial dilutions of 3D7 ring-stage *P. falciparum* parasites. Total nucleic acid was extracted from culture and serially diluted in ten-fold increments (red: 10^5 p/mL; yellow: 10^4 p/mL; light green: 10^3 p/mL; seafoam green: 10^2 p/mL; light blue: 10^1 p/mL; dark blue: 10^0 p/mL). Red horizontal line represents threshold cut-off (0.02). **(C)** Amplification and **(D)** regression curve from serial dilutions of a *P. vivax* plasmid surrogate. *P. vivax* plasmid surrogate was serially diluted in ten-fold increments (red: 10^6 copies/µL; yellow: 10^5 copies/µL; light green: 10^4 copies/µL; seafoam green: 10^3 copies/µL; light blue: 10^2 copies/µL; dark blue: 10^1 copies/µL).

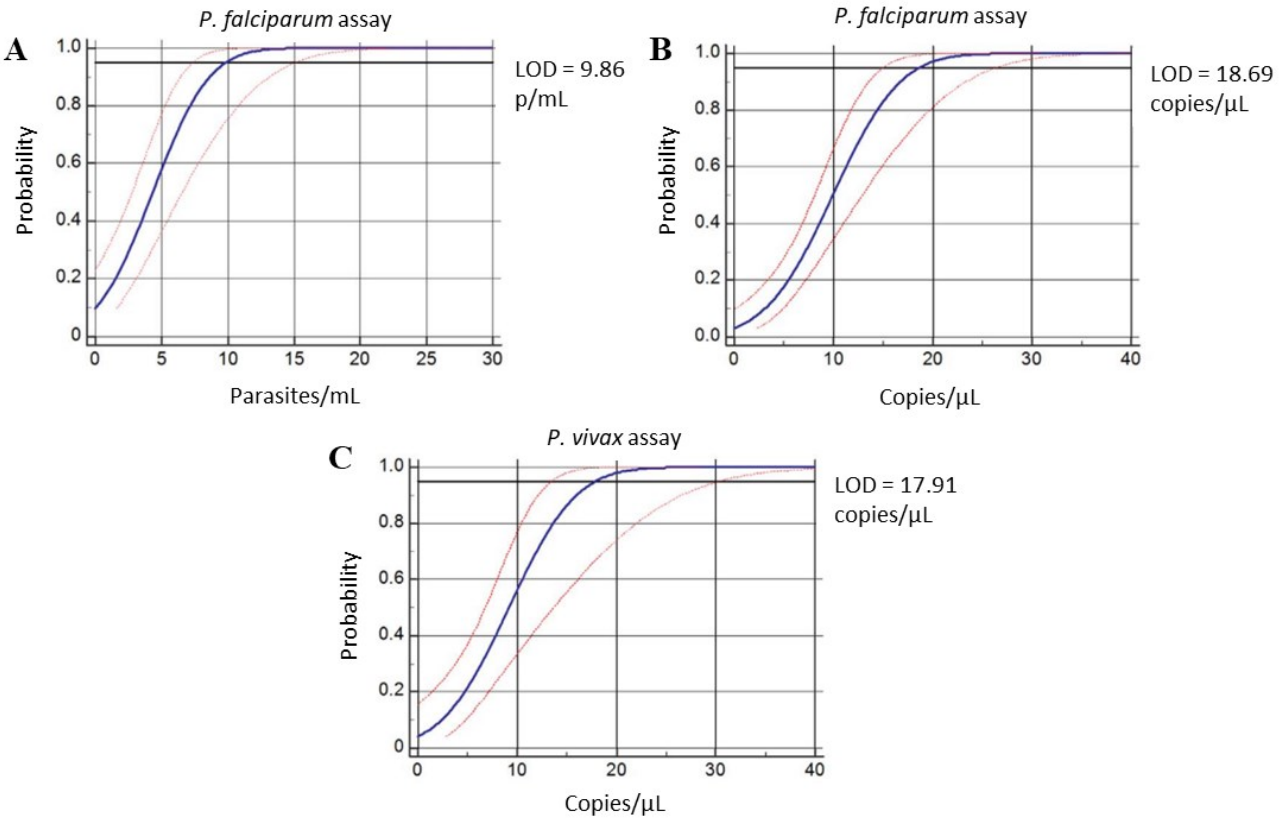


Fig. 3.2: Analytical sensitivity as determined by probit regression for **(A)** *P. falciparum* assay (*P. falciparum* 3D7 malaria parasites), **(B)** *P. falciparum* assay (plasmid surrogate), and **(C)** *P. vivax assay* (plasmid surrogate). The blue line depicts the regression curve and the red dotted line represents the 95% confidence intervals.

Analytical specificity was assessed using *P. falciparum* 3D7 parasites and *P. vivax* plasmids to test for potential cross-reactivity between the species-specific primers and probes (Table 3.1). Thirty-six replicates (18 replicates of 10^6 p/mL *P. falciparum* 3D7 and 18 replicates of 10^6 copies/µL *P. vivax* plasmid) were analyzed in both assays. All 18 *P. falciparum* replicates were positive for *P. falciparum* and negative for *P. vivax* by RT-qPCR. Similarly, all 18 plasmid replicates were positive for *P. vivax* and negative for *P. falciparum* by RT-qPCR, giving an analytical specificity of 100%. Additionally, we tested 25 patient blood samples from the ProvLab that were negative by microscopy and qPCR (Table 3.1). All samples were negative for *P. falciparum* and *P. vivax* when assessed by RT-qPCR.

3.3. Precision

3.3.1. Repeatability and reproducibility

Intra-assay variation (variation between replicates in the same experiment) and inter-assay variation (variation between replicates in different experiments) were assessed to determine the repeatability and reproducibility of the species-specific assays. Serial dilutions of positive controls (*P. falciparum* 10^5 to 10^1 p/mL and *P. vivax* 10^5 to 10^1 copies/µL) and three negative control samples were run in replicates of six across three different experiments performed on different days (Table 3.2). Precision analysis was performed on the basis of C_T values of the replicates and the coefficient of variation (%CV) was determined. Intra-assay %CV ranged from 0.01-0.03% and <0.01-0.02% for the *P. falciparum* and *P. vivax* samples, respectively. Inter-assay %CV ranged from 0.01-0.26% and <0.01-0.09% for *P. falciparum* and *P. vivax* samples, respectively.

Table 3.1: |Analytical specificity of RT-qPCR assays

Sample	*P. falciparum* Assay (n/N)[a]	*P. vivax* Assay (n/N)[a]
P.f. 10^6 p/mL	18/18	0/18
P.v. 10^6 copies/ µL	0/18	18/18
Negative	0/25	0/25

[a] n – number of samples with a positive result; N – total number of samples

Table 3.2: Reproducibility and repeatability of RT-qPCR assays

Sample	Intra-Assay Mean (C_T)	%CV[a]	Inter-Assay Mean (C_T)	%CV[a]
P.f. 10^5 p/mL	19.10	0.02	19.77	0.03
P.f. 10^4 p/mL	22.33	0.03	22.83	0.26
P.f. 10^3 p/mL	25.94	0.02	26.48	0.04
P.f. 10^2 p/mL	29.43	0.01	29.38	0.01
P.f. 10^1 p/mL	34.22	0.03	33.95	0.01
P.v. 10^5 copies/µL	25.70	0.01	25.77	<0.01
P.v. 10^4 copies/µL	28.79	<0.01	28.93	0.09
P.v. 10^3 copies/µL	32.18	<0.01	32.36	0.04
P.v. 10^2 copies/µL	35.16	0.02	35.23	0.01
P.v. 10^1 copies/µL	37.97	0.01	37.78	0.01
Negative	-	-	-	-
Negative	-	-	-	-
Negative	-	-	-	-

[a] %CV – coefficient of variation

3.4. Assay sensitivity for the detection of mixed infections

In addition to correctly diagnosing mono *P. falciparum* and mono *P. vivax* submicroscopic infections, we wanted to test the analytical sensitivity of our assays in varying ratios of mixed infections. To this end, we mixed plasmids containing the 18S rRNA genes from each species at different ratios and tested these mixtures in the species-specific assays (Table 3.3). We reproducibly detected both *P. falciparum* and *P. vivax* targets with ratios as low as 1:100 for each species.

Table 3.3: Detection of varying ratios of mixed *P. falciparum* and *P. vivax* plasmids

Ratio (*P. falciparum* : *P. vivax*)*	**Falcprobe (CT)**	**Vivprobe (CT)**
100:1	22.82	30.03
10:1	24.58	28.95
5:1	24.85	28.11
1:1	24.87	27.5
1:5	24.62	27.41
1:10	26.12	26.77
1:100	28.46	24.12

***** Samples were produced by combining *P. falciparum* and *P. vivax* plasmids

3.5. Sensitivity and specificity relative to qPCR

We directly compared species identification by RT-qPCR to qPCR using a dilution series of *P. falciparum* parasites (10^5 to 10^0 p/mL) to observe the differences in analytical sensitivity (Table 3.4). Using qPCR, we detected 10^1 p/mL (two of three replicates) with a mean C_T value of 38.49, whereas we detected 10^0 p/mL (two of three replicates) when using RT-qPCR. Further, the C_T values at all dilutions were lower with RT-qPCR compared with qPCR.

Table 3.4: Direct comparison of RT-qPCR and qPCR for species identification using a serial dilution of *P. falciparum* 3D7 parasites.

Dilution	**qPCR mean C_T**	**RT-qPCR mean C_T**	**P-value[a]**
10^5 parasites/mL	23.29	18.79	<0.0001
10^4 parasites/mL	26.84	22.31	<0.0001
10^3 parasites/mL	30.43	25.95	<0.0001
10^2 parasites/mL	33.89	29.18	<0.0001
10^1 parasites/mL	38.49*	33.62	0.0046
10^0 parasites/mL	ND[b]	39.54*	-

[a] Calculated by unpaired student's T-test, statistically significant results ($p<0.05$)
[b] None detected
* Only two of three replicates detected

We next determined the clinical sensitivity and specificity of the RT-qPCR assay compared with qPCR using a blind panel of patient samples collected from the Alberta ProvLab (Table 3.5). Of 77 samples, 42 (54%) were identified by qPCR as *P. falciparum*, 18 (23%) as *P. vivax*, and 17 as negative. When analyzed by RT-qPCR, all 60 positives were correctly identified; however, an additional *P. falciparum* and one *P. vivax* sample were also identified by RT-qPCR as mixed infections and confirmed by sequencing. Based on qPCR as the gold standard, the diagnostic sensitivity for both RT-qPCR assays was 100%, while diagnostic specificities were 97.20% and 98.33% for the *P. falciparum* and *P. vivax* assays, respectively.

Table 3.5: Diagnostic sensitivity and specificity of RT-qPCR compared to qPCR performed on 77 clinical samples

		qPCR*			
		P. falciparum		*P. vivax*	
		Positive	Negative	Positive	Negative
RT-qPCR	Positive	42	1	18	1
	Negative	0	34	0	58
		P. falciparum (%)	95% CI	*P. vivax* (%)	95% CI
Sensitivity		100	91.59-100	100	81.47-100
Specificity		97.20	85.47-99.93	98.33	91.06-99.96

* 17 of 77 samples were negative for both *P. falciparum* and *P. vivax* by qPCR

3.6. Evaluation of submicroscopic infections in febrile and asymptomatic populations from Colombia

To determine whether RT-qPCR provides enhanced species identification of submicroscopic infections, samples collected from two cohorts in Colombia were analyzed by both qPCR and RT-qPCR (Table 3.6). The first cohort included 274 selected participants who presented to the health facility with fever and suspected malaria. For the second cohort, samples were collected from 245 asymptomatic participants in community-based surveys. All samples from febrile and asymptomatic participants were negative by thick smear. In the febrile population, 52 samples were positive for *Plasmodium* (genus) DNA by qPCR. The species was identified for only 10 of these samples by qPCR compared with 34 samples analyzed by RT-qPCR. Similarly, 36 samples from the asymptomatic group were positive at the genus level by qPCR; the species could only be determined in one sample by qPCR, but 13 were identified at the species level by RT-qPCR. These results demonstrate that RT-qPCR outperformed qPCR for species identification of samples collected in a field setting.

Table 3.6: qPCR and RT-qPCR analysis of clinical samples from participants from Colombia

	Febrile Population (n=274)		Asymptomatic Population (n=245)	
	qPCR pos n (%[a])	RT-qPCR pos n (%[a])	qPCR pos n (%[a])	RT-qPCR pos n (%[a])
***Plasmodium* spp.**	52 (19%)	-	36 (14.7%)	-
P. falciparum	4 (1.5%)	16 (5.8%)	0	3 (1.2%)
P. vivax	6 (2.2%)	15 (5.5%)	1 (<0.1%)	3 (1.2%)
Mixed Species[b]	0	3 (0.1%)	0	7 (2.9%)

[a] Percent positive in the study population
[b] Positive for both *P. falciparum* and *P. vivax*

In comparing the two methods, 41 of the Colombian patient samples were positive at the genus level but the species was not identified. To address this discordance, we first ruled out possible *P. malariae* infection since this species also co-circulates in Colombia; all 41 samples were negative for this species by qPCR. Another explanation for the discrepancy between the genus and species detection is that the RNA was not sufficiently preserved in the field samples. RNA is highly labile and prone to degradation. Whole blood samples were collected from venipuncture and stored as frozen pellets prior to extraction, without the use of an RNA preservation solution. While the DNA may be preserved for detection by qPCR, the RNA template for species-identification may not. We tested this hypothesis on the 41 discordant samples that were positive by genus screening but negative for species identification, by re-screening duplicate whole blood samples that were pelleted and preserved in Trizol. These RNA samples were not initially collected with the intention of use for malaria screening but instead for an independent gene expression study. Purified RNA was extracted, quantified by nano-drop (ND-1000, Thermo Scientific, USA), and screened by RT-qPCR. Of the 41 discordant samples, five additional samples were species identified (two *P. falciparum* and three *P. vivax*) when analyzed from RNA extracted from Trizol. Although the sample collection method for the diagnostic aspect of our study was not initially designed for analysis of RNA, these results suggest that RNA preservation methods at the point of sample collection may increase the sensitivity of our method, closer to what we observed with the reference materials.

3.7. Summary and brief discussion

The ability to detect infections with low level parasitemia and the identification of the infecting species are two critical parameters guiding malaria treatment [49, 152]. In recent years, several methods have been developed to improve diagnostic sensitivity and specificity [75]. In this chapter, I describe a sensitive method to differentiate submicroscopic malaria infections as low as 10 p/mL and

18 copies/μL for *P. falciparum* and *P. vivax*, respectively. This method is based on a previous protocol developed in our lab [70] that was further optimized for detection of RNA to take advantage of the high copy numbers of 18S rRNA transcript per parasite and increase assay sensitivity. One consideration that could be addressed in this assay is the sampling volume used at the point of extraction. By using only 130 μL of whole blood, our minimum theoretical LOD is 7-8 p/mL. As a single *P. falciparum* parasite can contain 3,500-10,000 copies of 18S rRNA transcript per parasite [73], the LOD of 19 copies/μL determined by the *P. falciparum* plasmid surrogate corresponds to 19,000 copies/mL or 2-5 p/mL. This suggests that by increasing the sampling volume at the point of extraction, it is possible to further increase the sensitivity of this assay. The volume used here was selected to minimize potential cross-contamination during the high-throughput extraction process. While this assay provides excellent sensitivity for identification of malaria infections at the species level using well-controlled reference materials, due to the complex processing and equipment required, we believe that this method would require further improvement in order to be suitable for point of care testing.

While the specificity assessed with patient samples from ProvLab was high (97-98%), two mixed infections were detected by RT-qPCR but not by qPCR. These infections were confirmed by sequencing and can be explained by the improved sensitivity of the RT-qPCR compared with the qPCR species-specific assay. This was demonstrated with a direct comparison of RT-qPCR to qPCR using serial dilutions of parasites from culture. Furthermore, RT-qPCR was superior to qPCR for species identification with the patient samples from Colombia and detected 34/52 positive samples from the febrile cohort and 13/36 positive samples from the asymptomatic cohort. However, 41 discordant results were observed with samples that were genus positive by qPCR but species undetermined by RT-qPCR. We ruled out the possibility of *P. malariae* infection and demonstrated that the use of RNA preservation methods could account for some of the discrepancy.

Alternative explanations should also be considered. It is possible that these were mixed infections in which the concentration of template for each species was below the limit of detection for the species assay. This is consistent with our findings that the amplicons sequenced from a subset of these samples had overlapping electropherograms which could not be deciphered. Another possibility is that the target sequences for the primers and probes used in the species-specific assay are polymorphic in naturally circulating parasite populations. It is also possible that the genus reaction can detect non-*Plasmodium* sequences. Based on the published results of Rougemont *et al.* [62], the genus probe cross-reacted with other 18S rRNA genes from other pathogens (*Aspergillus, Toxoplasma, Neospora,* and Pneumocystis). However, the probes used in the species reactions were highly specific. This could account for a positive genus C_T, but no species identification.

In conclusion, we present a species-specific RT-qPCR method for improved detection of low-level *P. falciparum* and *P. vivax* infections when compared directly to species detection by qPCR. This method could also be potentially adapted for field surveillance upon validation with sample collection methods used in the field (eg, finger prick blood and preparation of dried blood spots) and appropriate preservation of RNA. Another application of this method could be for experimental human malaria infections in a controlled clinical environment. As molecular diagnostics are implemented more broadly for malaria surveillance, and particularly within the context of elimination [148], the increased sensitivity of this method for species identification could be important in epidemiological surveys and to define the submicroscopic burden of malaria.

Chapter 4 – Evaluating the impact of submicroscopic malaria infections in pregnant women from Colombia

Portions of this chapter have been published:

Gavina K, Gnidehou S., Arango E., Hamel-Martineau C., Mitran C., Agudelo O., Lopez C., Karidio A., Banman S., Carmona-Fonseca J., Salanti A., Tuikue-Ndam N., Maestre A., Yanow S.K. Clinical outcomes of submicroscopic infections and correlates of protection of VAR2CSA antibodies in a longitudinal study of pregnant women in Colombia. *IAI*. 2018:**86**(4): e00797-17.

Sample collection and microscopy were performed by local staff in Colombia under the supervision of Dr. Eliana Arango and Dr. Amanda Maestre. Placental histopathology and analyses were performed postpartum in Colombia under the supervision of Dr. Olga Agudelo, Carolina Lopez, and Dr. Jaime Carmona-Fonseca. Dr. Sedami Gnidehou, Chloe Hamel-Martineau, Aisha Karidio, and Shanna Banman performed the ELISA experiments on the serum samples from pregnant women from Colombia (section 4.2.4). Catherine Mitran cultured the *P. falciparum* parasites and performed the selection of CS2 parasites on CSA. Dr. Michael Hawkes assisted with the modelling and statistical analyses performed in this chapter. Dr. Ali Salanti and Dr. Nicaise Tuikue-Ndam both provided reagents for the study. I performed all other experiments in this chapter and wrote the original manuscript under the supervision of Dr. Stephanie Yanow.

4.1. Introduction

MiP can cause serious adverse outcomes such as placental malaria, maternal anemia, PTB, and LBW infants [79]. Most of our knowledge of MiP stems from research based in Africa, particularly where there is high transmission of *P. falciparum*. In sub-Saharan Africa, the risk of these outcomes is heightened in primigravid women infected with *P. falciparum* whereas multigravid women develop pregnancy-specific immunity from previous exposure to MiP [79, 153]. Acquisition of antibodies against the *P. falciparum* protein, VAR2CSA, is a key immune mechanism against MiP. Pregnant women acquire antibodies to VAR2CSA following exposure to MiP, usually in a parity-dependent manner [79]. These antibodies can block adhesion of infected erythrocytes (IEs) to CSA *in vitro* [104, 105], and are associated with protection from placental malaria and other adverse delivery outcomes [106, 107].

Far less is known about the impact of other *Plasmodium* species on MiP outcomes. Studies investigating the impact of *P. vivax* infection during pregnancy revealed conflicting results in terms of implications for adverse delivery outcomes [60, 93-99]. Many *Plasmodium* infections are often undetectable by microscopy and are only detected using molecular diagnostics, thereby considered SMIs. The application of molecular diagnostics for surveillance and screening has revealed a high prevalence of SMIs, particularly in lower transmission settings such as Latin America [154].

Few studies have examined the effects of SMIs in regions where *P. falciparum* and *P. vivax* co-circulate, these yielded mixed results. *P. falciparum* and *P. vivax* SMIs at delivery were associated with poor outcomes in one study from Papua New Guinea (PNG) [103] but not in studies conducted in Colombia and India [60, 117]. In a multi-centre study of pregnant women in Colombia, Guatemala, Brazil, India, and PNG, submicroscopic *P. falciparum* and *P. vivax* infections were not associated with

either maternal anemia or low birth weight [118]. These findings warrant further investigation to determine whether pregnant women with SMIs are at risk for adverse clinical outcomes and to identify possible immune mechanisms, including the role of VAR2CSA antibodies, in response to SMIs in pregnancy.

In this chapter, we describe the first prospective longitudinal study conducted in Latin America to determine the prevalence of SMIs in pregnancy and characterize the host anti-VAR2CSA antibody response to SMIs. Our primary objective was to compare the birth weight in infants born of pregnancies complicated by SMIs to pregnancies with no SMI. Secondary clinical outcomes of interest included PTB, babies small for gestational age, and maternal anemia. We examined antibody levels against VAR2CSA and functional inhibition of parasite binding *in vitro* among women with a SMI as predictors of clinical outcomes of interest.

4.2. Results

4.2.1. Study cohort

We followed a cohort of pregnant women longitudinally to evaluate the impact of SMIs on delivery outcomes. Of the 402 women recruited into the study, 187 women participated through to delivery, and 180 women were included in the data analysis (Figure 4.1). One-hundred forty-eight women were lost to follow up: sixteen women delivered in their home, 19 women delivered in their local village, 98 women delivered at a distant regional hospital, five women delivered when study staff were unavailable to collect samples, and ten women were lost to follow-up without any known reason. Of note, because of the focus on the impact of SMIs, seven women were excluded from downstream data analyses due to a smear-positive *Plasmodium* result by microscopy during pregnancy.

Characteristics of the 180 pregnant women included in the analysis are presented in Table 4.1. Most women were recruited during their second trimester (median = 19 weeks gestation, IQR 15-25) and the median number of antenatal visits attended by women was 2.2, ranging from one to five visits prior to delivery. There were no significant differences in characteristics at enrolment between women with and without a SMI in pregnancy.

4.2.2. Prevalence of MiP

Malaria diagnosis by microscopy and molecular tests were performed on all samples collected at enrolment, during subsequent antenatal visits (ANV), and at delivery. Of the 180 women included in the data analysis, 25% (n=45) were positive for a SMI at least once during pregnancy. Forty-seven percent (21/45) of these women were infected with *P. falciparum*, 33% (15/45) with *P. vivax*, and 20% (9/45) with mixed infections, which included eight simultaneous infections (positive for both *P. falciparum* and *P. vivax* within the same blood sample) and one sequential infection (positive for *P. falciparum* and *P. vivax* at different times within pregnancy).

The dynamics of infection during the course of pregnancy are detailed in Table 2, which presents the number of positive samples at enrolment, during follow-up, and at delivery in all pregnant women (n=1111 total samples). Sixteen women (8.6%, 16/187) had a positive malaria diagnosis at enrolment (four *P. falciparum*, 11 *P. vivax*, and one mixed spp), another 29 infections were diagnosed during follow-up (12 *P. falciparum* infections, 13 *P. vivax* infections, four mixed spp infections), and 12 infections detected at delivery (six *P. falciparum*, four *P. vivax*, and two mixed spp). In nine women, multiple samples were positive for *Plasmodium* by RT-qPCR (eight by the same species, one mixed infection); however, the parasite DNA levels were too low for genotyping to discriminate between new, chronic, or relapse infections (in the case of *P. vivax*).

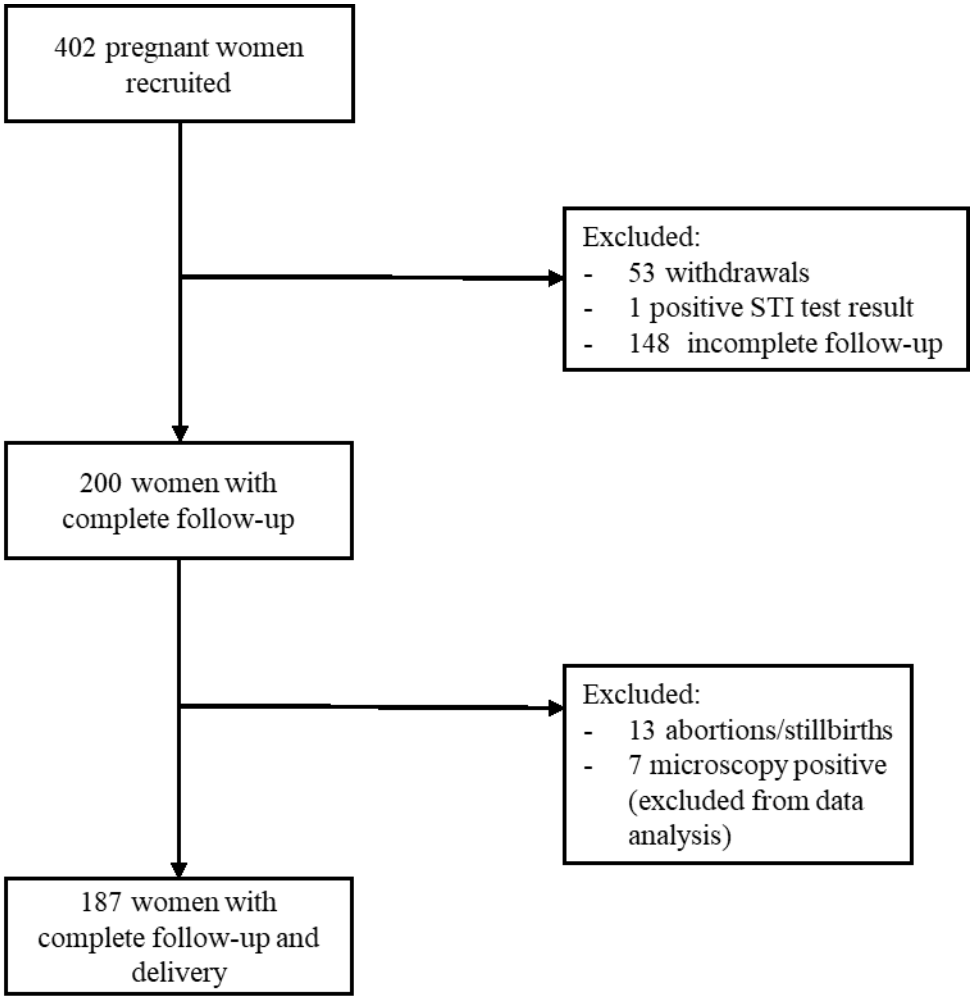


Figure 4.1: Flow chart of pregnant women recruited into the study.

Table 4.1: Characteristics based on submicroscopic malaria infection in pregnant women included in the study

Characteristic at enrolment	PCR positive* (n=45)	PCR negative (n=135)	P- value[a]
Age, years (median, (IQR))	21 (19-28)	22 (19-28)	0.96
Height, cm (median, (IQR))	157 (152-160)	156 (152-161)	0.88
Weight, kg (median, (IQR))	53 (50-62)	55 (50-62)	0.62
Gravidity (n,(%))			
Primigravid	18 (40)	40 (29.6)	0.27
Secundigravid	11 (24.4)	31 (23)	0.84
Multigravid	16 (35.6)	64 (44.4)	0.22
Gestational age, weeks (median, (IQR))[b]	21 (14-24)	19 (15-25)	0.75
Hb level, g/dL (median,(IQR))[c]	11.6 (10.7-12.6)	11.8 (11-12.55)	0.71
Use of bed nets (n,(%))[d]	25 (55.6)	63 (46.7)	0.31
Delivery outcome			
Weight of newborn, g (mean ± SD)[e]	3220 ± 510	3259 ± 451	0.63
Low birth weight babies, <2500g (n(%))	5 (11.1)	6 (4.4)	0.15
Gestational age, weeks (mean ± SD) [f]	38.5 ± 2.1	38.9 ± 2.1	0.25
Pre-term births, <37 weeks (n(%))	6 (13.3)	16 (11.9)	0.32
SGA births (n(%))[g]	5 (11.1)	14 (10.4)	0.59
Anemia, Hb level <11g/dL (n(%))[h]	16 (35.6)	30 (22.2)	0.11
APGAR score (median, (range))[i]	8.5 (5-9)	8.6 (4-10)	0.20

***** – only women positive for a SMI were included in the analysis, **[a] –** calculated by Fisher's exact test, unpaired T-test, or Mann-Whitney test, **[b]** – gestational age determined by ultrasound, data assessed for 171/180 women, **[c]** – data assessed for 167/180 women, **[d]** – data assessed for 179/180 women **[e]** – data for 177/180 women, **[f]** – data assessed for 171/180 women, **[g]** – data for 171/180 women, **[h]** – data for 164/180 women, **[i]** – APGAR index taken after first minute of birth, data assessed for 174/180 women

Table 4.2: Malaria prevalence by microscopy, qPCR, and histopathology in all samples in the study

Diagnostic method	Enrolment (n/N, (%))	Follow-up (n/N, (%))	Delivery (n/N, (%))
Peripheral microscopy			
P. falciparum	0/187, (0%)	1/368, (0.3%)	0/187, (0%)
P. vivax	5/187, (2.7%)	1/368, (0.3%)	0/187, (0%)
Mixed infection[a]	0/187, (0%)	0/368, (0%)	0/187, (0%)
Peripheral RT-qPCR			
P. falciparum	4/187, (2.1%)	12/368, (3.3%)	6/187, (3.2%)
P. vivax	11/187, (5.9%)	13/368, (3.5%)	4/187, (2.1%)
Mixed infection[a]	1/187, (0.5%)	4/368, (1.1%)	2/187, (1.1%)
Placental microscopy			
P. falciparum	N/A	N/A	0/182, (0%)
P. vivax	N/A	N/A	0/182, (0%)
Mixed infection[a]	N/A	N/A	0/182, (0%)
Placental RT-qPCR			
P. falciparum	N/A	N/A	4/182, (2.2%)
P. vivax	N/A	N/A	2/182, (1.1%)
Mixed infection[a]	N/A	N/A	3/182, (1.6%)
Placental histopathology	N/A	N/A	0/187, (0%)
Acute infection[b]	N/A	N/A	0/187, (0%)
Chronic infection[c]	N/A	N/A	12/187, (6.4%)
Past infection[d]			

[a] –defined as the simultaneous infection with both *P. falciparum* and *P. vivax*, [b] – defined as the presence of parasites, [c] – defined as the presence of parasites and hemozoin, [d] – defined as the presence of hemozoin only

Placental malaria was not observed in this population by microscopy; however, placental blood from nine women was positive by RT-qPCR. Based on histopathology, active placental infections were not observed among the 180 women but hemozoin was detected in the placentas of 12 women (6.7%), indicating a past *Plasmodium* infection in pregnancy. This was further confirmed by RT-qPCR; six of the twelve women were positive by RT-qPCR at an earlier time in pregnancy (two *P. falciparum* infections, one *P. vivax* infection, and three mixed infections). In an analysis to investigate whether SMIs during pregnancy were associated with placental malaria (past placental infection by histopathology or placental SMI by RT-qPCR), we observed no significant association between SMIs during pregnancy and presence of hemozoin in the placenta (p=0.19) or submicroscopic placental infection by RT-qPCR (p=0.08).

4.2.3. Clinical outcomes at delivery

Overall, few differences were observed in the delivery outcomes of pregnant women who were infected with submicroscopic malaria during pregnancy compared with those who were not (Table 4.1). The mean ± SD birth weight of infants born to mothers with an SMI was 3220 g ± 510 g, compared to 3259 g ± 451 g among those without a SMI (difference of means 38.4 g, 95% CI -121 to 198, p=0.63).

Mono-infection with different species of *Plasmodium* was not associated with decreases in birth weight, gestational age, weight-for-age Z-scores, and maternal Hb levels at delivery (Figure 4.2). However, we observed that babies born to mothers who had a mixed infection during pregnancy, compared to uninfected mothers, had a significantly lower gestational age at birth (mean difference 2.3 weeks, 95% CI 35 to 38, p=0.0033) (Figure 4.2B). In a multivariable linear regression model adjusting for maternal age and parity as significant independent covariates, mixed infections remained a significant predictor of gestational age at delivery (p=0.0074). Expressed as a relative risk, mothers who were exposed to both *P. falciparum* and *P. vivax* during pregnancy were three times more likely (p=0.005) to deliver a pre-term infant (<37 weeks gestational age) compared to mothers who were not infected during pregnancy (Table 4.3). In the nine women with a mixed infection, we investigated whether placental malaria affected the risk for PTB. No significant association was observed with PTB and past placental infection (presence of hemozoin by placental histopathology) (p=0.69, 95% CI -3.1 to 4.5).

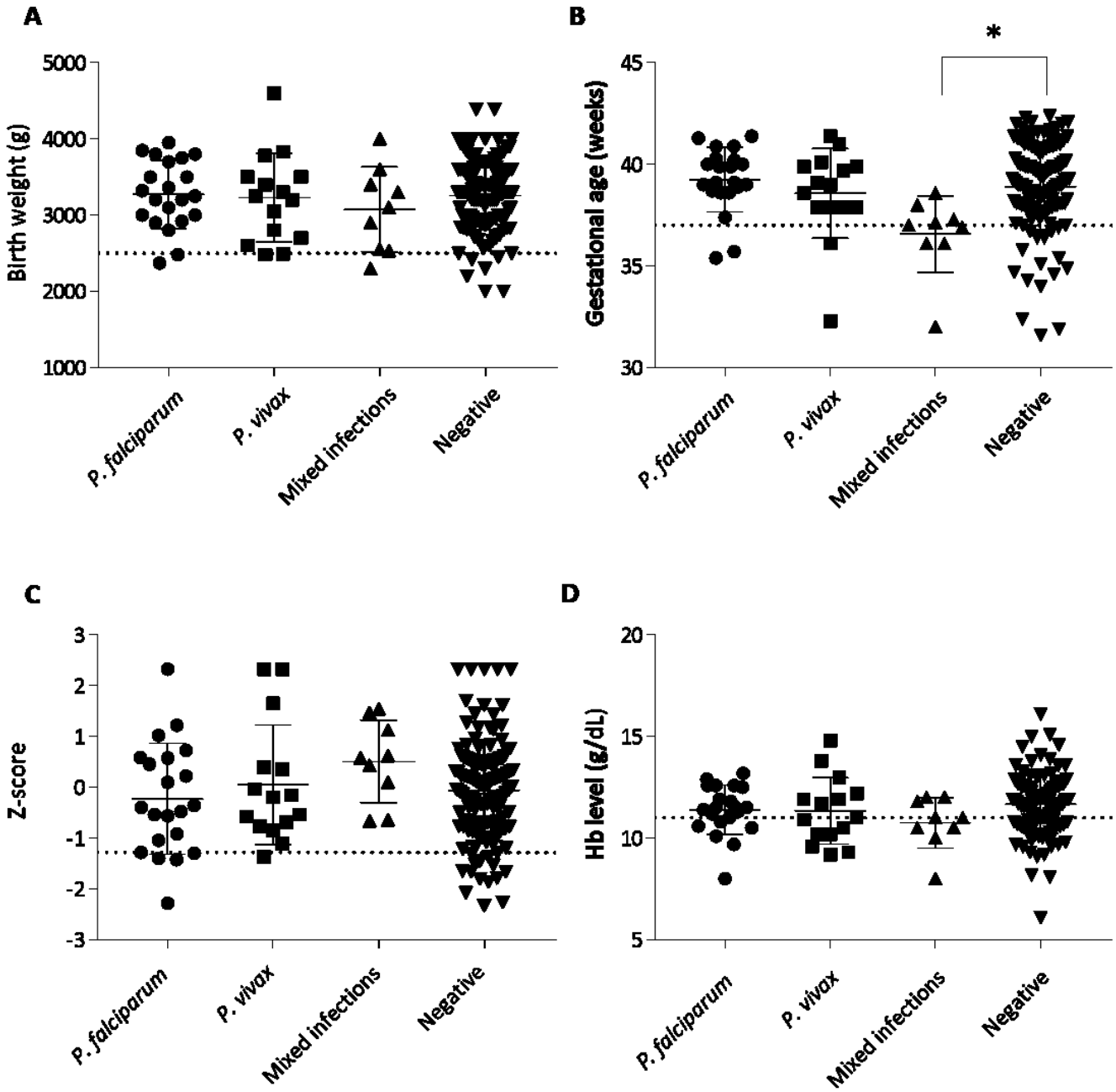


Figure 4.2: Submicroscopic malaria infections during pregnancy are not generally associated with changes in outcomes at delivery. (A) Infant birth weight, (B) gestational age at delivery, (C) Z-scores of babies to determine small for gestational age (SGA), and (D) maternal hemoglobin (Hb) levels at delivery. Solid horizontal lines indicate the mean for each group, error bars indicate standard deviation (SD), and dotted horizontal lines represent the threshold for low birth weight (2,500 g), pre-term birth (37 weeks), SGA (10th percentile), and anemia (11 g/dL). *p=0.0033; Mann-Whitney test.

Table 4.3: Risk factors associated with adverse delivery outcomes

Risk factors	Small for gestational age		Low birth weight		Pre-term birth		Maternal anemia	
	RR (95% CI)	P-value	RR (95% CI)	P-value	RR (95% CI)	P-value	RR (95% CI)	P-value
Submicroscopic infection								
None	1		1		1		1	
P. falciparum	1.57 (0.59-4.17)	0.36	1.93 (0.54-6.90)	0.31	0.73 (0.19-2.88)	0.66	0.71 (0.28-1.76)	0.46
P. vivax	0.80 (0.20-3.13)	0.75	1.93 (0.54-6.90)	0.31	0.76 (0.19-3.02)	0.70	1.13 (0.53-2.43)	0.74
Both species[a]	n/a[b]	-	2.43 (0.35-16.82)	0.30	**3.40 (1.45-7.97)**	**0.005**	1.37 (0.55-3.37)	0.50
Timing of infection								
None	1		1		1		1	
1st Trimester	n/a[b]	-	n/a[b]	-	n/a[b]	-	0.80 (0.14-4.46)	0.80
2nd Trimester	0.56 (0.08-3.91)	0.56	1.49 (0.19-11.55)	0.70	0.53 (0.08-3.74)	0.53	0.64 (0.22-1.83)	0.40
3rd Trimester	1.26 (0.49-3.22)	0.63	2.71 (0.81-9.05)	0.11	1.70 (0.76-3.78)	0.20	1.06 (0.61-1.86)	0.83
Gravidity								
Multigravid	1		1		1		1	
Primigravid	1.38 (0.87-2.17)	0.17	1.63 (0.91-2.91)	0.10	1.00 (0.57-1.75)	0.99	0.99 (0.60-1.66)	0.99

[a] – Includes both simultaneous (detected in the same blood sample) and sequential (at different times within pregnancy) infections of *P. falciparum* and *P. vivax*

[b] – Zero patients based on exposure, unable to calculate relative risk

In those women with repeated parasite positivity by RT-qPCR (n=9) during the course of pregnancy, we did not observe any significant association with LBW (p=1.0), PTB (p=0.83), SGA (p=0.45), or maternal anemia at delivery (p=0.51) compared to women with only one positive sample during pregnancy.

4.2.4. VAR2CSA-specific antibodies and correlates of protection

We hypothesized that the lack of adverse birth outcomes following mono-species SMI, particularly with *P. falciparum*, could be attributed to protective antibodies in pregnant women. To test this hypothesis, we measured VAR2CSA-specific IgG and observed that 65% (117/180) of women had antibodies at enrolment (Figure 4.3A). No significant difference was observed in the antibody levels between women with an SMI compared to healthy women (p=0.6885). In a regression analysis to test if antibody levels were associated with clinical outcomes at delivery, no significant association was observed between VAR2CSA-specific IgG levels and birth weight (Figure 4.4A, p=0.58), gestational age (Figure 4.4B, p=0.67), SGA (Figure 4.4C, p=0.58), or maternal Hb levels at delivery (Figure 4.4D, p=0.54).

Antibody levels in primigravid and multigravid women were similar (p=0.76), as were the proportions of primigravid (64%) and multigravid (65%) women with antibodies, consistent with our previous observations in pregnant women from this region [109]. The proportion of women seropositive for anti-VAR2CSA antibodies was similar at enrolment and delivery (p=0.67); however, anti-VAR2CSA antibody levels decreased from enrolment to delivery by a mean of 7.2 AU (p<0.0001) in all women (Figure 4.3B). In a sub-analysis by gravidity, the anti-VAR2CSA antibody levels of primigravid and multigravid women had a mean decrease of 8.2 AU (p<0.001) and 6.8 AU (p<0.0001) from enrolment to delivery, respectively.

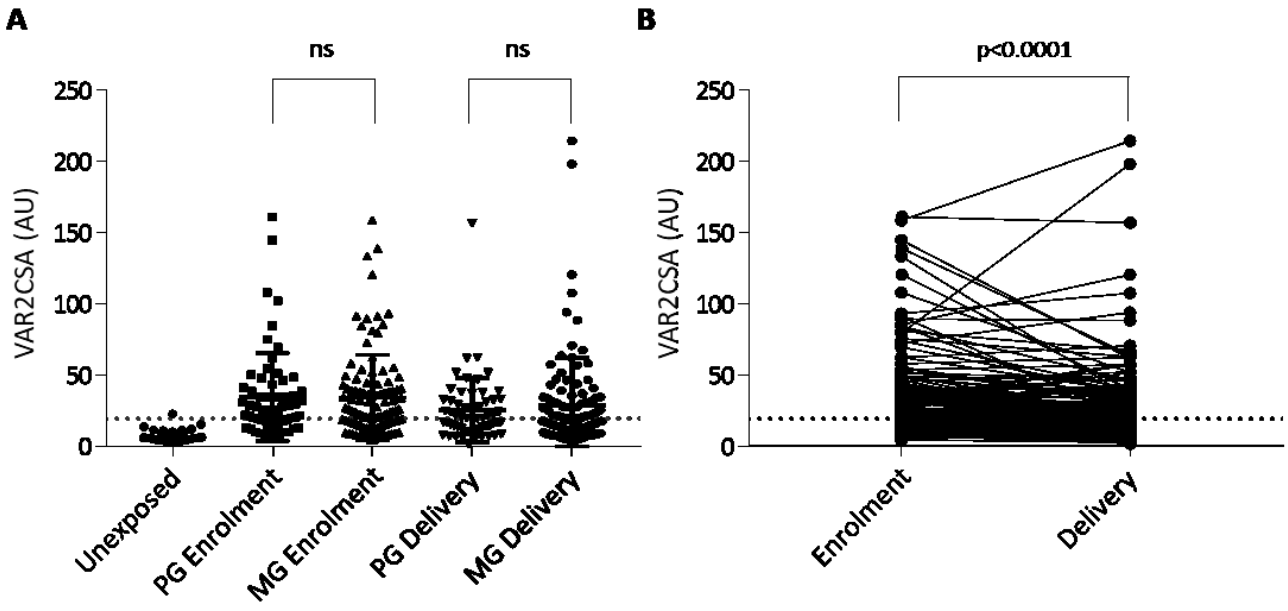


Figure 4.3: Anti-VAR2CSA antibody levels are independent of parity. (A) Anti-VAR2CSA antibody levels were measured by ELISA at inclusion and at delivery in sera from Colombian primigravid (PG) and multigravid (MG) women and compared to a Colombian malaria-unexposed control group. (B) Matched antibody levels measured at enrolment and at delivery in individual subjects. Horizontal lines indicate the mean for each group, error bars indicate SD, and dotted horizontal lines mark the cut-off for seropositivity (AU= 19.7). ns – not significant.

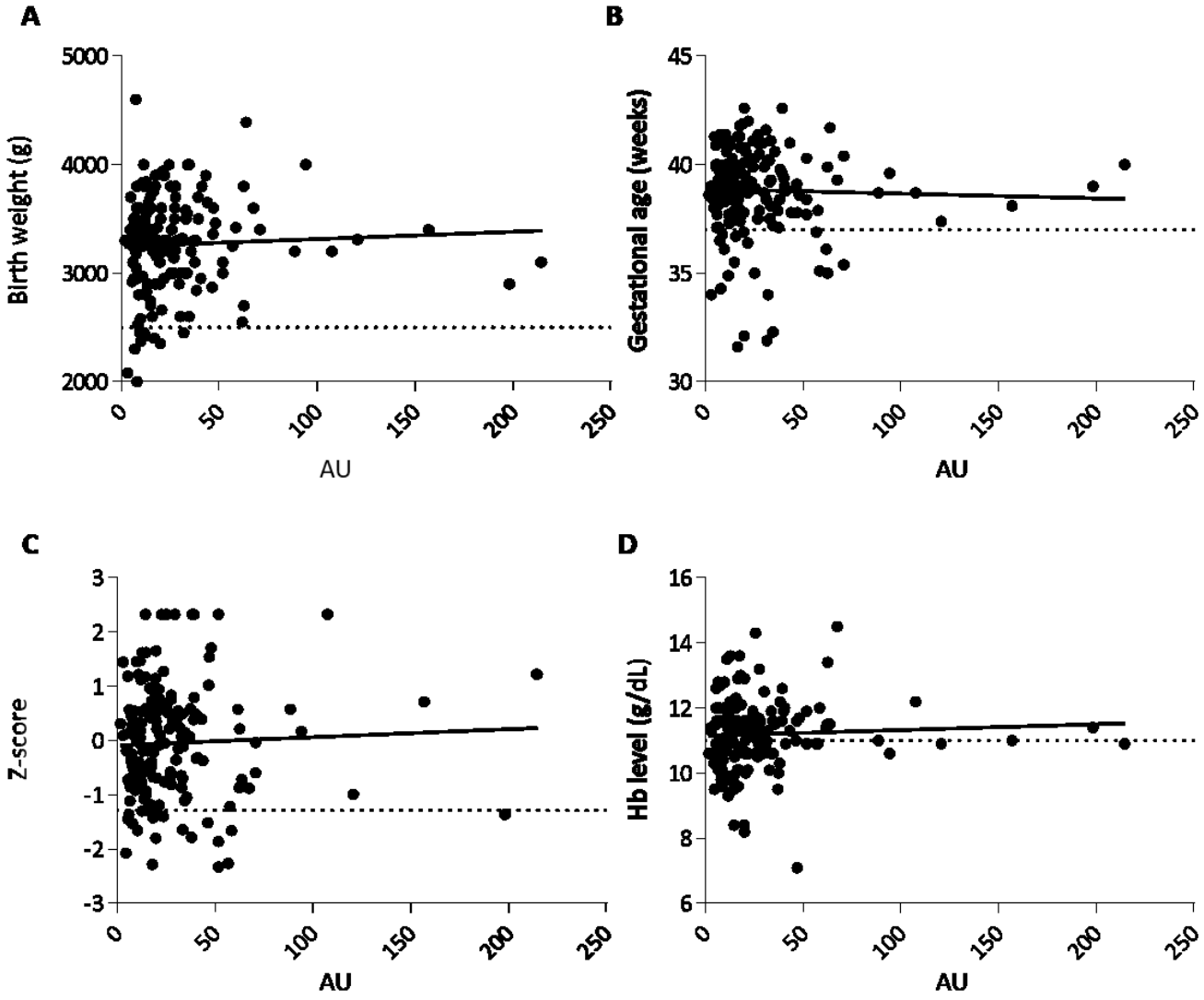


Figure 4.4: Anti-VAR2CSA antibody levels are not associated with pregnancy outcomes at delivery. Linear regression analysis of the association of serum VAR2CSA-specific antibody levels and birth weight (A), gestational age at delivery (B), Z-scores of SGA (C), and maternal hemoglobin levels at delivery (D). Horizontal dotted lines indicate the threshold for low birth weight (2,500 g), pre-term birth (37 weeks), SGA (10^{th} percentile), and for anemia (11 g/dL). p-value determined by linear regression.

Infection with *P. falciparum* in pregnancy typically results in boosting of VAR2CSA-specific antibodies [107, 155]. We therefore analysed antibody levels in SMI samples with a positive diagnosis by RT-qPCR, and in samples collected in subsequent ANVs or at delivery to test whether SMIs boosted VAR2CSA-specific antibodies. There was no significant boosting of antibody levels following *Plasmodium* infection, irrespective of the species (p=0.66, p=0.21, p=0.25, for *P. falciparum, P. vivax*, and mixed infections, respectively). A subset of samples from nine women were selected to illustrate VAR2CSA-specific antibody dynamics (Figure 4.5).

To determine whether the high frequency of anti-VAR2CSA antibodies at enrolment contributed to positive pregnancy outcomes despite SMI during pregnancy, we measured the functional activity of antibodies at enrolment in the 45 women with SMI. Antibody function was assessed using the IBA, which measures the ability of antibodies to block binding of *P. falciparum* CS2 IEs to CSA *in vitro*. Parasites were selected to express VAR2CSA and bound strongly to CSA in the presence of pooled sera from unexposed Colombians, which served as a negative control. The percent inhibition of individual sera from malaria-exposed women was determined relative to this unexposed pool. Samples that inhibited IE binding to CSA by >39.1% were considered functional. Of the 45 women with a SMI, 40% (18/45) had functional antibodies at enrolment (Figure 4.6A); 28% (5/18) were primigravid and 48% (13/27) were multigravid (Figure 4.6B). A significant difference in the level of inhibition was observed between primigravid and multigravid women (31.5% vs 40%, mean difference 8.5%, p=0.049).

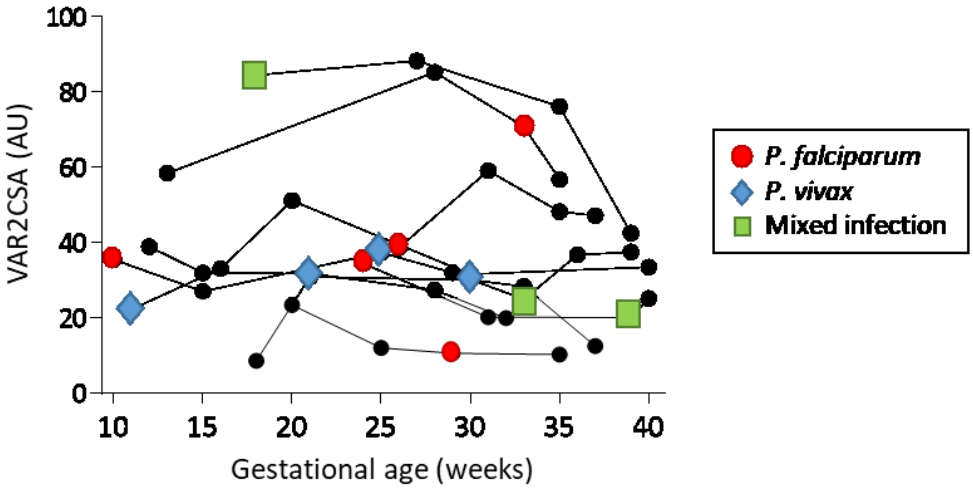


Figure 4.5: Anti-VAR2CSA antibody levels are not boosted by submicroscopic *Plasmodium* infections during pregnancy. Anti-VAR2CSA antibody levels in sera from nine selected pregnant women with exposure to *P. falciparum*, *P. vivax*, and mixed infections. Black dots indicate serum measurements from sample obtained at antenatal care. Coloured symbols indicate infection at time of antenatal visit (red circle; *P. falciparum* infection, blue diamond; *P. vivax* infection, and green square; mixed infection).

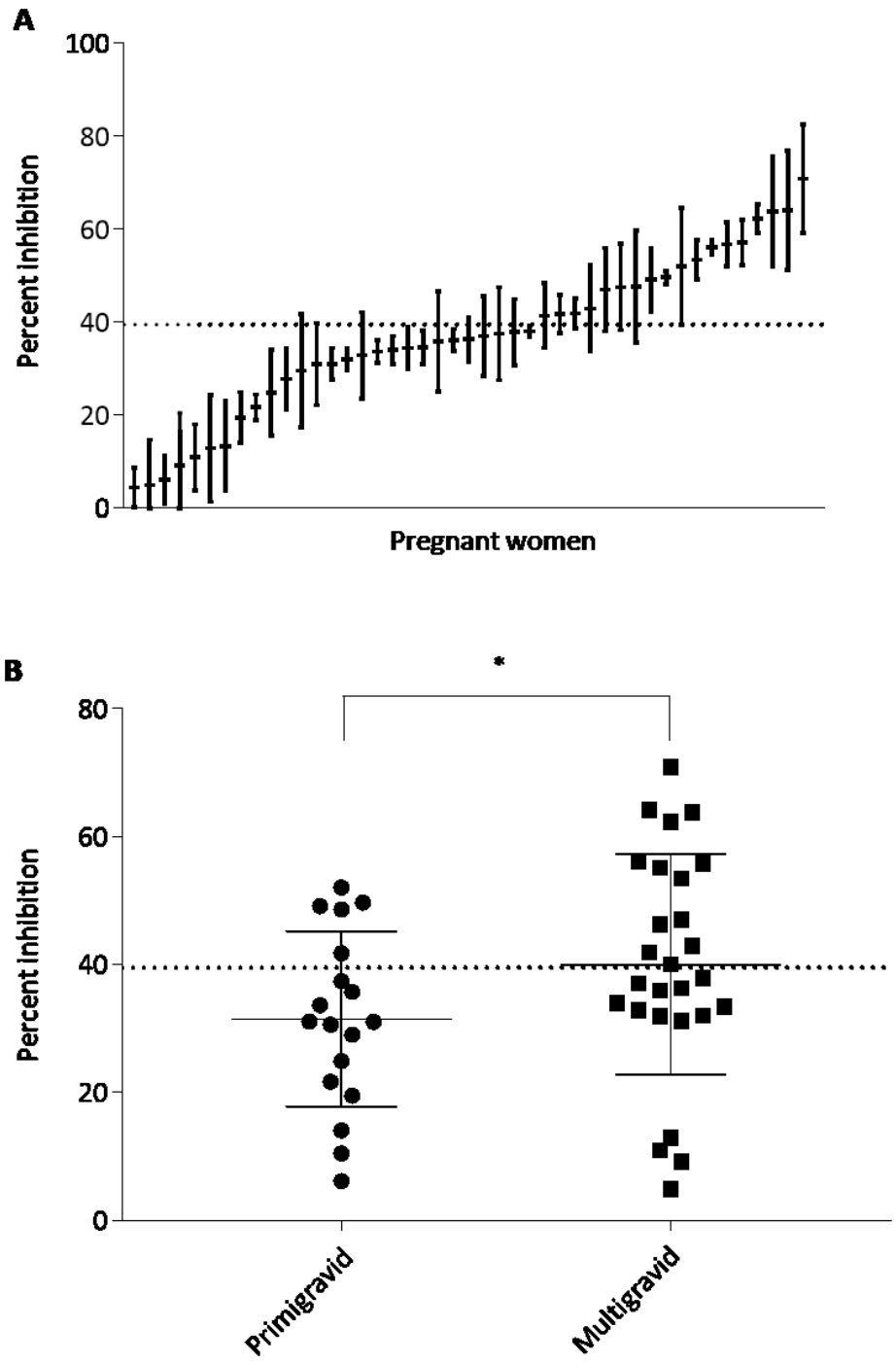


Figure 4.6: CSA adhesion blocking antibodies are observed in sera from Colombian pregnant women infected with submicroscopic malaria. (A) Binding inhibition profile for pregnant women infected by *Plasmodium spp.* (n=45). Horizontal dash indicates the mean of three replicate measurements, error bars indicate SD, horizontal dotted line represents cut-off for inhibition (39.1%). (B) Inhibitory activity of antibodies from primigravid and multigravid women at enrolment. Horizontal dotted line represents cut-off for inhibition (39.1%). *p=0.049; Mann-Whitney test.

We next performed linear regression analysis to test if inhibitory antibody function correlated with clinical outcomes (Figure 4.7). No significant association was observed between the percent inhibition and birth weight (p=0.85) (Figure 4.7A), gestational age (p=0.80) (Figure 4.7B), or Z-scores for SGA (p=0.33) (Figure 4.7C). However, in a multivariable linear regression model adjusting for the potential confounding effects of covariates, inhibitory antibody function was a significant independent predictor of higher maternal Hb levels at delivery (p=0.0086) (Figure 4.7D). Furthermore, both primigravid and multigravid women who did not have functional antibodies were at a significantly higher risk for anemia (Hb <11g/dL) (RR=1.87, 95% CI 1.13-2.79, p=0.014) (Table 4.4).

Table 4.4: Relative risk of delivery outcomes in women without CSA adhesion blocking antibodies compared to women with functional antibodies

Outcome	Relative risk[a]	95% Confidence interval	p-value
Low birth weight	1.08	0.19-5.93	0.93
Pre-term birth	0.59	0.13-2.65	0.49
Small for gestational age	1.62	0.36-7.29	0.53
Maternal anemia	**1.87**	**1.13-2.79**	**0.014**

[a] – relative risk of women without functional antibodies vs women with functional antibodies (reference group); function defined as being seropositive (AU $\geq$ 19.7) with a % inhibition $\geq$ 39.1%.

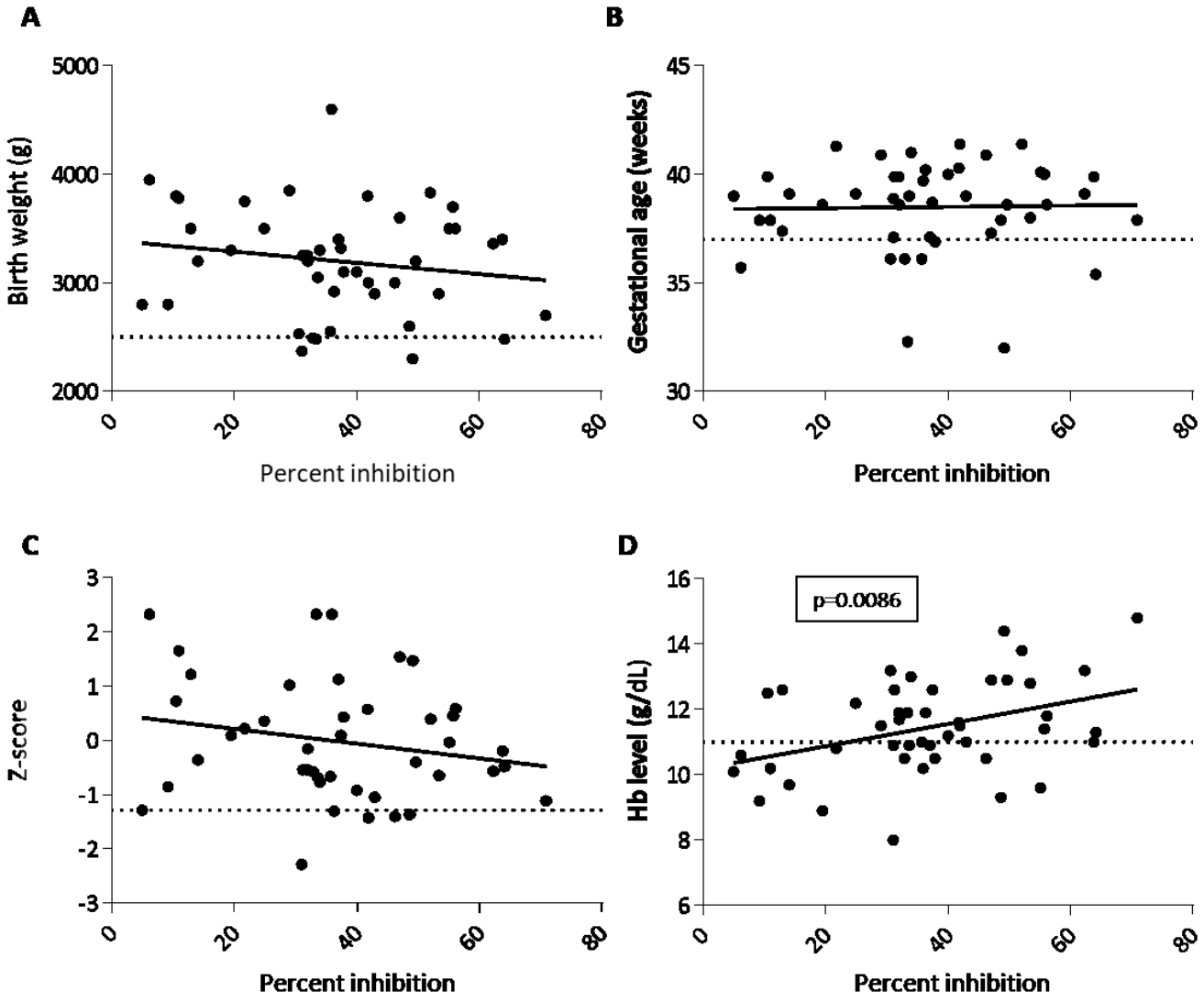


Figure 4.7: CSA adhesion blocking antibodies correlate with hemoglobin levels at delivery but not with other delivery outcomes in women with a submicroscopic infection. Linear regression analysis of the association of inhibitory antibodies and birth weight (A), gestational age at delivery (B), Z-scores of SGA (C), and maternal hemoglobin levels at delivery (D). Horizontal dotted lines indicate the threshold for low birth weight (2,500 g), pre-term birth (37 weeks), SGA (10^{th} percentile), and for anemia (11 g/dL). p-value determined by linear regression.

4.3. Summary and brief discussion

In this chapter, the results of a prospective longitudinal study of MiP in Latin America are presented to assess the impact of SMIs in pregnancy on delivery outcomes. We observed a high frequency of submicroscopic MiP (25%, 45/180). We did not detect a statistically significant difference in birth weight between infants born of pregnancies complicated by submicroscopic MiP and babies born from healthy mothers. Of note, our study had adequate statistical power (80%) to detect a clinically meaningful difference in birth weight (250 g), and the mean difference observed in our cohort was 38 g, based on the 95% CI. We can therefore exclude an effect of SMI on infant birth weight in this Colombian cohort. However, in a secondary analysis looking at other delivery outcomes, women with mixed *P. falciparum* and *P. vivax* SMIs were at increased risk of PTB. These findings reveal a novel association of *P. falciparum* and *P. vivax* mixed SMIs and adverse birth outcomes that is particularly relevant to regions outside of Africa. While this result has not been reported previously in longitudinal studies of MiP, it is in keeping with findings from non-pregnant populations that mixed *P. falciparum* and *P. vivax* infections can have deleterious outcomes, including anemia in infants in Papua, Indonesia [156] and severe malaria in children in PNG [157]. The mechanism underlying the pathogenesis of mixed infections is not known.

The women in our study had no other negative delivery outcomes associated with submicroscopic malaria. Our data are consistent with the findings of a multi-centre study, which included Colombia, that submicroscopic *P. falciparum* and *P. vivax* infections had no significant association with LBW or maternal anemia [118]. In contrast, several studies in Africa reported associations between SMIs and placental malaria, anemia, PTB and LBW [111-116]. In our study, women with SMIs were not treated with antimalarials, as RT-qPCR was performed retrospectively, and no IPTp program is available in the study communities, suggesting that these SMIs resolved naturally during the course of pregnancy. Our data suggest that women had immunity that facilitated parasite

clearance and prevented sequestration of IEs in the placenta. Antibodies that blocked adhesion of IEs to CSA were associated with reduced risk of anemia in women exposed to SMIs during pregnancy, suggesting a role for these antibodies in opsonisation of IEs [108]. Opsonizing antibodies to variant surface antigens were associated with reduced risk of anemia in pregnant women in Malawi, suggesting this is an important immune mechanism in pregnancy.

Antibodies that protect women from adverse outcomes in pregnancy predominantly target the VAR2CSA surface antigen [79, 153]. A majority of women in our cohort were positive for anti-VAR2CSA antibodies at enrolment (65%). Furthermore, VAR2CSA antibody levels were independent of parity and 64% of primigravid women were seropositive at enrolment. These results are consistent with our previous observations in this region that antibodies to the DBL5ε domain of VAR2CSA were not parity-dependent [109]. In fact, we observed similar levels of antibodies to DBL5ε in non-pregnant populations (men and children), as well as antibodies to the DBL3X domain and the minimal CSA-binding domain, ID1-ID2. This prior work, in conjunction with the data presented here, suggest that in this population and malaria transmission area, antibodies to the full-length VAR2CSA protein may be acquired outside of pregnancy. One hypothesis is that the antibodies arose against an antigen from *P. vivax* and cross-reacted with VAR2CSA. This is supported by our findings that pregnant women, men and children who were infected with only *P. vivax* had antibodies that cross-reacted with the DBL5ε, DBL3X and ID1-ID2 domains of VAR2CSA with similar frequencies [109].

The study had certain limitations. First, the final sample size was affected by the loss to follow-up in the early stages of the study. Nevertheless, the study was adequately powered not to reject the null hypothesis of our primary objective to test whether SMIs during pregnancy are significantly associated with birth weight at delivery. Additionally, not all women attended monthly antenatal visits and infections may have been missed during follow-up.

It is estimated that 4.3 million pregnancies are at risk of malaria in Latin America [122]. Our results with molecular diagnostics confirm the high prevalence of submicroscopic MiP in Colombia despite a reduction in the overall burden of malaria in recent years [158]. In this chapter, the results shed light on the clinical outcomes of SMIs with *P. falciparum*, *P. vivax* and mixed infections in pregnancy in this lower transmission region and identifies an important role for host immunity in protecting pregnant women from SMI-related anemia.

Chapter 5 – Development of a direct-from-blood species-specific malaria molecular diagnostic to be used at point-of-care

Experiments in this chapter are unpublished.

Dr. Ninad Mehta helped with troubleshooting and calibration of the CHAI Open-qPCR machines. Dr. Abu Kausar produced and performed QC for the hydrogels. I performed all experiments in this chapter under the supervision of Dr. Stephanie Yanow.

5.1. Introduction

In field settings where resources are limited, microscopy is often the gold standard for malaria diagnosis at, or near, the point-of-care. RDTs, based on immunochromatography, provide an alternative method for diagnosing malaria in these settings. While convenient, RDTs are limited by decreased sensitivity at low levels of parasitemia, inhibition at high levels of parasitemia, and often the inability to distinguish malaria species or mixed infections [159, 160]. DNA-based methods, such as qPCR and LAMP, are diagnostic tools that are both highly specific and sensitive [161]. However, some of the drawbacks to these methods include the requirement of cold storage and the pre-processing or extraction of template from patient samples, making field-use implementation a challenge. To overcome these limitations, our lab developed methods in which qPCR-based malaria diagnostics can be performed directly from blood on a portable PCR platform [142, 143, 162]. The method involved the use of a desiccated hydrogel, which allows for the preservation of PCR reagents at ambient temperatures. Eliminating the need for cold storage of reagents and streamlining experimental preparation overcomes one of the major challenges for implementing molecular diagnostics in the field or at POC.

In Chapter Three, I showed that RNA-based methods were necessary to identify the *Plasmodium* species in low-level submicroscopic malaria infections. The work in this chapter describes, firstly, the optimization of a direct-from-blood qPCR screen to detect *P. falciparum* and *P. vivax* directly from blood; and secondly, the adaptation of this assay to an RNA-based platform to improve sensitivity for the detection of submicroscopic infections at the species level. The assays were developed for use on a portable real-time PCR machine in the field. The RT-qPCR assay was validated with a panel of blood samples from non-pregnant patients in Colombia, confirmed by the RT-qPCR

method described in Chapter Three, and the results were directly compared to probe-based RT-qPCR and direct-from-blood qPCR.

5.2. Results

5.2.1. DNA-based detection of *P. falciparum* and *P. vivax* direct-from-blood

The species-specific assays in this section were performed on two different operating platforms: a BioRad CFX Connect and CHAI Biotechnologies Open-qPCR. Additionally, assays were performed under two separate reaction conditions: in a conventional liquid reaction (section 5.2.1.1) and in a desiccated hydrogel reaction (section 5.2.1.2).

5.2.1.1. Detection of *P. falciparum* and *P. vivax* from blood in the liquid reaction

These assays used species-specific primers, which are listed in Table 2.1 in section 2.3.2. The primers used in this method target the 18S ribosomal RNA gene and were highly specific to *P. falciparum* and *P. vivax*. They were designed with the intention of multiplexing the two species-specific reactions together. A titration of primer concentrations was tested to determine the optimal concentration for the detection of *P. falciparum* and *P. vivax* directly from blood. Three concentrations were tested: 100 nM, 200 nM, and 400 nM. Amplification and melting curves were observed for all three concentrations; however, 400 nM primer concentrations yielded a higher relative fluorescence and had the least variation between replicates.

Using *P. falciparum* 3D7 parasites and a *P. vivax* plasmid surrogate spiked into negative blood, we prepared two dilution series (from 10^7 p/mL to 10^0 p/mL and 10^6 copies/µL to 10^0 copies/µL,

respectively) to determine the analytical sensitivity of these assays (Figure 5.1). Dilutions were run in triplicate and were performed on a BioRad CFX Connect. For the *P. falciparum* assay, all triplicate reactions produced an amplification and melting curve down to a dilution of 10^3 p/mL, with two of three replicates detected at 10^2 p/mL. For the *P. vivax* assay, all triplicate reactions produced an amplification and melting curve down to a dilution of 10^3 copies/µL. Based on these results, the LOD for these assays when performed on the CFX is 10^3 p/mL for *P. falciparum* and 10^3 copies/µL for *P. vivax*.

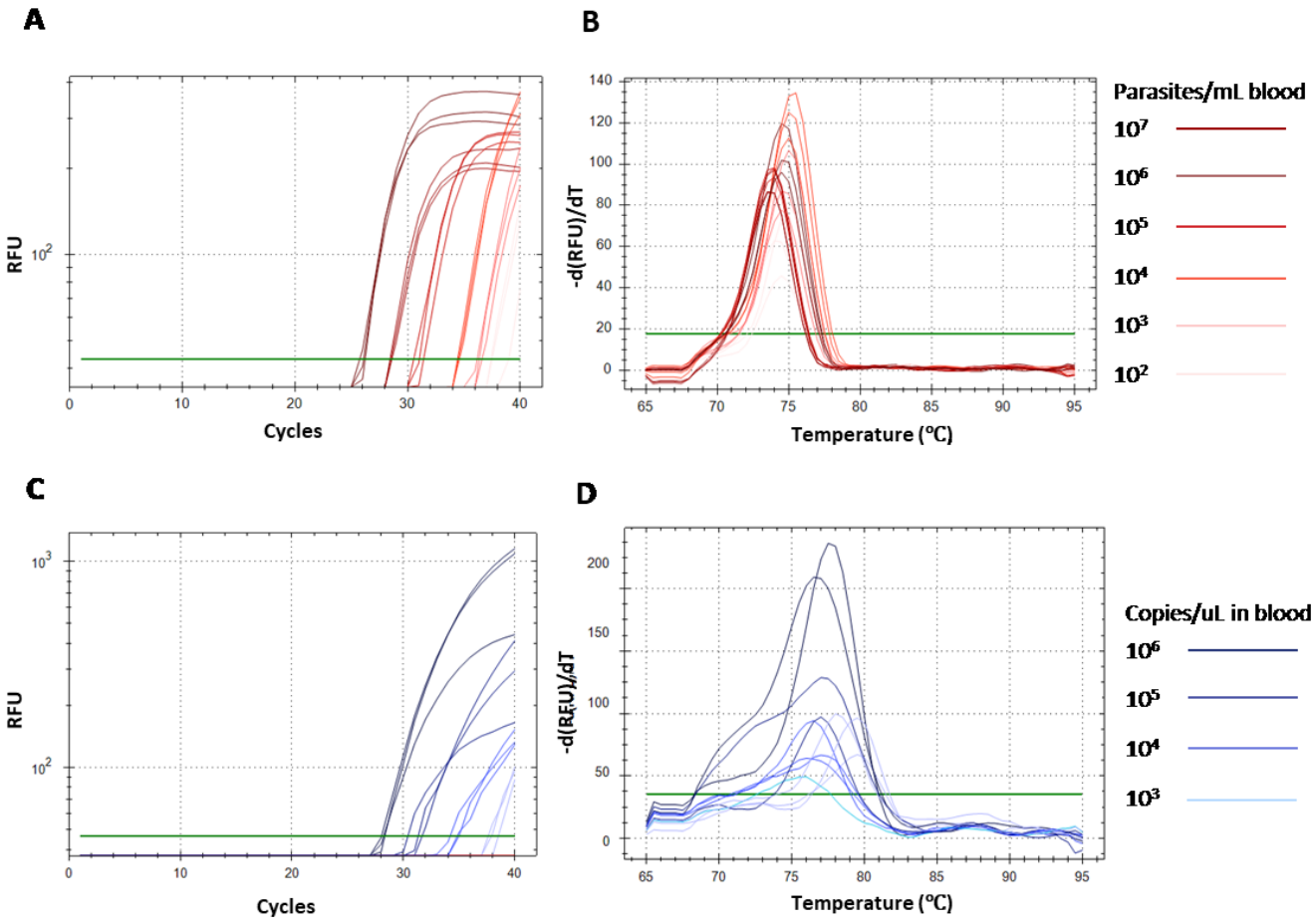


Figure 5.1: Detection of *Plasmodium falciparum* and *Plasmodium* vivax in blood by real-time PCR (qPCR) on a BioRad CFX Connect. **(A)** Real-time amplification and **(B)** melting curves of 3D7 ring-stage *P. falciparum* parasites cultured in blood and tested by qPCR. **(C)** Amplification and **(D)** melting curves of a *P. vivax* plasmid surrogate spiked into negative blood and tested by qPCR. Threshold (horizontal line) was set using the automatic call by CFX software.

Based on the results from the singleplex species reactions, we next tested whether it was possible to multiplex the two reactions together. Method optimization included running a temperature gradient from 55°C to 65°C to test different annealing temperatures, as well as a primer titration ranging from a final concentration of 100 nM to 400 nM, with the goal of maximizing separation between the *P. falciparum* melting temperature (T_M) and *P. vivax* T_M in the melting curve analysis. To test this, a *P. falciparum* and a *P. vivax* clinical sample (confirmed by microscopy and qPCR) were run in triplicate on the CFX (Figure 5.2). An annealing temperature of 58°C with a primer concentration of 200 nM yielded the least variation among replicates with maximal separation in the melting curve analysis. The melting temperature for the *P. falciparum* product was 76°C and the melting temperature for the *P. vivax* product was 80.2 ± 0.3°C.

5.2.1.2. Detection of *P. falciparum* and *P. vivax* in the hydrogel reaction

We tested whether the species-specific reactions could be performed with mastermix formulated in the desiccated hydrogel. We tested the *P. falciparum* and *P. vivax* assay on six patient samples (two *P. falciparum*, two *P. vivax*, and two negative) obtained from the ProvLab that have been confirmed by both microscopy and qPCR (Figure 5.3). Primers were multiplexed and samples were run in triplicate. The mean melting temperature for the *P. falciparum* and *P. vivax* samples were 75.75 ± 0.27°C and 78.78 ± 0.52°C, respectively. Both *Plasmodium* negative blood samples and NTCs were negative for amplification.

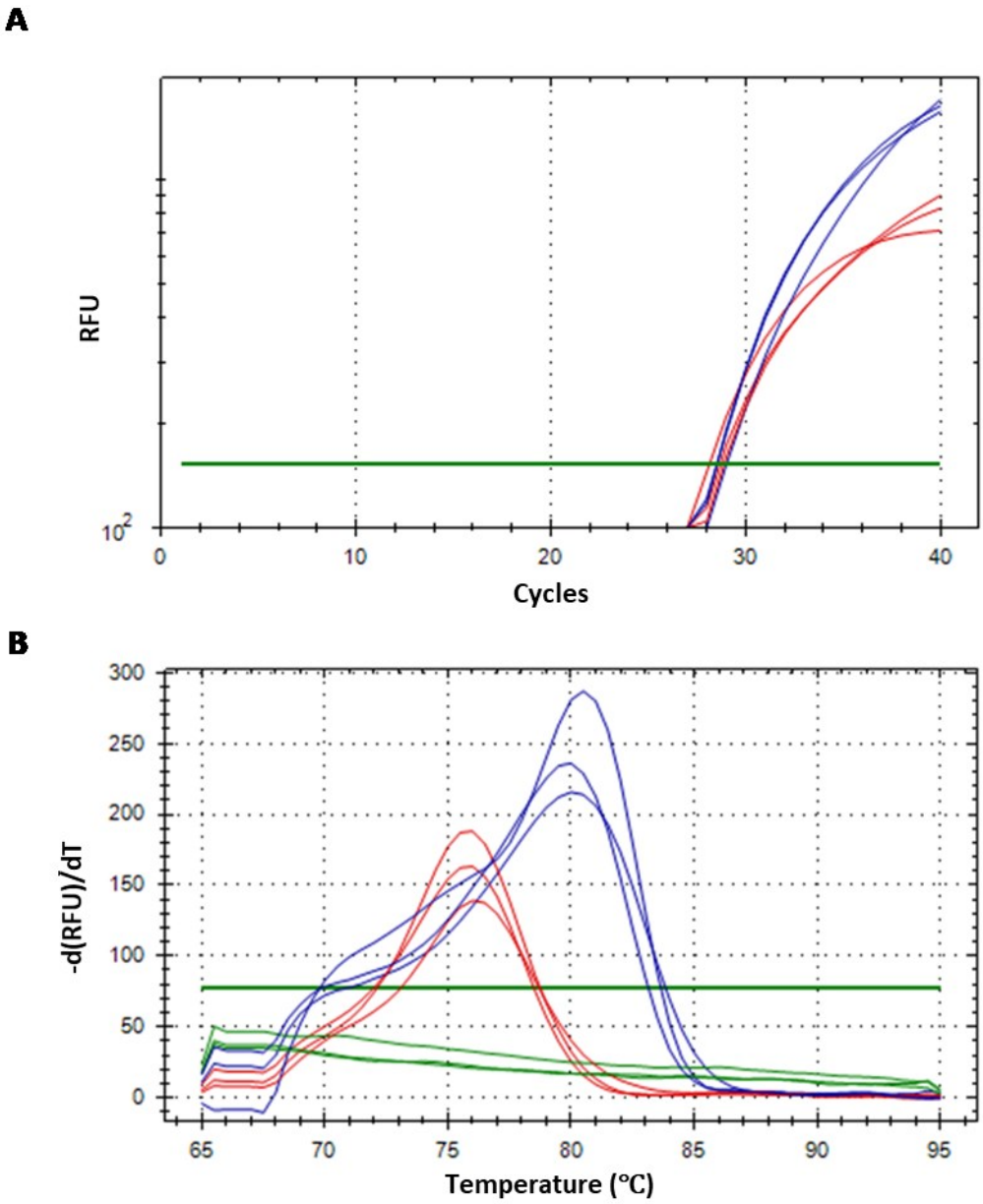


Figure 5.2: Multiplexed detection of *Plasmodium falciparum* and *Plasmodium vivax* in blood by real-time PCR on a BioRad CFX Connect. (A) Amplification and **(B)** melting curves from a blood sample of a patient infected with *P. falciparum* (red), blood from a patient infected with *P. vivax* (blue), and negative blood (green). Blood samples were tested in triplicate and were previously confirmed by reference qPCR and microscopy. Threshold (horizontal line) was set using the automatic call by CFX software.

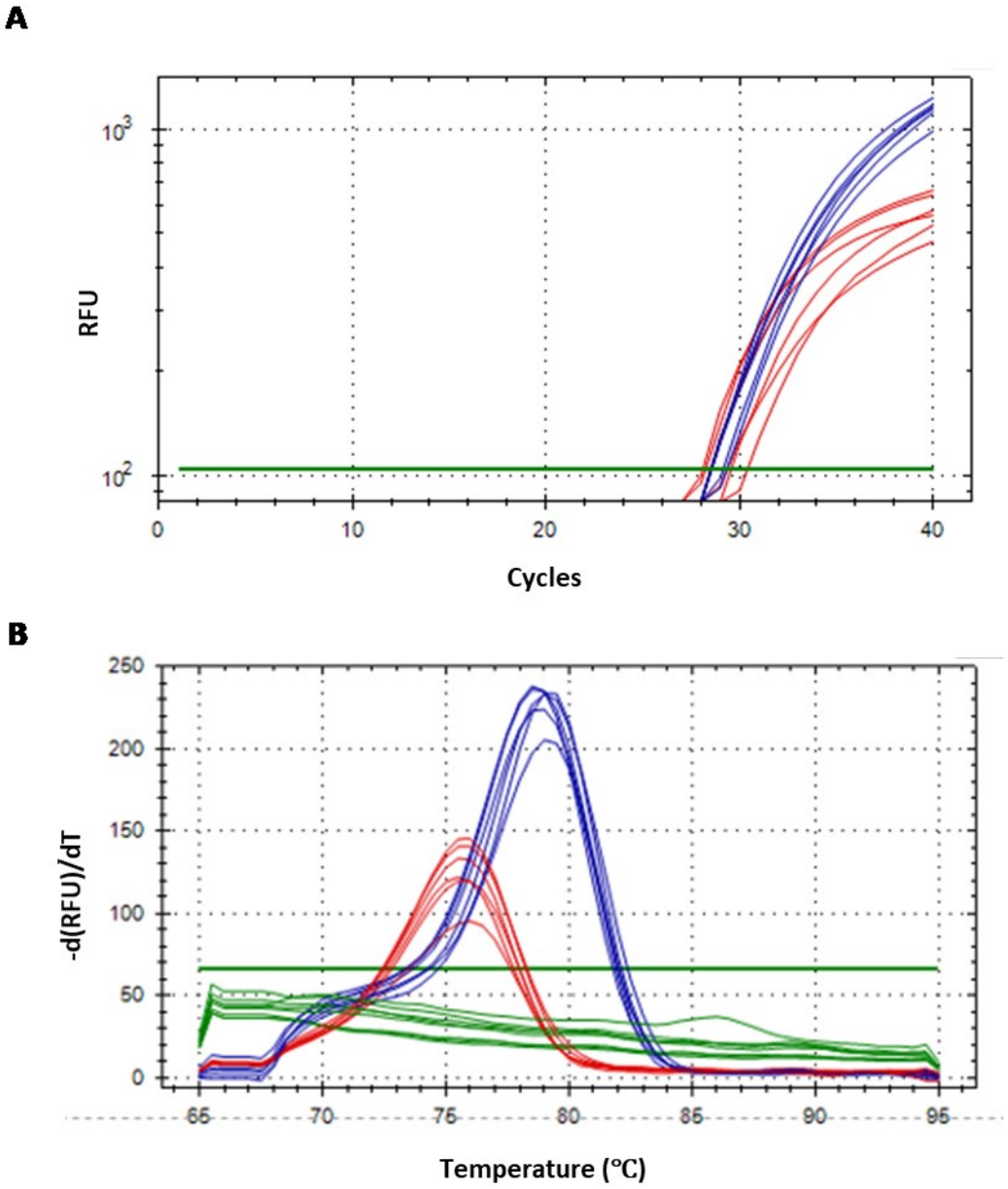


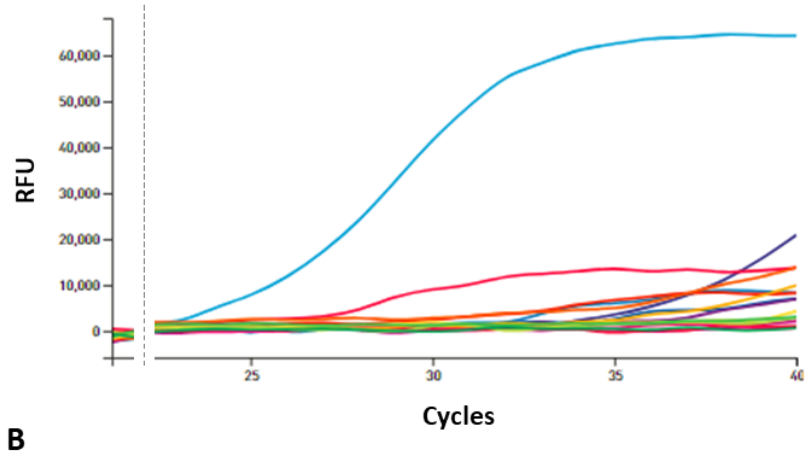
Figure 5.3: Multiplexed detection of *Plasmodium falciparum* and *Plasmodium vivax* in blood by real-time PCR in hydrogel on a BioRad CFX Connect. (A) Amplification and **(B)** melting curves from a blood sample of patients infected with *P. falciparum* (red), blood from patients infected with *P. vivax* (blue), and negative blood (green). Blood samples were tested in triplicate for two patients infected with *P. falciparum*, two patients infected with *P. vivax*, and two *Plasmodium* negative patients. All samples were previously confirmed by reference qPCR and microscopy. Threshold (horizontal line) was set using the automatic call by CFX software.

5.2.1.3. Detection of *P. falciparum* and *P. vivax* on a portable qPCR platform

We used the same dilution series described in section 5.2.1.1 for *P. falciparum* and *P. vivax* to determine the analytical sensitivity of these assays in hydrogel when using the CHAI open-qPCR, a portable qPCR platform. For the *P. falciparum* assay, dilutions from 10^6 to 10^0 p/mL were run in duplicate (Figure 5.4). Amplification for duplicate samples was observed down to a dilution of 10^3 p/mL, with a mean T_M of 79.7 ± 1.2°C. For the *P. vivax* assay, a dilution range of 10^6 to 10^0 copies/µL from a plasmid surrogate spiked into negative blood was run in duplicate (Figure 5.5). Amplification of duplicate samples was observed down to a dilution of 10^3 copies/µL, with a mean T_M of 77.9 ± 0.9°C. NTCs run on both assays were negative for amplification. Similar to the BioRad CFX, the LODs for the *P. falciparum* and *P. vivax* assays when performed on a portable PCR machine were 10^3 p/mL and 10^3 copies/µL, respectively.

In addition to the singleplex *P. falciparum* and *P. vivax* reactions, we also tested whether multiplexing the species reactions could be performed on the portable qPCR machine in hydrogel. To this end, we tested a *P. falciparum* patient sample, a *P. vivax* patient sample, and a negative patient sample (confirmed by microscopy and qPCR) obtained from the ProvLab (Figure 5.6). All patient samples were performed in duplicate. *P. falciparum* 3D7 parasites from culture (10^5 p/mL) were used as a positive control, and negative blood was used as an NTC. While amplification of both *P. falciparum* and *P. vivax* replicates was observed, the T_Ms of each product in the melting curve analysis could not be discerned from one another (*P. falciparum* T_M = 83.9 ± 0.08°C; *P. vivax* T_M = 83.6 ± 1.5°C). These results suggest that further assay optimization is required for multiplexing the two species-specific reactions.

A



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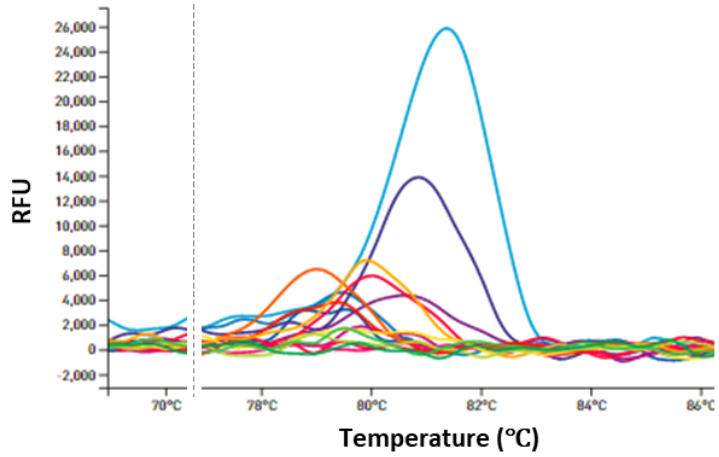
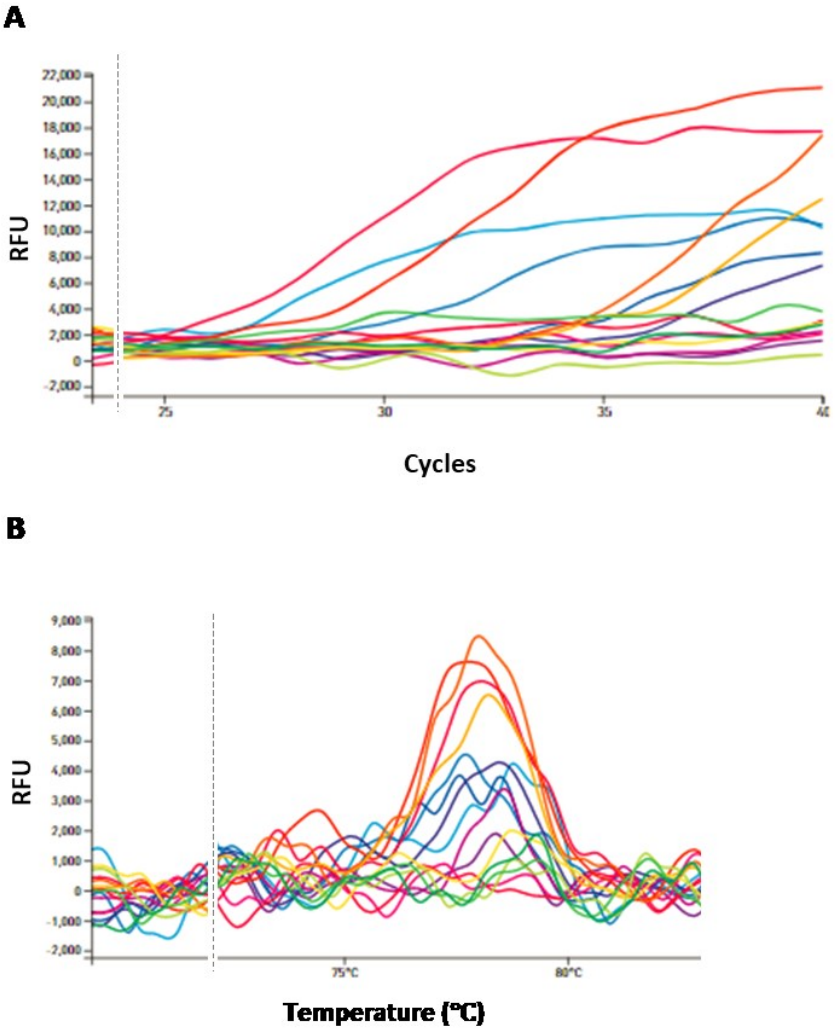


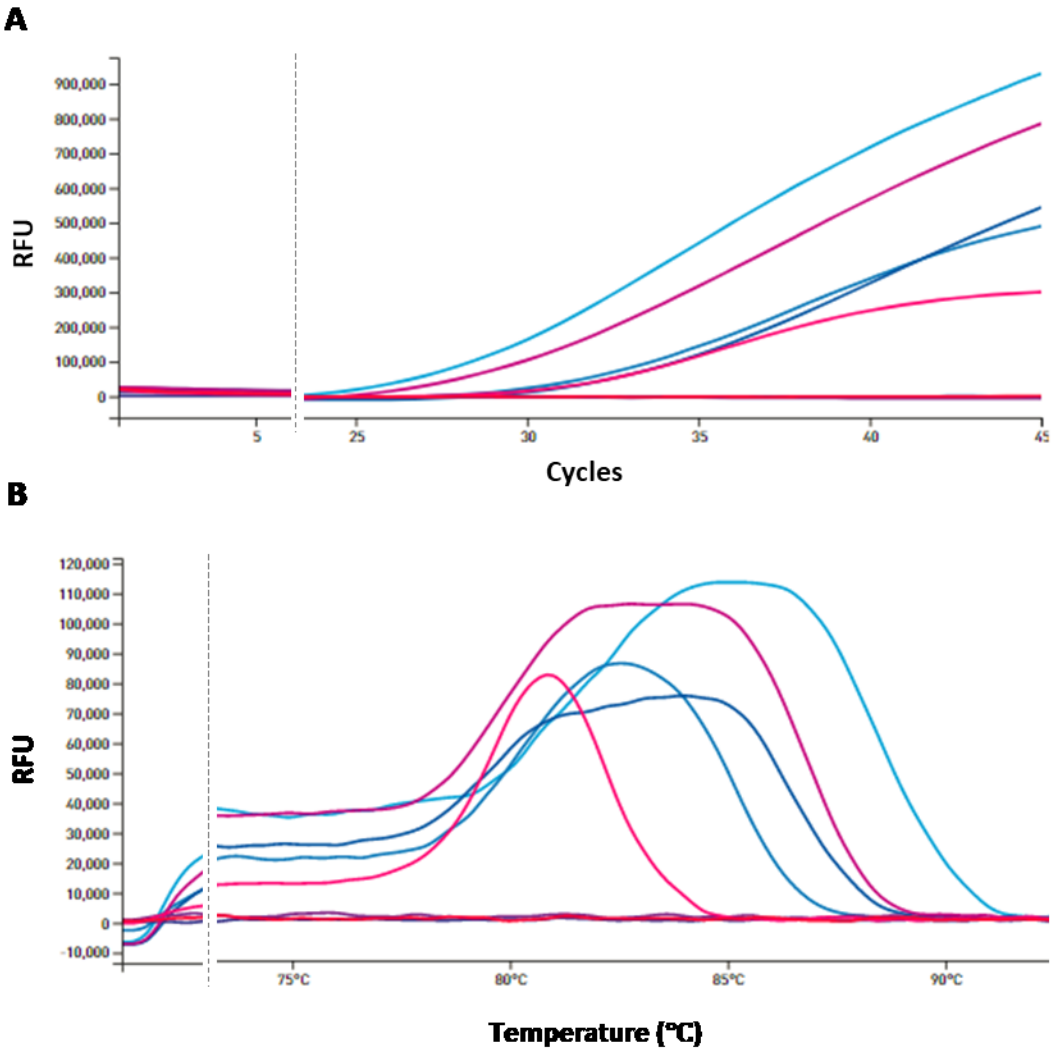
Sample	Well	C_T	T_M
10^6	A1	25.14	81.38
10^6	B1	25.05	80.01
10^5	A2	30.18	79.46
10^5	B2	28.71	79.38
10^4	A3	33.92	77.41
10^4	B3	33.79	79.04
10^3	A4	36.60	80.95
10^3	B4	36.06	79.92
10^2	A5	-	80.58
10^2	B5	-	87.02
10^1	A6	-	61.24
10^1	B6	-	92.28
10^0	A7	-	-
10^0	B7	-	61.16
NTC	A8	-	-
NTC	B8	-	-

Figure 5.4: Detection of *Plasmodium falciparum* in serially diluted blood by real-time PCR in hydrogel on a CHAI Open-qPCR machine. (A) Amplification and **(B)** melting curves from 3D7 ring-stage *P. falciparum* parasites cultured in blood and tested by qPCR. Parasites were serially diluted in increments of ten-fold dilutions in negative blood. Blood samples were tested in duplicate.



Sample	Well	C_T	T_M
10^6	A1	25.68	75.67
10^6	B1	26.01	78.06
10^5	A2	28.77	77.72
10^5	B2	28.28	77.97
10^4	A3	33.16	78.76
10^4	B3	34.50	77.98
10^3	A4	35.02	78.54
10^3	B4	35.16	78.25
10^2	A5	-	88.51
10^2	B5	-	39.89
10^1	A6	-	65.12
10^1	B6	-	-
10^0	A7	-	-
10^0	B7	-	91.49
NTC	A8	-	-
NTC	B8	-	-

Figure 5.5: Detection of *Plasmodium vivax* in serially diluted blood by real-time PCR in hydrogel on a CHAI Open-qPCR machine. (A) Amplification and **(B)** melting curves from a *P. vivax* plasmid surrogate spiked into negative blood. Samples were serially diluted ten-fold in negative blood. Blood samples were tested in duplicate.



Sample	Well	C_T	T_M
P. vivax	A1	28.20	84.65
P. vivax	A2	31.97	82.53
P. falciparum	A3	33.21	83.92
Negative	A4	-	66.41
Negative	A5	-	-
P. falciparum	A6	29.42	84.03
Positive	A7	31.42	80.90
NTC	A8	-	

Figure 5.6: Multiplexed detection of *Plasmodium falciparum* and *Plasmodium vivax* in blood by real-time PCR in hydrogel on a CHAI Open-qPCR machine. (A) Amplification and **(B)** melting curves from a blood sample of a patient infected with *P. falciparum*, blood from a patient infected with *P. vivax*, and negative blood. Blood samples were tested in duplicate and all samples were previously confirmed by reference qPCR and microscopy.

5.2.2. RNA-based detection of *P. falciparum* and *P. vivax* directly from blood

We next tested whether RT-qPCR could be used to detect *P. falciparum* and *P. vivax* RNA targets to further improve the sensitivity of the direct-from-blood assays. Method optimization included titrations of: the VitaNavi enzyme (final volumes ranging from 0.5 µL to 2.5 µL), RNase inhibitor (final concentrations ranging from 1.6 units/µL to 6.4 units/µL), and primers (final concentrations ranging from 100 nM to 400 nM) to determine optimal concentrations for the detection of *P. falciparum* and *P. vivax* directly from blood. Amplification was observed at all ranges; however, peak heights for melting curves were highest and variation between replicates was lowest when 0.5 µL of enzyme, 3.2 units/µL of RNase inhibitor, and 400 nM of primers were used per reaction.

5.2.2.1. Sensitivity of direct-from-blood RT-qPCR for the detection of P. falciparum and P. vivax

To determine the analytical sensitivity of the direct-from-blood RT-qPCR assay, we tested serial dilutions of *P. falciparum* and *P. vivax* to determine the LOD. For the *P. falciparum* control, serial dilutions of 3D7 parasites from culture ranged from 10^7 to 10^0 p/mL. For the *P. vivax* control, we used a clinical sample from a patient infected with *P. vivax* (0.1% parasitemia) obtained from the ProvLab. The patient sample was serially diluted in uninfected blood across a range of eight log dilutions (10^0 to 10^{-7}). Dilutions were run on the BioRad CFX in triplicate, from 10^6 to 10^0 p/mL for the *P. falciparum* assay and 10^0 to 10^{-7} log dilutions for the *P. vivax* assay (Figure 5.7). Amplification was observed in all triplicates down to a dilution of 10^2 p/mL and 10^{-4} log dilutions for the *P. falciparum* and *P. vivax* assays, respectively. Additionally, amplification was observed in two of three replicates at a dilution of 10^1 p/mL for the *P. falciparum* assay and in one of three replicates at 10^{-5} log dilution for the *P. vivax* assay.

The serial dilutions were also tested on the portable PCR platform (Figure 5.8 and Figure 5.9). Dilutions of 3D7 *P. falciparum* parasites (10^7 to 10^1 p/mL) and a clinical sample of a patient infected with *P. vivax* (10^0 to 10^{-6} log dilutions) were performed in duplicate. Amplification was observed in all duplicate samples down to a dilution of 10^2 p/mL and 10^{-4} log dilutions for the *P. falciparum* and *P. vivax* assays, respectively. The mean T_M for *P. falciparum* products was 79.8 ± 0.4°C, while the mean T_M for *P. vivax* products was 81.8 ± 0.9°C.

We compared the direct-from-blood RT-qPCR results of the 3D7 *P. falciparum* parasite dilution series to the direct-from-blood qPCR assay (Table 5.1). Our results suggest that the RT-qPCR method has superior sensitivity compared to the qPCR method on both the BioRad CFX platform and CHAI Open qPCR machine. The mean C_T values were significantly different between the two methods on both instruments (CFX 10^6 p/mL mean difference = 2.13 C_T, p-value = 0.05; Open-qPCR 10^6 p/mL mean difference = 6.94 C_T, p-value = 0.0001; Open-qPCR 10^5 p/mL mean difference = 4.91 C_T, p-value = 0.02).

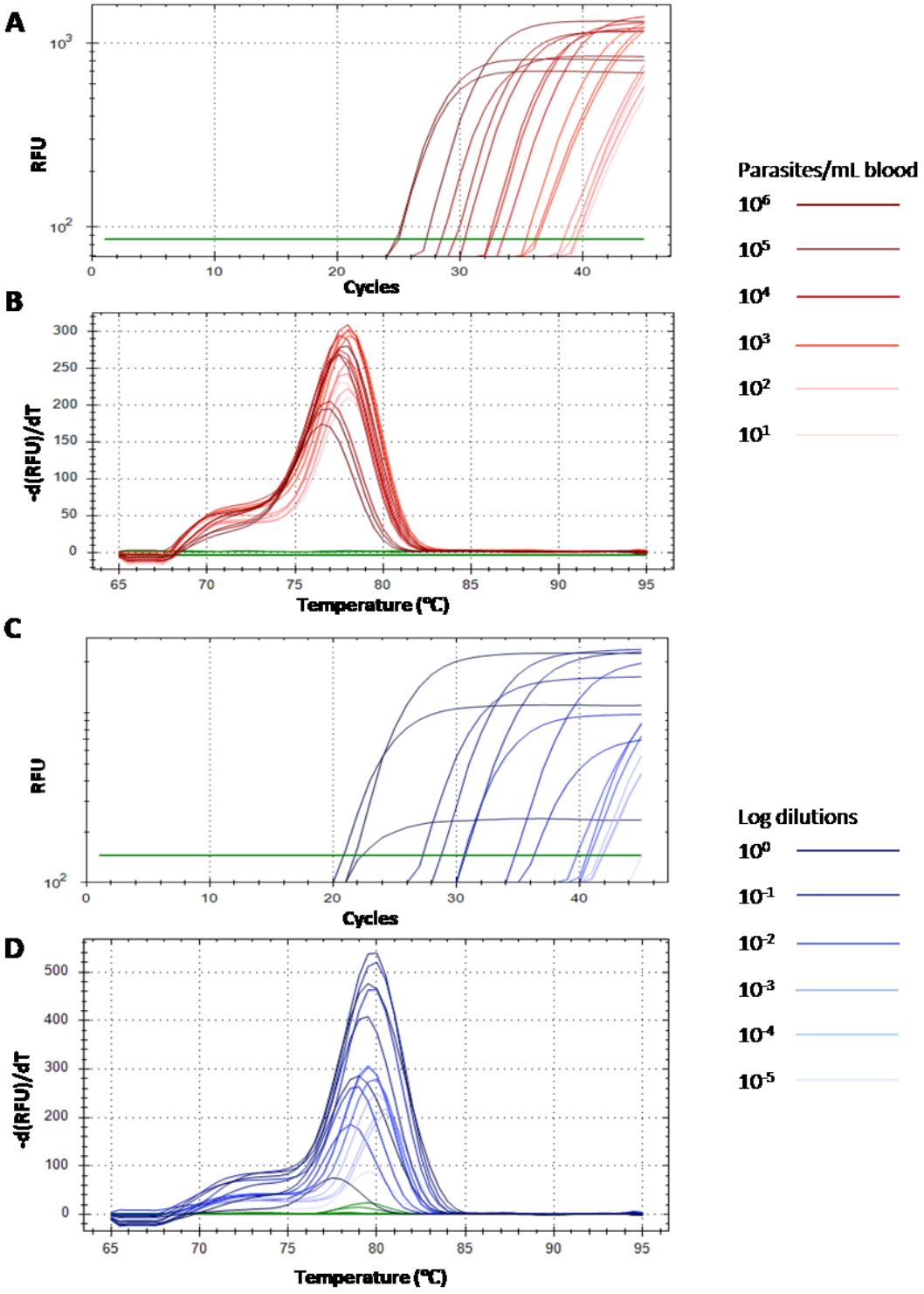
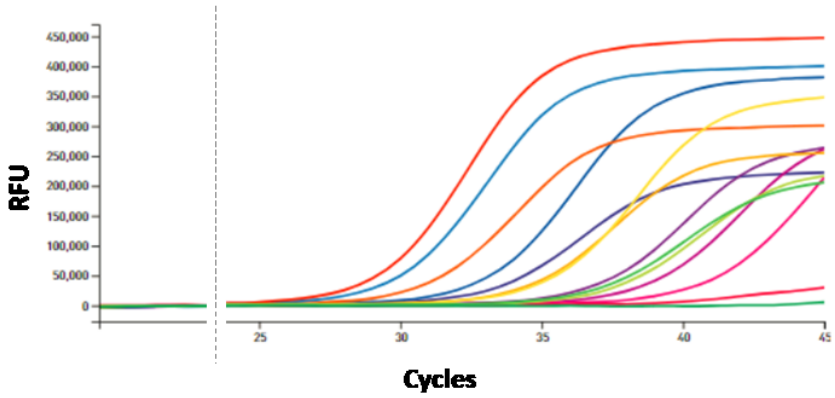
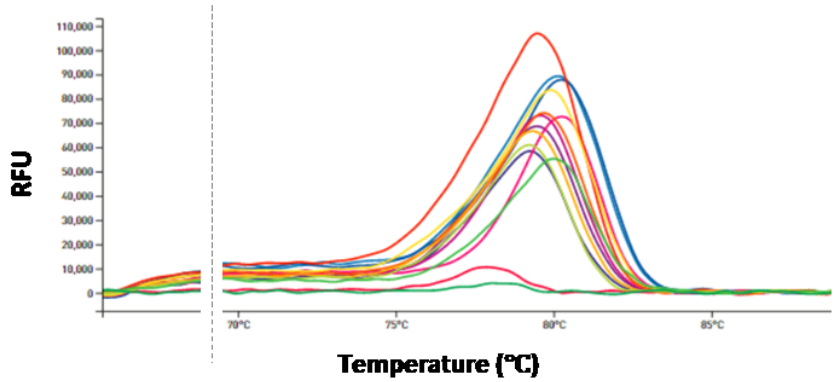


Figure 5.7: Detection of *Plasmodium falciparum* and *Plasmodium vivax* in blood by reverse-transcription real-time PCR (RT-qPCR) on a BioRad CFX Connect. (A) Real-time amplification and **(B)** melting curves from serially diluted 3D7 ring-stage *P. falciparum* parasites cultured in blood and tested by RT-qPCR. **(C)** Amplification and **(D)** melting curves from a *P. vivax* patient sample serially diluted ten-fold in negative blood and tested by RT-qPCR.

A

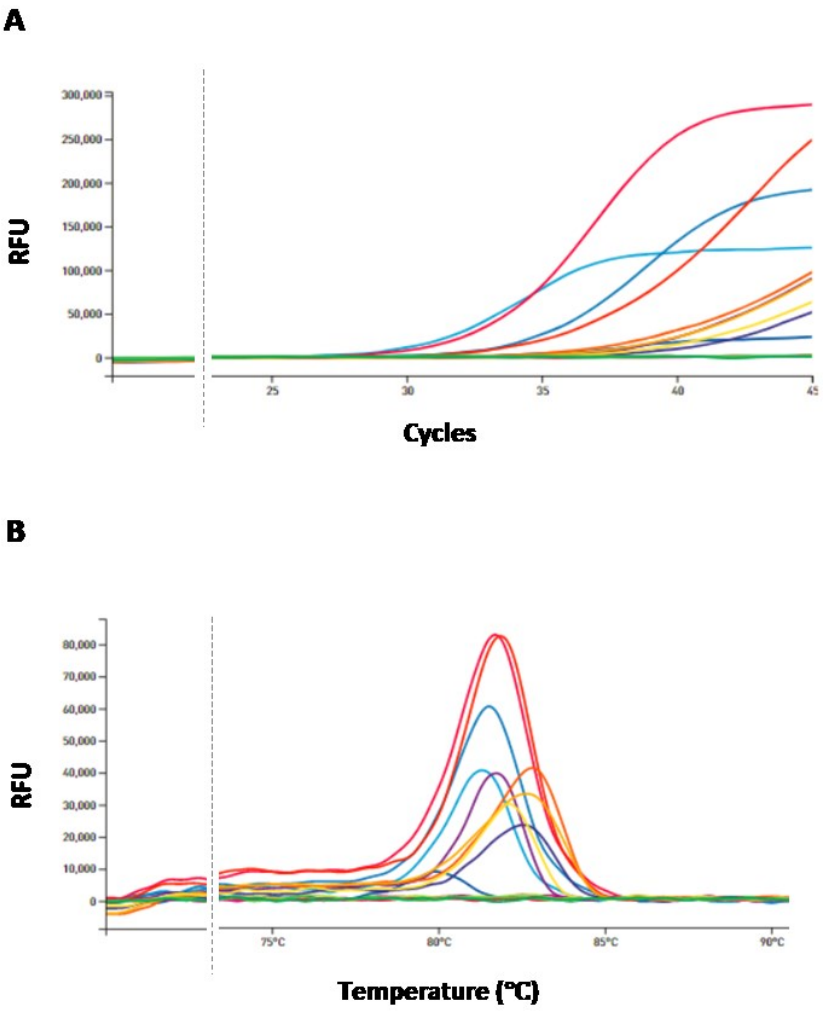


B



Sample	Well	C_T	T_M
NTC	A1	-	-
NTC	B1	-	-
10^7	A2	27.99	80.03
10^7	B2	28.36	79.49
10^6	A3	32.10	80.18
10^6	B3	31.97	79.71
10^5	A4	34.24	79.44
10^5	B4	34.48	79.42
10^4	A5	36.94	79.56
10^4	B5	35.28	80.02
10^3	A6	38.99	80.28
10^3	B6	37.42	79.34
10^2	A7	40.31	80.28
10^2	B7	38.84	80.06
10^1	A8	-	77.16
10^1	B8	-	89.84

Figure 5.8: Detection of *Plasmodium falciparum* in serially diluted blood by reverse-transcription real-time PCR in hydrogel on a CHAI Open-qPCR machine. (A) Amplification and **(B)** melting curves from 3D7 ring-stage *P. falciparum* parasites cultured in blood and tested by qPCR. Parasites were serially diluted in increments of ten-fold dilutions in negative blood. Blood samples were tested in duplicate.



Sample	Well	C_T	T_M
10^0	A1	30.69	81.21
10^0	B1	32.06	81.73
10^{-1}	A2	33.82	81.45
10^{-1}	B2	36.11	81.92
10^{-2}	A3	35.20	79.81
10^{-2}	B3	38.45	82.91
10^{-3}	A4	39.47	82.53
10^{-3}	B4	40.01	82.76
10^{-4}	A5	41.53	81.72
10^{-4}	B5	41.13	82.25
10^{-5}	A6	-	-
10^{-5}	B6	-	64.67
10^{-6}	A7	-	61.87
10^{-6}	B7	-	-
NTC	A8	-	-
NTC	B8	-	-

Figure 5.9: Detection of *Plasmodium vivax* in serially diluted blood by reverse-transcription real-time PCR in hydrogel on a CHAI Open-qPCR machine. (A) Amplification and **(B)** melting curves from a *P. vivax* patient sample. Samples were serially diluted in increments of ten-fold dilutions in negative blood. Blood samples were tested in duplicate.

Table 5.1. Direct comparison of direct-from-blood qPCR and direct-from-blood RT-qPCR for species identification using a serial dilution of *P. falciparum* 3D7 parasites.

Instrument	Dilution	qPCR mean C_T	RT-qPCR mean C_T	p-value[a]
CFX[b]	10^6 parasites/mL	27.82	25.69	0.05
	10^5 parasites/mL	30.14	29.49	0.3223
	10^4 parasites/mL	33.69	32.79	0.0973
	10^3 parasites/mL	35.48	35.9	0.2362
	10^2 parasites/mL	38.65[c]	38.95	0.5569
	10^1 parasites/mL	ND	40.72[c]	-
	10^0 parasites/mL	ND	ND	-
Open-qPCR[d]	10^6 parasites/mL	25.1	32.04	0.0001
	10^5 parasites/mL	29.45	34.36	0.0222
	10^4 parasites/mL	33.86	36.11	0.1136
	10^3 parasites/mL	36.33	38.21	0.1524
	10^2 parasites/mL	ND	39.58	-
	10^1 parasites/mL	ND	ND	-

[a] Calculated by unpaired Student's T test
[b] Samples were performed in triplicate
[c] Only two of three replicates detected
[d] Samples were performed in duplicate

5.2.2.2. Precision of direct-from-blood RT-qPCR on a portable PCR platform

We determined the %CV for serially diluted *P. falciparum* and *P. vivax* samples (tested in replicates) and performed a precision analysis on the basis of C_T values to determine the variation in the assays (Table 5.2). *P. falciparum* assay %CV ranged from 0.29 – 2.63, while *P. vivax* assay %CV ranged from 0.68 – 4.63.

Table 5.2: Variation of *P. falciparum* and *P. vivax* direct-from-blood RT-qPCR assays as performed on a CHAI open-qPCR.

Sample	Mean C_T	%CV[a]
Pf 10^6 p/mL	32.04	0.29
Pf 10^5 p/mL	34.36	0.49
Pf 10^4 p/mL	36.11	3.25
Pf 10^3 p/mL	38.21	2.91
Pf 10^2 p/mL	39.58	2.63
Pf 10^1 p/mL	ND	-
Pv Clinical sample 10^0	31.38	3.09
Pv Clinical sample 10^{-1}	34.97	4.63
Pv Clinical sample 10^{-2}	36.825	6.24
Pv Clinical sample 10^{-3}	39.74	0.96
Pv Clinical sample 10^{-4}	41.33	0.68
Pv Clinical sample 10^{-5}	ND	-

a %CV – coefficient of variation

5.2.3. Assessment of a panel of blind clinical samples by direct-from-blood RT-qPCR

To assess clinical sensitivity, we tested a panel of blood samples from non-pregnant patients from Colombia (Table 5.3). We then compared the results from the direct-from-blood RT-qPCR to results from the probe-based RT-qPCR method described in Chapter Three as the gold standard. In the *P. falciparum* assay, 29 (41%) of the 70 samples were positive by probe-based RT-qPCR. In the *P. vivax* assay, 37 (53%) of the 70 samples were positive by probe-based RT-qPCR. When analyzed directly from blood, only 13/29 *P. falciparum* infections and 27/37 *P. vivax* infections were detected. In addition, 11 false positives and 26 false negatives were observed in the direct-from-blood RT-qPCR method. Based on the probe-based RT-qPCR method as the gold standard, the diagnostic sensitivities and specificities were 44.83 % and 85.37% for the *P. falciparum* assay, and 72.97% and 84.85% for the *P. vivax* assay.

Table 5.3: Diagnostic sensitivity and specificity of direct-from-blood RT-qPCR compared to nucleic acid RT-qPCR performed on 70 clinical samples.

		Probe-based RT-qPCR[a]			
		P. falciparum		*P. vivax*	
		Positive	Negative	Positive	Negative
Blood RT-qPCR[b]	Positive	13	6	27	5
	Negative	16	35	10	28
		P.falciparum (%)	95% CI[c]	*P. vivax* (%)	95% CI[c]
Sensitivity		44.83	26.45-64.31	72.97	55.88-86.21
Specificity		85.37	70.83-94.43	84.85	68.10-94.89

[a] Probe-based RT-qPCR performed on purified total nucleic acid
[b] SYBR-based RT-qPCR performed directly from blood
[c] CI – confidence interval

Whereas table 5.3 describes the results as prevalence of *P. falciparum* and *P. vivax* infection for each sample, we also compared direct-from-blood RT-qPCR results for each individual patient in terms of diagnostic outcome (i.e. a positive result for just *P. falciparum* or just *P. vivax* in single sample would be a mono-infection, but a positive result for both *P. falciparum* and *P. vivax* in a single sample would be a mixed infection)to the probe-based results (Table 5.4). Of the 70 patient samples, 40/70 diagnostic outcomes (57.1%) were concordant. The breakdown of concordant samples by species is as follows: 7/17 (41.2 %) *P. falciparum* samples, 17/25 (68%) *P. vivax* samples, 4/12 (25%) mixed species, and 12/16 (75%) negative samples. We performed a direct-from-blood *Plasmodium* genus screen [143] on the discordant *P. falciparum*, *P. vivax*, and mixed species samples (blood samples that were incorrectly diagnosed by the direct-from-blood RT-qPCR method when compared to the probe-based RT-qPCR method) to test whether the disparity in sensitivity from direct-from-blood to purified nucleic acid was the reason for discordance. Of the 26 discordant samples (10 *P. falciparum*, eight *P. vivax*, and eight mixed species), only two samples (both *P. falciparum*) were detected by the genus-specific assay.

Table 5.4: Analysis of 70 clinical samples based on direct-from-blood RT-qPCR and nucleic acid RT-qPCR.

	RT-qPCR method		
	Direct-from-blood, n (%[a])	Probe-based, n (%[a])	Concordant results, n/N (%[a])[b]
P. falciparum	12 (17.1%)	17 (24.3%)	7/17 (41.2%)
P. vivax	25 (35.7%)	25 (35.7%)	17/25 (68%)
Mixed species[c]	7 (10%)	12 (17.1%)	4/12 (25%)
Negative	26 (37.1%)	16 (22.9%)	12/16 (75%)

[a] Percent positive in panel of samples
[b] n – number of positive samples by direct-from-blood RT-qPCR in agreement with probe-based RT-qPCR, N – number of positive samples positive by probe-based RT-qPCR
[c] Positive for both *P. falciparum* and *P. vivax*

5.3. Summary and brief discussion

In this chapter I describe the methodology to detect *P. falciparum* and *P. vivax* directly from blood using a DNA-based qPCR and RNA-based RT-qPCR platform. The Global Technical Strategy (GTS) for Malaria is a report released by the WHO to provide a technical framework and guide regional and country programs towards malaria control and elimination [163]. Some of the ambitious goals included in the GTS are the total reduction of malaria incidence by at least 90% and the elimination of malaria in at least 35 countries by 2030. For these goals to be achieved, highly sensitive and affordable diagnostics must be available for field surveillance and at point-of-care. The work from this chapter offers insight into the potential of a field-friendly molecular diagnostic that may be used for surveillance. Laboratory-grade real-time PCR machines can cost as much as $30,000 USD; in contrast, the portable real-time PCR instrument used in this chapter retails at under $4,000 USD per machine. Additional areas of cost-savings include that this methodology is designed for DNA amplification directly from blood, eliminating the need for sample processing or nucleic acid extraction, and that the use of a desiccated hydrogel eliminates the need for cold storage.

While cost-effectiveness is an important factor for implementing any diagnostic method for field surveillance, diagnostic sensitivity is another integral aspect that must be considered. In the hands of an expert microscopist, microscopy offers a limit of detection in the range of 5,000 p/mL to 50,000 p/mL, limiting its effectiveness for detection of low density parasitemias [51, 164]. We demonstrate that DNA-based amplification directly from blood has a sensitivity as low as 10^3 p/mL for *P. falciparum* and 10^3 copies/µL for *P. vivax* when performed in hydrogel on the portable PCR platform. While this is a dramatic improvement in sensitivity compared to microscopy, the LOD may still be insufficient for the detection of low-level submicroscopic infections. Based on the validation results

presented in chapter three, we demonstrated that a LOD of 10 p/mL for *P. falciparum* and 18 copies/µL were sufficient for detecting low-level SMIs in the Latin American region.

By optimizing the assay on an RNA-based platform to achieve a lower LOD, we showed that direct-from-blood RT-qPCR had a sensitivity of 10^2 p/mL for *P. falciparum* and a 4-log dilution of a patient sample for *P. vivax*, which is approximately the equivalent of 0.00001% parasitemia based on the original parasitemia of the patient sample. However, much work remains before this method may be viable in the field. This method requires cold storage for both reagents and preservation of sample RNA. In future work, we could adapt the desiccated hydrogel to the RNA-based platform. Additionally, I believe further optimization of this method is required to improve both reaction efficiency and sensitivity. In comparing the direct-from-blood RT-qPCR to the direct-from-blood qPCR performed on the portable PCR platform, we could detect a lower parasite dilution using the RNA-based method but the mean C_T values of the DNA-based method were generally lower. One potential explanation is the difference in reagents used between the two assays. The RT-qPCR assay uses a proprietary buffer already containing dNTPs and $MgCl_2$ that differs from the concentration used in the qPCR assay. Also, while multiple primers and a titration of primer concentrations were tested as part of the method validation, a titration of final blood concentration was not performed, which could reveal a more optimal final blood concentration than the 10% used in these assays.

When analyzing the results of the panel of clinical samples, I conclude that the direct-from-blood RT-qPCR was greatly outperformed by the probe-based RT-qPCR from purified nucleic acid. This is not a surprising result considering that the LOD of the probe-based assay is much lower than the direct-from-blood assay. Given that the majority of infections detected by the probe-based assay had a C_T value > 35, detection by the direct-from-blood RT-qPCR was limited by the sensitivity of the assay and the Poisson distribution of template in solution. In the context of surveillance or surveying in the

field, samples would assumingly be systematically screened first by a genus-specific assay before being species determined. Simulating these conditions, we tested the discordant samples with a genus-specific direct-from-blood RT-qPCR assay [143] and were only able to identify 2/26 (7.7%) positive for *Plasmodium* spp. These results suggest that while the direct-from-blood assays provide a superior sensitivity compared to microscopy, if these methods were implemented for surveillance, 24 infections (identified by probe-based RT-qPCR) would be missed at the species level, with 24 infections missed at the genus level.

In conclusion, the methodology described in this chapter supports the framework for the development of a direct-from-blood RT-qPCR method to detect *P. falciparum* and *P. vivax*. Two advantages of this assay include the increased sensitivity over current field diagnostics and the elimination of a nucleic acid extraction step. Additional optimization and the adaptation of the hydrogel to overcome the need for cold storage could allow for the validation of this method to be used in the field. As several countries and regions continue to approach elimination of malaria, sensitive molecular diagnostics, such as the ones described here, will be important for accurate screening of asymptomatic infections and defining the burden of submicroscopic malaria [148].

Chapter 6 – Discussion

6.1. Is there a need for molecular diagnostics to screen for MiP?

Pregnant women represent a particularly vulnerable population in the context of malaria. The work presented in this thesis has provided some insight into MiP in a Latin American context, specifically with regards to the impact of SMIs on delivery outcomes. Multiple studies conducted in Africa, an area of high *P. falciparum* transmission, have shown significant associations of submicroscopic MiP with negative delivery outcomes [111-116], including LBW, PTB, risk for placental malaria, and maternal anemia. Studies in Latin America, particularly in regions where *P. falciparum* and *P. vivax* co-exist, have shown conflicting results whether SMIs are detrimental to pregnancy outcomes [60, 95, 118, 137, 154]. Through collaborative research, I have shown that submicroscopic MiP in a Colombian context is generally not associated with adverse delivery outcomes. An exception to this is the significant association of mixed *P. falciparum* and *P. vivax* SMIs and gestational age at delivery. This finding reveals a novel association of *P. falciparum* and *P. vivax* mixed SMIs and adverse birth outcomes that is particularly relevant to regions outside of Africa. This observation is in agreement with findings from other non-pregnant populations in which mixed *P. falciparum* and *P. vivax* infections can have adverse consequences such as severe malaria [157] and anemia [156].

6.1.1. Clinical perspective of MiP molecular screening

The results from our study demonstrated a significant association with mixed SMIs of *P. falciparum* and *P. vivax* and PTB at delivery. From a clinical perspective, does this adverse association warrant the costly implementation of molecular diagnostics for screening MiP in Colombia? Only nine pregnant women in the cohort were diagnosed with a mixed SMI during their follow up, representing 20% (9/45) of the infected population, and only 5% (9/180) of the total cohort. Additionally, according

to the WHO Global Survey on Maternal and Perinatal Health [165], patent malaria infection in pregnancy is not considered a risk factor for PTB in Latin America; this report, compacted with the small numbers of mixed SMIs in our study, make it difficult to justify the financial costs of performing molecular screening at antenatal visits. While the study was adequately powered to test the primary objective of whether SMIs are significantly associated with birth weight at delivery, the numbers were inherently small due to loss of follow-up. The sample size becomes even smaller when evaluating only mixed infections from this cohort. Further work must be done to investigate whether the impact of mixed SMIs and PTB can be replicated in this population, potentially through a case-controlled study of mixed infections in this population.

6.1.2. Elimination perspective of MiP molecular screening

Colombia is currently not in the pre-elimination or elimination phases of malaria control and remains endemic for both *P. falciparum* and *P. vivax* malaria [26, 121]. For Colombia to move into pre-elimination phase, there are several key challenges that must be addressed. The pregnant population represent an important demographic for targeting asymptomatic and submicroscopic malaria that is likely key to reducing transmission [166]. IPTp (most often antenatal treatment with SP) is often reserved for areas of high transmission and is not currently implemented by the government of Colombia. As such, control efforts for MiP are focussed on antenatal care and accurate diagnostic screening. For control efforts without the use of IPTp, two important factors must be considered: access to antenatal care and the sensitivity of the malaria diagnostic [119]. Antenatal care coverage in Colombia is high, with more than 88% of women attending at least four antenatal care visits [135]. Microscopy currently remains the gold standard for diagnosis of malaria in Colombia. However, previous studies [60, 129, 130] and my work have shown that diagnosis by microscopy alone grossly underestimates the prevalence of malaria. In the context of elimination, missing SMIs as a result of not

using a molecular diagnostic screening approach would be detrimental to control and elimination efforts since these infections are known to contribute to local transmission [167]. A study by Okell *et al.* demonstrated that while SMIs diagnosed by qPCR have a 4- to 16-fold reduction in infectivity compared to microscopy positive infections, they nevertheless can contribute as much as 50% of mosquito infections in very low-transmission areas [33]. Further, studies conducted in Kenya and Burkina Faso demonstrated that gametocyte densities as low as one gametocyte/µL, well below the LOD of microscopy, were capable of infecting *Anopheles* mosquitoes [168]. The average volume of blood consumed by *Anopheles* mosquitoes during a blood meal ranges from one to five µL [169], suggesting that despite the low volume of blood consumed by the mosquito, transmission of gametocytes at submicroscopic levels can continue to persist. By this rationale, sensitive molecular diagnostics must absolutely be used for the screening of SMIs in pregnancy.

6.1.3. Application of molecular diagnostics in the field

An overarching theme in my validation work has been: in the context of a field setting, how sensitive do we need these tests to be and at what cost? From a clinical perspective (section 6.1.1), the implementation of molecular diagnostics in the field may be unnecessary. However, from an elimination perspective (section 6.1.2), how can we evaluate the appropriateness of a molecular diagnostic to be used in the field? Two factors that we must consider are the sensitivity of the method and cost-effectiveness.

Microscopy and RDTs are two diagnostic methods that are currently employed for use in the field. The LODs of these two methods range from $5x10^3$ to $1x10^5$ p/mL [52, 53, 57, 170]. The method I validated in chapter three, while highly sensitive (LOD: 10 p/mL for *P. falciparum* and 18 copies/µL for *P. vivax*), is limited for field use by the need for expensive equipment and cold chain for TNA

extraction and sample processing prior to RT-qPCR. In chapter five, I presented an RNA-based diagnostic method that may be performed directly from blood (LOD: 10^2 p/mL for *P. falciparum* and 0.00001% parasitemia for *P. vivax*), eliminating the need for sample processing. However, cold storage is still required for reagents and preservation of RNA integrity in blood samples. Further work into the optimization of these assays to be performed using desiccated hydrogels could provide an ideal molecular diagnostic method for field screening. Currently the hydrogel has been validated for DNA-based molecular diagnosis and has a LOD of 10^3 p/mL for *P. falciparum* and 10^3 copies/µL for *P. vivax*, when performed on a portable PCR platform. However, results from the panel validation in chapter three suggest that these LODs may not be sensitive enough to detect SMIs at the species level in this region.

Cost of the diagnostic test is an important consideration in field settings, which are often resource limited. The average cost for microscopy and RDTs are $1.53USD unit cost/test and $1.29USD unit cost/test, respectively [52, 53, 57, 170]. RT-qPCR from purified nucleic acid would not be feasible for use in the field due to the requirement of expensive equipment and the exorbitant costs of sample preparation. Direct-from-blood RT-qPCR (approximately $4USD cost/test) represents another alternative diagnostic for surveillance in the field with improved sensitivity, but is limited by the requirement of cold chain for reagents. The use of desiccated hydrogel technology directly from blood samples would eliminate the need for cold storage and nucleic acid extraction. Aquila diagnostics estimated the cost of the hydrogel test per sample to be approximate $1USD [142] when it reaches mass production. While this approximation of cost may change, the streamlining of sample preparation and performance on a portable PCR machine (retailing at under $4000 per unit) makes this option very cost-effective. As molecular diagnostics are implemented more broadly for malaria surveillance, and particularly within the context of elimination [148], the increased sensitivity gained

by adapting this method for RNA detection could be important for epidemiological surveys and defining the submicroscopic malaria burden at the species level.

6.2. Outstanding questions and knowledge gaps in MiP

The work presented in my research represents the first prospective longitudinal evaluation of MiP in Latin America to determine the impact of SMIs on delivery outcomes and characterize host anti-VAR2CSA antibody responses to SMIs. The results from this study have shed some novel insight into our understanding of MiP, while raising some questions about our current knowledge gap.

6.2.1. Alternative risks and adverse outcomes

It has been well described that the timing of malaria infection during pregnancy can affect the clinical outcomes at delivery [80, 171]. In our study, most pregnant women were recruited during their second or third trimesters resulting in a small sample size for analysis. This begs the question whether we would observe any differences comparing the impact of SMIs early in pregnancy to SMIs late in pregnancy. However because few adverse outcomes were observed in our population, it is unlikely the timing of SMI would be a significant factor. Studies in Africa and Asia have demonstrated that malaria infections that occur in the first trimester are significantly associated with LBW, maternal anemia, fetal growth restriction, and miscarriage [171-174]. In other studies from Benin, Brazil, the Democratic Republic of Congo, Malawi and Thailand, malaria infections in both early and late pregnancy were associated with LBW and fetal growth restriction [173, 175-179]. Further, fetal growth restriction can occur either immediately or with a delay after malaria infection [175, 179, 180], highlighting the importance of antenatal screening and compliance of follow-up throughout the course of pregnancy.

In our follow-up, 13 women were excluded from analyses due to abortion or stillbirth; samples were not collected at delivery from these women. The association of *P. falciparum* and *P. vivax* infections in pregnancy and stillbirth has been described extensively [181]. An outstanding question from this study would be whether or not these miscarriages or stillbirths were due to SMIs. Another knowledge gap is our understanding of relapse *P. vivax* infections in the context of MiP in Colombia. A limitation of our study, due to the low levels of parasite infection, was the inability to discriminate between relapse, chronic or new *P. vivax* infections. One possibility of overcoming this limitation is the use of a whole genome capture (WGC) method [182]. The use of WGC and RNA baits has demonstrated that parasite DNA content can be enriched from 1% total DNA content to up to 20-40% DNA content [182]. This could allow for sufficient DNA levels for sequencing and single nucleotide variance analysis to differentiate between new infections, chronic infections, and relapse infections.

Inflammation in the placenta has been significantly associated with maternal anemia and fetal growth restriction in Africa [79], and with LBW in Papua New Guinea [103]. A previous study in Colombia identified that SMIs were significantly associated with placental damage but not associated with adverse outcomes at delivery [60]. A more recent study investigating the impact of *P. falciparum* and *P. vivax* maternal, placental, and congenital malaria, also confirmed no association between SMIs and adverse outcomes at delivery [183]. While no active placental infections were detected in our study by histopathology, few women were diagnosed with a past placental infection (presence of hemozoin by histopathology); however, no significant association was observed between delivery outcomes and placental histopathology. Elucidating the outcomes of placental inflammation in different malaria-endemic regions warrants further investigation.

6.2.2. MiP immunity

One outstanding question is how VAR2CSA-specific antibodies are acquired in primigravid women. My data are consistent with a previous study in this region suggesting that these antibodies may be acquired outside of pregnancy [109]. One hypothesis is that the antibodies arose against an antigen from *P. vivax* and cross-reacted with VAR2CSA. This is supported by the findings that pregnant women, men and children who were infected with only *P. vivax* had antibodies that cross-reacted with the DBL5ε, DBL3X and ID1-ID2 domains of VAR2CSA with similar frequencies [109]. In another study from our lab conducted in populations from Brazil and Colombia, we showed that non-pregnant individuals exposed only to *P. vivax* had antibodies that recognized VAR2CSA (data unpublished). Specifically, exposure to the *P. vivax* protein PvDBP induced antibodies that functionally recognize the DBL domains of VAR2CSA and inhibited the binding of parasites to CSA *in vitro*. It is possible that the functional antibodies observed in the primigravid women from the pregnant Colombian cohort originate from exposure to the PvDBP protein.

While both primigravid and multigravid women possessed antibodies capable of functionally blocking iRBCs to CSA *in vitro*, it was also shown that multigravid women possessed a higher degree of functional inhibition, a result that was also observed in a previous study in Benin [106]. This could be explained due to affinity maturation of antibodies, which develop greater avidity with increased exposures to VAR2CSA in pregnancy. This would not be a surprising result, as high avidity antibodies resulting from affinity maturation are often correlated with strong activity against other pathogens [184, 185]. In a study conducted by Tuikue-Ndam *et al.* [106], VAR2CSA-specific antibodies had higher functional inhibition at delivery compared to enrolment, which could also be a result of antibody maturation. It had been demonstrated that pregnant women are more often infected with placental-type parasites later in pregnancy and that exposures increase with gestational age [101]. From this, the authors rationalize that this could explain the increase in functional inhibition at delivery compared to the functional inhibition at inclusion [106]. It would be of interest to test if these results could be

replicated in our Colombian cohort of pregnant women, which would give novel insight into the function of VAR2CSA in a Latin American setting.

A thorough understanding on the role of VAR2CSA-specific antibodies and protection remains unclear and is an area of importance that requires further investigation. In terms of the association between the functional inhibition of iRBCs to CSA and their protective role in delivery outcomes, studies in Africa have shown conflicting results; in Benin, functional inhibition was associated with LBW, but not PTB [106]; in Malawi, functional antibodies were not associated with birth weight [186]; whereas in Kenya, functional antibodies were both positively associated with birth weight and gestational age [187]. The results from my work demonstrated that the functional inhibition of iRBCs to CSA was significantly associated with protection from maternal anemia. This supports the finding that IgG antibodies against MiP-specific variant surface antigens (VSA) were significantly associated with maternal Hb levels [188]. However, my data contradict results shown in studies from Malawi, Benin, and Kenya, where the adhesion blocking activity of antibodies were not significantly associated with anemia or changes in Hb level [106, 186, 187]. One consideration that should be taken into account is that the malaria infections identified in my study were submicroscopic, whereas many of the comparative studies in Africa were from women diagnosed with placental malaria or positive by microscopy. Exploration of differences in VAR2CSA-specific responses in different areas of high and low submicroscopic endemicity, and their impact on outcomes at delivery, would provide more insight into the role of functional inhibition in protection from MiP.

Functional inhibition of iRBCs binding to CSA is just one possible mechanism of immune protection of VAR2CSA antibodies. A study in Malawi demonstrated that opsonisation of iRBCs for clearance by phagocytic cells are also associated with protection from maternal anemia [108]. This was further supported by another study investigating severe malaria in Sudan, which found that maternal

Hb levels were significantly associated with the opsonizing activity of VSA-specific antibodies [189]. One area of future study would be to perform phagocytosis assays to determine whether sera from the Colombian cohort have opsonising activity and are also associated with protection by this mechanism.

6.3. Concluding remarks

My initial work aided in the validation of a highly sensitive RT-qPCR method to be used for screening submicroscopic *P. falciparum* and *P. vivax* infections. This method became the standard diagnostic screen that was used to describe the prevalence and assess the impact of SMIs in pregnancy in our longitudinal study of pregnant women from Colombia. Submicroscopic MiP has been described extensively in studies based in Africa [111, 113-116] and in cross-sectional studies in Colombia [60, 95, 130]. My research describes results from the first longitudinal evaluation of submicroscopic MiP in Latin America, investigating the clinical impacts on delivery outcomes, as well as characterizing the role of antibodies against the *Pf*EMP1 protein VAR2CSA. Additional work I completed for the validation of a direct-from-blood RT-qPCR method for the detection of SMIs at the species level will lay the ground work for further development into a diagnostic method that may be used for diagnosis at POC or epidemiological surveying in a field setting.

In conclusion, my research has enhanced our understanding of the clinical impact of SMIs in pregnancy in Latin America. In addition to using molecular diagnostics to define the prevalence of SMIs in pregnancy, I also demonstrated novel associations between mixed *P. falciparum* and *P. vivax* infections with both gestational age at delivery and risk for PTB. My finding that VAR2CSA-specific antibodies that can inhibit the binding of iRBCs to CSA are associated with protection from maternal anemia supports the view of VAR2CSA being a major target for protective immunity and a vaccine candidate for MiP. My work on defining the impact of SMIs on delivery outcomes and the protective

role of anti-VAR2CSA antibodies will contribute to a more comprehensive understanding of MiP biology.

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