Reducing the burden of malaria and curable sexually transmitted and reproductive tract infections during pregnancy in an area of high co-infection

MIKE CHAPONDA

Thesis submitted in accordance with the requirements for the degree of Doctor of Philosophy

University of London

22nd JANUARY 2025

Disease Control Department
Faculty of Infectious and Tropical Diseases
LONDON SCHOOL OF HYGIENE AND TROPICAL MEDICINE

Funded by the Commonwealth Scholarship Commission

Declaration by Candidate

I Mike Chaponda confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed: ---------- Date: ----------

Full Name: Mike Chaponda

Table of Contents

List of Tables

List of Figures

Scope of work conducted by the PhD candidate

1. Contributed to the development of the study protocol.
2. Developed some of the standard operating procedures and study forms.
3. Contributed to the translation and back translation of the information sheet and consent forms.
4. Applied for and obtained ethical clearance from the Tropical Diseases Research Centre Ethics Review Committee, The Zambia Medicines Regulatory Authority, and the National Health Research Authority
5. Obtained permission to conduct the study from the provincial Office of the Ministry of Health and the district Health office administrators in Zambia
6. Conducted pre-study meetings with the Community Advisory Board (CAB)
7. Recruited study team members.
8. Trained team members.
9. Managed the day to day running of the project entitled "Effects of metronidazole plus intermittent preventive treatment of malaria in pregnancy on birth outcomes: a randomized controlled trial in Zambia" (Clinical trials.gov NCT04189744). My role as the principal investigator for the trial was largely to oversee the conduct of the trial in general. Specifically, I was responsible for ensuring that the trial met all ethical and regulatory standards and was conducted according to GCP-ICH standards. I was also responsible for communication with local ethics committees and with the Zambia Medicines Regulatory authority (ZAMRA) including processing the application for the material transfer agreement at the National Health Research Authority (NHRA). At the height of the covid-19 pandemic in 2021, the NHRA gave guidance to halt all research activities as part of the measures to combat the pandemic. After three weeks, I entered discussions with the NHRA to give a special dispensation to allow the ASPIRE trial to continue recruiting and following up participants. The NHRA gave permission to with stringent conditions to observe infection control measures to prevent the transmission of covid-19. I provided leadership to ensure this was achieved.
10. I was also responsible for ensuring that the study protocol was adhered to including consenting of the study participants, recruitment according to the eligibility criteria, and follow-up of the study participants. I was also responsible for participant's safety

and reporting of serious adverse events to the sponsor, ethics committee and ZAMRA. In addition, I was responsible for ensuring that biological samples were collected at the correct visits, processed, and transported from the study sited to the laboratory in a timely manner. I provided supervision to the study team to ensure that all processes of sample collection and processing were according to the set standard.

11. Coordinated study team members.
12. Supervised the screening of eligibility of participants, obtaining written consent and administration of study drugs while coordinating the rest of the activities during the enrolment and sample collection process.
13. I personally conducted 305 (5%) DNA extractions from dried blood samples or diagnosis of malaria and for the three sexually transmitted infections.
14. I personally conducted 540 (10%) of molecular diagnoses for malaria, Chlamydia, gonorrhoea and trichomoniasis infection by standard polymerase chain reaction.
15. Stained and read over 5% of slides for diagnosis of bacterial vaginosis.
16. Coordinated data entry, regularly entered data, and conducted data entry checks.
17. Conducted data cleaning.
18. Contributed to the developed of the analysis plan.
19. Processed and analysed data.
20. Wrote the thesis.

Activities coordinated by the candidate but done by other study staff

1. Preparation of blood slides for diagnosis of malaria and reading of slides
2. The genotyping work conducted in Copenhagen was analysed by the team at the University of Copenhagen
3. Processing of placental tissue and slide preparation
4. Reading of slides for diagnosis of placental malaria
5. Rapid plasma reagin (RPR) and *Treponema pallidum* haemagglutination (TPHA) assay testing.
6. Gestational age measurement by ultra-sound

Abstract

Background

Malaria in pregnancy causes deleterious effects on the mother and has devastating consequences such as low birthweight (LBW), small-for-gestational age (SGA), or preterm birth. In addition, sexually transmitted and reproductive tract infections (STIs/RTIs) are also associated with adverse birth outcomes similar to malaria. A recent observational cohort study of pregnant women conducted in Nchelenge, Zambia, reported that 37.0% (345/941) of pregnant women were co-infected with malaria and at least one curable STI/RTI, 25.4% (237/941) had a curable STI/RTI alone, and 19.5% (182/941) had malaria alone. Of five common curable STIs/RTIs, *Trichomonas vaginalis* (TV) and bacterial vaginosis (BV) were most often implicated in co-infection with malaria. This established the biological basis for combination therapy among pregnant women aimed at reducing the dual burden of malaria and STIs/RTIs with a particular focus on TV and BV.

The World Health Organization (WHO) recommends intermittent preventive treatment in pregnancy with sulfadoxine-pyrimethamine (IPT-SP) for asymptomatic women, but high-level parasite resistance to SP threatens its efficacy. Thus, it is applicable] to replace SP with a combination treatment that contains a highly efficacious antimalarial compound alongside an efficacious anti-STI/RTI therapy to produce better birth outcomes. Dihydroartemisinin-piperaquine (DP), has the potential to replace SP for IPTp. The WHO recommends metronidazole (MTZ) for the treatment of TV and BV in pregnancy which can be provided as a single observed treatment alongside IPTp with SP or DP. The objectives of my thesis were:

1. to determine the protective efficacy of IPTp with SP plus MTZ, or DP plus MTZ, was superior to SP alone in protecting against adverse pregnancy outcomes among pregnant women in Nchelenge district.

2. to determine the day-28 treatment efficacy against *Plasmodium falciparum* infection among pregnant women in who received either SP alone, SP plus MTZ, or DP plus MTZ

3. to determine the prevalence of molecular markers of resistance to sulphadoxine-pyremethmine in *P. falciparum* samples found among pregnant women in Nchelenge district who received either SP alone, SP plus MTZ, or DP plus MTZ.

4. to compare the prevalence of STIs/RTIs at first and second antenatal care visits among pregnant women in Nchelenge district who received either SP alone, SP plus MTZ, or DP plus MTZ.

Methods

The data for this thesis were part of the individually randomized, 3-arm, partially-placebo controlled superiority trial comparing the efficacy, safety, and tolerance of IPTp-SP versus IPTp-SP plus MTZ, or IPTp-DP plus MTZ to reduce adverse birth outcomes attributable to malaria and curable STIs/RTIs in the Nchelenge District of Zambia. Eligible and consenting HIV-uninfected pregnant women (N=5,436) with gestational age between 16 and 28 weeks measured by ultrasound were enrolled consecutively and allocated to treatment groups in a 1:1:1 ratio during antenatal booking. Women received IPTp based on the arm to which they were allocated at each antenatal care visit from enrolment until delivery at monthly intervals. Biological samples for diagnosis of malaria and STIs/RTIs were collected for retrospective analyses. The primary endpoint was based on a composite measure of adverse pregnancy outcome: spontaneous abortion, stillbirth, preterm birth, low birthweight, and neonatal death. In addition, a sub-group of samples that were PCR-positive for *P. falciparum malaria* at enrolment were analysed to measure the frequency of point mutations on the *dhps* and *dhfr* genes for resistance for SP.

Results

Protective efficacy of IPTp

In the SP group, 18.8% (287/1,524) of women had an adverse pregnancy outcome compared to 19.4% (292/1,501) in the SP-MTZ group [RR 1.04, 95% CI 0.90 to 1.20], p=0.62) and 18.6% (280/1,504) in the DP-MTZ group [RR 0.99 95% CI 0.85 to 1.14, p=0.76]. The risks of individual adverse pregnancy outcomes of spontaneous abortion, stillbirth, preterm birth, low birthweight, and neonatal death did not differ between the treatment groups.

Day-28 SP efficacy against Plasmodium falciparum infection

Plasmodium falciparum infection prevalence at visit 1 (day 0) and visit 2 (day 28) by treatment group were as follows: SP 45.7% (844/1,847) and 20.5% (336/1,640), p=<0.001; SP+MTZ 46.4% (840/1,810) and 19.1% (311/1,628) at visit 2, p=<0.001; DP+MTZ 47.1% (850/1,806) and 5.3% (87/1,642) at visit 2, p=<0.001.

Genotypes of *P. falciparum*

The genotypes present in *P. falciparum* samples were analysed among two separate sub-groups of women who were parasite-positive at the enrolment visit. In a sub-group of 200 women who had malaria parasitaemia at enrolment, the prevalence of *dhps* 540 mutations and *dhfr*581 mutation was 66.8% (133/199, 95% CI: 59.8, 73.3) and 5.0% (10/199, 95% CI: 2.4, 9.0), respectively. The second set of samples consisted of 176 *falciparum* PCR-positive samples collected that were analysed at the University of Copenhagen. The *Pfdhfr* SNPs in codons 51,59, 108 and 164 were observed at 84.09% and 0% respectively. In the *Pfdhps* gene, codons 431, 436, 437, 540, 581 and 613 were analyzed. While the rest neither showed any mutations nor had any insignificant proportions. Codons 540 and 581 showed much higher prevalence point mutations at 74.24% and 9.45% respectively. Ultimately, the study showed quintuple and sextuple mutations at 66% and 11% respectively.

Prevalence of curable STIs/RTIs at first and second antenatal care visits

The prevalence of STIs/RTIs between visit 1 (day 0) and visit 2 (day 28) was significantly lower across treatment groups except for *Chlamydia trachomatis* in the DP-MTZ arm. For TV visit 1 versus visit 2 the prevalence was as follows: SP group 13.7% (248/1,807) versus 10.5% (100/955, p=0.014); SP-MTZ group 13.7% (248/1,810) versus 7.6% (72/945, p=<0.001); DP+MTZ 14.3 (258/1,806) versus 8.4% (80/957, p=<0.001). For BV visit 1 versus visit 2 was as follows: SP arm 35.5% (644/1,807) versus 29.7% (488/1,643, p=<0.001); SP-MTZ arm 34.6% (626/1,810) versus 23.1% (377/1,632, p=<0.001); DP-MTZ arm 33.4% (606/1,806) versus 19.2% (315/1,640, p=<0.001).

For *Neisseria gonorrhoeae*, visit 1 versus visit 2 prevalence was as follow: SP group 13.1% (237/1,807) versus 6.8% (65/955, p=<0.001); SP-MTZ group 12.7% (229/1,807) versus 7.4% (70/945, p=<0.001); DP-MTZ group 11.7% (211/1,806) versus 6.8% (65/957, p=<0.001). Chlamydia infection was similar in the DP-MTZ arm between visit 1 and visit 2, 5.7%

(103/1,806) versus 4.4% (42/957, p=0.14), respectively. However, in the SP group prevalence was 7.1% (129/1,807) versus 2.1% (20/955, p=<0.001), and the SP-MTZ group 6.0% (109/1,080) versus 3.0% (28/945, p= <0.001) respectively.

Conclusions

Despite the parasite resistance to SP, current WHO guidelines do not recommend withdrawing IPTp-SP even when there is a high prevalence of mutations associated with SP resistance (In 2013, the WHO recommended that countries consider discontinuing IPTp-SP once the population prevalence of the *pfdhps* 540E mutation is 95% and the prevalence of the *pfdhps* 581G mutation is 10%) and despite DP being better at reducing maternal malaria, SP was not inferior in protecting against adverse pregnancy outcomes. SP significantly reduced the day 28 population prevalence of STIs/RTIs suggesting specific non-malarial pathways to reducing adverse pregnancy outcomes. Alternative regimens to address the dual burden of malaria and STIs/RTIs are still needed in this setting.

Abbreviations

ABO	Adverse birth outcomes
95% CI	95 percent confidence interval
ACT	Artemisinin-based combination therapy
AE	Adverse event
AL	Artemether-lumefantrine
ANC	Antenatal care
AZ	Azithromycin
BV	Bacterial vaginosis
CRF	Case report form
CT	*Chlamydia trachomatis*
DBS	Dried blood spot (on filter paper)
DHFR	Dihydrofolate Reductase
DHPS	Dihydropteroate Synthase
DMEC	Data monitoring and ethics committee
DP	Dihydroartemisinin-piperaquine
DMEC	Data safety and monitoring board
ERG	Evidence review group from WHO
Hb	Haemoglobin
HIV	Human immunodeficiency virus
IPTp	Intermittent preventive treatment in pregnancy
IQR	Interquartile range
IRR	Incidence rate ratio
IRS	Indoor residual spray
ITN	Insecticide treated bed net
ITT	Intention to treat
LBW	Low birthweight
LSHTM	London School of Hygiene and Tropical Medicine
MoH	Ministry of Health
MTZ	Metronidazole
NAAT	Nucleic Acid Amplification Test

NG	*Neisseria gonorrhoeae*
PCR	Polymerase chain reaction
PCR -RFLP	Polymerase chain reaction restriction fragment length
PTB	Preterm birth
RCT	Randomized controlled trial
RDT	Rapid diagnostic test
RR	Risk ratio
RTI	Reproductive tract infection
SAE	Serious adverse event
SGA	Small-for-gestational age
SOP	Standard operating procedure
SP	Sulfadoxine-pyrimethamine
STI	Sexually transmitted infection
STIs/RTIs	Sexually transmitted and reproductive tract infections
TDRC	Tropical Disease Research Centre
TV	*Trichomonas vaginalis*

Acknowledgement

I would like to thank my supervisor Professor Matthew Chico for the advice whenever I needed direction throughout the programme. I also wish to thank most sincerely Professor Daniel Chandramohan who was the co-supervisor for helping me navigate difficult situations during my training. I wish to also express my gratitude to Jane Bruce for guiding me during data analysis. Many thanks to the Commonwealth Scholarship Commission in the UK for the scholarship to study at LSHTM. I also wish to thank the Tropical Diseases Research Centre for the support provided to me to enable me take up full time studies and for the institutional labs used for analysing the samples.

I am grateful to my colleagues at the Tropical Diseases Research Centre (TDRC) in Ndola for helping me in a big way in conducting the trial from which I derived the analysis for my PhD thesis. I also want to thank the scientific and technical staff in the TDRC labs for helping me with analysing a huge number of samples from the trial. I also thank the ASPIRE project team both at TDRC and in Nchelenge for their tireless work during the trial. All the staff at Kabuta, Kafutuma, Kashikishi and Nchelenge rural health Centres and at St Pauls' hospital. I also wish to thank Dr Sergio Muwowo of the Pathology and Microbiology Department at the University Teaching Hospital for reading the histopathology slides of the placental tissue. To my wife, Abigail, I would like to say thank you for supporting me in everything I do and for being the shock absorber in my rollercoaster life in the pursuit of academic excellence. Thank you for taking care of the family during my time away from home and for being a pillar of strength.

Summary of content

The thesis attempts to provide knowledge on whether intermittent preventive treatment with SP, IPTp plus MTZ, or DP plus MTZ were superior to SP alone in protecting against adverse pregnancy outcomes among pregnant women. In addition, the thesis fills the knowledge gap concerning the day-28 treatment efficacy against *Plasmodium falciparum* infection among pregnant women who receive either SP alone, SP plus MTZ, or DP plus MTZ. This is also closely related to the prevalence of 540 and 581 biomarkers of resistance in *P. falciparum* found among pregnant women in Nchelenge district who receive either SP alone, SP plus MTZ, or DP plus MTZ. Furthermore, the thesis provides new knowledge on the day 28 treatment efficacy against *T. vaginalis* and bacterial vaginosis among pregnant women who receive either SP alone, SP plus MTZ, or DP plus MTZ are compared.

The thesis is organised as follows:

- Chapter 1 gives the background to the study by highlighting the burden of malaria, curable STIs/RTIs in pregnant women and their effect on birth outcome and the antenatal care package for their management. The background also highlights the gap in knowledge on malaria and curable STI/RTI coinfection and points out the weaknesses of the intermittent preventive treatment in pregnancy with SP (IPTp-SP) strategy for the alleviation of the consequences of malaria infection in pregnancy in the face of emerging parasite resistance to SP. The background also points out the inaccuracy of the syndromic approach for the management of STIs/RTIs. The chapter also gives the rationale for the study and the objectives.

- Chapter 2 describes the methods employed including the study design, a description of the study area, identification and recruitment of participant, and tools used in the collection of data and samples, detection of infections, data processing and analyses.

- Chapter 3 presents the results of the protective efficacy of IPTp-SP compared to IPTp-SP with MTZ or IPTp-DP with MTZ. The occurrence of adverse pregnancy outcomes in each arm is described here. Thereafter, the individual occurrence of adverse pregnancy outcomes in the SP arm were compared with adverse pregnancy outcomes

in the SP plus MTZ arm and the DP plus MTZ arm. The chapter also describes the trial profile, the baseline characteristics, and the participant distribution and maternal weight distribution in the three study arms.

- Chapter 4 describes the factors associated with the efficacy against malaria-related outcomes of IPTp-SP compared with IPTp-SP plus MTZ or IPTp-DP plus MTZ using the WHO protocol. Outcomes related to malaria infection during pregnancy and at delivery are also described in this chapter.

- Chapter 5 describes results on the estimates of dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) mutations associated with *P. falciparum* resistance to SP and gives estimates of the *in vivo* efficacy and the therapeutic and prophylactic failure of IPTp-SP. The idea behind this chapter is to give details of interest that is give an estimation of the level of SP resistance and the efficacy of SP in the study population.

- Chapter 6 presents the results of the comparison of the prevalence of STIs/RTIs at first and second antenatal care visits among women who received SP alone, SP plus MTZ, or DP plus MTZ.

- Chapter 7 discusses all the results and gives the limitations of the study, conclusions, and policy recommendations.

- Chapter 8 is the list of references.

- Chapter 9 contains the appendices.

1 Introduction

1.1 Background

Pregnancy causes a transient depression of cell-mediated immunity which increases the likelihood of foetal retention and development, but this also interferes with maternal responses to various infectious diseases (1, 2). Infections in the placental tissue are associated with histopathological features which may result in the inflammation of the chorionic villi associated with lower birth weight (3). Erythrocytes infected by *Plasmodium falciparum* manifest the surface antigen, VAR2CSA, which is responsible for mediating sequestration of parasitized red blood cells in the placenta. There are then host responses that lead to increased inflammation in the placenta, abnormal placental development, and impairment of placental functions, affecting foetal development (4). When the innate immune system is regulated so as not reject the foetus, the adaptive immune response toward foetal antigens is reduced. There is an increase in the numbers of monocytes and granulocytes that are produced due to activation of the immune system in the maternal systemic circulation, comparable to that observed in women with sepsis (5). In addition, components of the humoral immune system are activated (6).

The disease burden among pregnant women in low- and middle-income countries is high, particularly where malaria is endemic. In sub-Saharan Africa, *Plasmodium falciparum*, is the most common of malaria species (7). *Plasmodium falciparum* sequesters in the placenta in the maternal blood which prevents the immune system from clearing it (8, 9). Red blood cells which are infected by *P. falciparum* sequester in vascular spaces in the placenta mediated by adhesion to chondroitin sulphate A (10), leading to placental inflammation (11, 12) and complications associated with malaria in pregnancy (13).

Curable sexually transmitted and reproductive tract infections (STIs/RTIs), particularly bacterial vaginosis (BV) and *Trichomonas vaginalis* in pregnant women, are also important causes of adverse pregnancy outcomes (14). Traditionally, BV is defined as a dysbiotic disruption of the normal balance of the vaginal microbiota (15). The polymicrobial dominated by Gardnerella spp. biofilm reduces the viscosity of the cervicovaginal discharge leading to changing of the epithelial homeostasis and impairing the mucosal barrier and promoting coinfections and ascending infections in the upper genital tract. This increases the risk of

infections during pregnancy including risks for endometritis, salpingitis, and neonatal complications (16). The polymicrobial biofilm provide for a complex interaction between co-aggregation and metabolic cooperation which happens between the species in BV. This leads to the resistance of BV to host immune responses and antibiotic resistance. Pathogens of sexually transmitted infections also benefit from ecological interactions with the BV biofilm (17). Women with BV are twice as likely to acquire *C. trachomatis, M. genitalium, N. gonorrhoeae, T. vaginalis,* HIV, and HPV, compared to women without BV when exposed to these STIs (18-20).

Trichomonas vaginalis has been recognized as an STI since the 1800s (21). However, the factors that influence its virulence including the interaction between the host and the parasite are not fully understood (22). There are two important virulence factors that are known in the function of TV (23). Cytoadherence to epithelial cells is a critical step in the initial phase of TV infection and is important in subsequent pathogenesis (24). This process is able to induce gene upregulation in both the host cell and the parasite and is species-specific (25, 26).

The World Health Organization (WHO) recommends intermittent preventive treatment with sulfadoxine-pyrimethamine (IPTp-SP) for reducing the burden of malaria in pregnancy and adverse pregnancy outcomes (27). However, resistance to SP by malaria parasites has compromised the effect of IPTp-SP (28, 29). Previous systematic reviews and meta-analyses of clinical trials have demonstrated the beneficial impact of women receiving three doses of IPTp-SP including in areas with high SP resistance (30). This, however, may be different in areas with very high SP resistance (31). Therefore, alternatives to SP are needed for IPT to address the burden of malaria during pregnancy.

The WHO has provided syndromic management of STIs/RTIs to guide diagnosis and treatment in low-resource settings (32, 33). This is because testing women for STIs/RTIs and providing specific treatment is challenging and requires huge laboratory resources and skilled human resources. However, the syndrome-based approach to diagnosis and treatment of vaginal discharge associated with STIs/RTIs has poor sensitivity in detecting cervical infections

because the majority are asymptomatic (34). Therefore, a considerable burden of STIs/RTIs in pregnancy remains undetected and untreated.

A systematic review and meta-analysis of studies published in 2012 reported a significant prevalence of malaria and curable STIs/RTIs in pregnant women attending ANC in sub-Saharan Africa (35). This suggested the need to integrate approaches to address malaria and STIs/RTIs in sub-Saharan Africa. However, of 171 studies that met inclusion criteria, not one reported the prevalence of malaria and curable STI/RTI co-infection. A cohort study of pregnant women conducted in Nchelenge, Zambia involving 1,086 first ANC attendees between 2013-14, found that more pregnant women were co-infected with malaria and curable STI/RTI (38.7%, 95% CI= 35.7–41.6) than had malaria alone (18.9%, 95% CI=16.5–21.2) or curable STIs/RTIs alone (26.0%, 95% CI = 23.5–28.8). The STIs/RTIs that were most common in this setting were TV and BV (36). The malaria was diagnosed using PCR methods.

This thesis will focus on malaria and STIs/RTIs, in particular TV and BV, in pregnancy and potential interventions to mitigate them. Much of the data are from a clinical trial conducted in Nchelenge, Zambia, aimed at determining which combination of antimalarial and antimicrobial drugs might be more effective at reducing adverse the pregnancy outcomes. The thesis has four objectives:

Objective 1. To determine if Intermittent preventive treatment with sulfadoxine-pyrimethamine with metronidazole, or dihydroartemisinin-piperaquine with metronidazole was superior to sulfadoxine-pyrimethamine alone in protecting against adverse pregnancy outcomes among pregnant women in Nchelenge district;

Objective 2. To determine the day-28 treatment efficacy against *Plasmodium falciparum* infection among pregnant women in Nchelenge district who received either sulfadoxine-pyrimethamine alone, sulfadoxine plus metronidazole, or dihydroartemisinin-piperaquine plus metronidazole;

Objective 3. To determine the prevalence of 540 and 581 markers of resistance to sulfadoxine-pyrimethamine in *Plasmodium falciparum* samples among pregnant women in

Nchelenge district who received either sulfadoxine-pyrimethamine alone, sulfadoxine-pyrimethamine plus metronidazole or dihydroartemisinin-piperaquine plus metronidazole;

Objective 4. To compare the prevalence of STIs/RTIS at the first and second antenatal visits among pregnant women in Nchelenge district who received sulfadoxine-pyrimethamine alone, sulfadoxine-pyrimethamine plus metronidazole, dihydroartemisinin-piperaquine plus metronidazole.

1.2 Malaria in pregnancy

1.2.1. Prevalence of malaria in pregnancy

Malaria in pregnancy causes a considerable burden of maternal and foetal morbidity and mortality (37, 38). The WHO reported that, in 2021, in 38 moderate and high transmission countries in the WHO African region, there were an estimated 40 million pregnancies, of which 13.3 million (32%) were exposed to malaria infection during pregnancy. West Africa and Central Africa had the highest prevalence of exposure to malaria during pregnancy, 40.7% and 39.8%, respectively. There was a prevalence of 20% in East and Southern Africa. Without intervention, it is estimated that malaria infection during pregnancy in these 38 malaria endemic countries would have resulted in 961,000 children delivered with low birth weight (<2.5 kg). It follows that in the 33 countries in which IPTp was implemented, an estimated 457,000 of LBWs were averted (39). In African countries where malaria is endemic, *P. falciparum* infections are responsible for 11% of neonatal deaths commonly attributable to LBW (40, 41). Other complications include spontaneous abortions and intrauterine growth restriction. These complications are in part due to the accumulation of parasitized red blood cells in the placenta (12). Malaria in pregnancy may be reflected in different ways as shown in Table 1.

Table 1. Summary of the types of malaria infection in pregnancy

Type of malaria	Definition
Peripheral malaria	Malaria infection detected by a peripheral blood smear
Symptomatic malaria infection	Malaria infection with symptoms
Asymptomatic malaria infection	Malaria infection without symptoms
Microscopic malaria infection	Malaria infection diagnosed by microscopy of a blood smear
Sub-microscopic malaria infection	Malaria infection with parasites not detectable by microscopy but detectable by Nucleic Acid Amplification Test (NAAT)
Placental malaria	Malaria infection characterized by the accumulation of Plasmodium-infected red blood cells in the placental intervillous space

1.2.2 Importance of sub-microscopic malaria infection during pregnancy

A meta-analysis was conducted by van Eijk and colleagues published in 2023 which included studies conducted between 1995 and 2017 in 27 countries and included 15 trials, 39 surveys and 14 cohort studies. The meta-analysis which was conducted to compare the epidemiology of submicroscopic malaria to that of microscopic malaria infection using both individual participant data (45 studies, 48,869 participants) and aggregated data (23 studies, 11,863 participants) in pregnant women in Asia, America and in Africa, showed that infections which were detected using NAAT were submicroscopic, ranging from 59% during pregnancy to 71–74% at delivery. The pooled prevalence of submicroscopic infections in study participants who were NAAT-positive was 62.5% (95% CI 56.5–68.3) during pregnancy. However, at delivery, it was 72.3% (CI= 67.1–77.2) in peripheral blood and 72.7% (CI= 66.8–78.3) in placental blood. The Americas had the highest proportion of submicroscopic infections at delivery among women who had NAAT-positive infections. During pregnancy, the median prevalence for microscopic infections was 8% (0.0-50.6, 66 sub-studies) and 13.5% (0.0-55.9, 66 sub-studies) for submicroscopic infections. Infections were less likely to be submicroscopic in Africa than in Asia and the Americas. In areas of high malaria transmission coupled with high SP resistance, submicroscopic infections were found to predict future episodes of submicroscopic or microscopic infections. Submicroscopic infections were also associated with fever in Africa

(adjusted odds ratio1.32, 95% CI 1.02-1.72; p<0.0001). When peripheral blood was tested for submicroscopic malaria, the pooled prevalence was estimated to be 14.6% (95% CI 12.1–17·4) for submicroscopic malaria and 9.7% (CI= 6.9–13.0) for microscopic malaria. For maternal peripheral blood at delivery, the pooled prevalence estimates were 10.1% (95% CI =8·0–12.4) for submicroscopic and 4·0% (2.9–5·4) for microscopic infection. Unsurprisingly, Africa had the highest prevalence of both submicroscopic and microscopic malaria in the high transmission areas (42). In a hospital-based case–control study conducted in Sudan, cases and controls were investigated for malaria using microscopy, placental histology, PCR. The study showed that submicroscopic *P. falciparum* infection alone was significantly associated with LBW (OR = 6.89, 95% CI = 2.2–20.8; P = 0.0001), just like the combination of histologically diagnosed and submicroscopic infections (OR = 2.45, 95% CI = 1.2–4.9; P = 0.012) (43).

1.2.3 Risk factors for malaria in pregnancy

The seriousness of malaria in pregnancy is affected by several factors including environmental, parasite, and maternal factors. In high-transmission areas, primigravidae have the highest burden of malaria, whilst in-low transmission areas, all gravidities are at risk. Primigravidae in areas of high transmission develop antibodies to VAR2CSA protein produced by malaria parasites leading to partial protection during subsequent pregnancies. However, this does not seem to happen in areas of low transmission (44). In general, pregnant women who reside in low or epidemic transmission areas have low immunity to malaria resulting in a two-to-three-fold increase in the risk of severe malaria compared to women who are not pregnant. The age of the pregnant women also plays a role in malaria in pregnancy. Greater risk of severe malaria is associated with younger mothers compared to older women who appear to have some protection from severe disease (45). In addition, malnutrition in pregnant women increases the risk for adverse pregnancy outcomes (46). Using individual patient data from 14,633 pregnant women in Africa and Western Pacific between 1996 to 2015, a meta-analysis showed that malaria and malnutrition are common exposures, with 35% of women having either of those exposures (46). Recent data suggested that reduced intake of L-arginine as one of the mechanisms through which malnutrition contributes to low birth weight (47, 48).

1.2.4 Effects of malaria on pregnancy outcomes

Pregnant women living in malaria endemic areas are a high-risk group for *P. falciparum* infection and related consequences (49) such as maternal anaemia, intrauterine growth restriction (IUGR) (50, 51), preterm delivery (51, 52), stillbirth (53, 54) and LBW (49, 51). Low birth weight is associated with a marked increase in neonatal death (55-58). In sub-Saharan Africa, malaria in pregnancy accounts for 20% of stillbirths and 11% of deaths in neonates (40, 59). It is also associated with small-for-gestational age (SGA) (60, 61), and premature birth (60, 61). In the long term, LBW is known to contribute significantly to long-term morbidity (62) commonly due to increased episodes of diarrhoeal diseases and respiratory infections. Malaria in pregnancy can also cause maternal anaemia which in turn is associated with postpartum haemorrhage, preterm labour, LBW, SGA babies, and perinatal death (63, 64). Maternal malaria is also an important risk factor in stillbirth (OR, 3.0; 95% CI, 1.0-8.9; P=0.04) (53). Table 2 shows a summary of the adverse effects of malaria in pregnancy.

Table 2. Summary of effects of malaria on pregnancy outcomes

Maternal effect of malaria	Remarks	Foetal effect of malaria	Remarks
Maternal anaemia	In one estimate in sub-Saharan Africa, the population attributable fraction of malaria to severe anaemia during pregnancy was 26% (45).	Stillbirths	12–20% of stillbirths in endemic regions of sub-Saharan Africa (65)
Severe malaria	Pregnant women are three times more likely to be affected by severe malaria (66)	Low birthweight	approximately 20% of cases of LBW in malaria-endemic areas are attributed to placental infection with malaria (40)
		Preterm birth	up to 36% of prematurity in malaria-endemic areas attributable to Plasmodium infection(40, 67)
		Neonatal death	Acute placental malaria infection has been associated with increased one-year mortality in infants(68)

A systematic review and meta-analysis was conducted by Thompson *et al*, and published in 2020, to examine the relationship between malaria during pregnancy and adverse pregnancy outcomes. In total, 18 studies were included, of which eight had examined associations between pregnancy associated malaria preterm birth, and 10 examined associations between malaria and LBW (population size ranging from 35 to 9,956 women). The overall risk of LBW was 63% higher among women who had malaria during pregnancy compared with women without (RR 95% CI 1.48–1.80) and the risk of preterm birth was 23% higher among women with malaria during pregnancy compared with women without (RR 95% CI 1.07–1.41).

The results indicated that malaria infection during pregnancy was associated with preterm birth and LBW (69).

Another meta-analysis was conducted by Moore *et al*. published in 2017 and included 141,415 women and 3,387 stillbirths from 59 studies. Of the studies included, 46 were conducted in Africa, ten were in Asia, and two were conducted in the Americas. The aim of the meta-analysis was to quantify the association between malaria in pregnancy and stillbirth and showed that there was a relationship between malaria infection and stillbirth. In addition, there was also a relationship between the risk of stillbirth and endemicity. The odds of stillbirth were increased in participants in whom *plasmodium falciparum* infection was detected at delivery using peripheral samples [OR 1·81 (95% CI 1·42–2·30)]. Areas of high endemicity were found to have a lower risk of stillbirth when compared to areas of low-to-intermediate endemicity (OR 1·96 [95% CI 1·34–2·89]) showing an increased risk of malaria-associated stillbirth as endemicity declined (65).

Concerning malaria infection and preterm delivery, the risk for preterm delivery increased in women who had malaria. This had been shown in studies most of which were conducted in Africa and one particularly in Tanzania in a syphilis screening study in which it was found that women who had malaria had an increased risk of preterm delivery, LBW and intrauterine growth restriction [(aOR 3.2; 95% CI, 1.9-5.2), (aOR 5.4; 95% CI, 3.1-9.5) and (aOR 2.8; 95% CI, 1.2-6.7)] respectively (51). Risk factors for preterm birth in a population with high incidence of preterm birth and HIV infection, were assessed in another study in Malawi and showed an association between persistent malaria with late preterm birth (aOR 1.99, 95% CI, 1.05-3.79) (70). A study conducted in 1987-1990 in women in a high malaria transmission area in Malawi to compare the efficacy of chloroquine with mefloquine in preventing LBW showed that malaria infection was associated with LBW (aOR: 1.71) (60). Concerning the association of stillbirths with malaria, a study in Ghana demonstrated the increased risk of stillbirths with malaria infection (aOR 1.9; 95% CI, 1.2-9.3) (54).

1.2.5. The malaria situation and treatment policy in Zambia

Despite the important strides made in addressing malaria in the past decade, malaria remains a major public health problem in Zambia with varying rates of prevalence across districts and

causing approximately 6 million cases and 2,000 deaths per year (71). The malaria control strategy in Zambia includes the distribution of long-lasting insecticide-treated mosquito nets, increased indoor residual spaying, improved case management using rapid diagnostic tests, and treatment with artemisinin-based combination therapy (72).

Most cases are caused by *P. falciparum* though all the four main species of Plasmodium are found in Zambia (73, 74). Artemisinin-based combination therapies are recommended for the treatment of uncomplicated malaria in Zambia. By combining two active ingredients with different mechanisms of action, ACTs are the most effective antimalarial medicines currently available (75-77). The recommended ACTs are artemether-lumefantrine (AL) and dihydroartemisinin-piperaquine (DP). ACTs were adopted as first line treatment for uncomplicated malaria in Zambia in 2005 (78). The national policy in Zambia is to administer oral quinine for the treatment of uncomplicated malaria during the first trimester of pregnancy. During the second and third trimester of pregnancy, AL and DP are recommended in the usual adult dose. For complicated falciparum malaria, parenteral quinine is provided during all trimesters and SP is recommended for routine IPTp from the second trimester to delivery at each ANC visit as recommended by the WHO (79). Pregnant women are encouraged to sleep under an insecticide-treated net (ITN) to protect them from exposure to malaria (80).zs. Three tablets of SP each containing 500 mg of sulfadoxine and 25 mg of pyrimethamine are given orally whether the woman has parasites or not, and as directly observed therapy (81).

The 2021 Zambia malaria indicator survey showed 86.3% of women who had reported being pregnant in the past year took at least one dose of IPTp, and 67.9% of women reported taking three doses. Geographic characterization indicated that the third dose of IPTp was higher in urban areas, 76.0%, compared to 65.2% in rural areas (82). The previous malaria indicator survey which was conducted in 2018 showed that 81% of the pregnant women received at least 2 doses of IPTp during their most recent pregnancy and 67% reported getting 3 doses (82). However, just about 5% of women reported taking at least 4 doses of IPTp which the Zambian strategic plan advocates for (71).

1.2.5.1 Prevalence of malaria in pregnancy in Zambia

Studies in Zambia have reported the prevalence of malaria in pregnant women. As noted earlier in this chapter, a pregnancy cohort study from 2013-14 found the prevalence of malaria at enrollment by microscopy was 31.8 % (95% CI, 29.0–34.5; N = 1079) and by polymerase chain reaction (PCR) was 57.8 % (95% CI, 54.9–60.8; N = 1074) in the Nchelenge district (83). Siame *et al* showed the prevalence of malaria in pregnant women by RDT was 30%, by microscopy 15%, and 22% by PCR in the same district in 2013 in a cross-sectional study (84). Old data from a study conducted 47 years ago in Nchelenge district in northern Zambia, reported the prevalence of peripheral parasitaemia to be 22% in primigravidae and 11% in multigravidae; placental malaria was detected in 15 (27.2%, 95% CI, 16.1-41.0) of 55 primiparous and 13 (13.0%, 95% CI 7.1-21.2) of 100 multiparous women (85). Peripheral parasitaemia was shown to be at 63.5% (95% CI, 50.4-75.2) in a study conducted during the peak of transmission in the rainy season in Choma district in southern Zambia in 1989 (86). This prevalence of malaria is comparable to what is seen in other regions in Africa among pregnant women (35).

1.2.5.2 Prevalence of *Plasmodium falciparum* resistance markers in Zambia

The parasite clearance demonstrated by SP in countries where IPTp is part of the ANC package is highly compromised based on the prevalence of resistance markers. Consequently, surveillance of SP resistance markers is essential (28, 29, 31). In a study conducted in the north-western province in Zambia between March and June 2002 with 169 participants, assessed resistance markers associated to SP resistance. The point mutations on *dhfr* and *dhps* (540 and 581) were 92.0% (95% CI, 85.4-96.3) and 44.2% (95% CI, 34.9-53.9) respectively of the PCR samples analysed (87). In another study published in 2014, a study conducted in Mansa in 2010 and 2011, 84 women with complete haplotype data of the *dhfr* and *dhps genes*, 63% (95% CI, 50-70) showed quintuple mutants and 2.4% (95% CI, 0.3-8.3) had an additional mutation in A581G of *dhps* (88). These were varied results of prevalence rates over the two time points possibly due to the small sample sizes.

A cross-sectional study conducted in 2013 in Nchelenge district showed a high number of mutations in the *dhfr* and *dhps* genes. Of the 72 PCR-positive samples examined, there was a

high prevalence of *dhfr* triple (Asn-108 + Arg-59 + Ile-59) mutant (68%, 95% CI 56.0-78.6) and *dhps* double (Gly -437 + Glu-540) mutant (21%, 95% CI 12.2-32.0). The quintuple haplotype was found in 17% (95% CI, 8.9-27.3) with 2 samples (2.8%, 95% CI, 0.3-9.7) with an additional Gly-581mutation (84). In the cohort study conducted in 2013-14 in Nchelenge district, 96 malaria-positive samples were analysed from a pregnancy cohort of 1,084 women. At that time, 70.8% (95% CI, 60.7-79.7) of samples expressed the *dhps* 540 mutation, and 9.4% (95% CI, 4.4-17.1) had the *dhfr* 581 mutation (36). The prevalence estimates of these two studies were similar and with comparable sample sizes and studies were conducted around the same period.

1.2.5.3 Prevalence of adverse birth outcomes in Zambia

In the 2018 Zambia Demographic and Health Survey (ZDHS), data on birthweight was obtained from 80% of the deliveries which occurred in the previous 5 years. The survey showed that 9% of the babies from the 80% of childbirths with a reported birth weight were LBW. Infants weighing less than 2.5 kilograms (kg) at birth or who are reported to be very small or smaller than average are at increased risk of early childhood death (89). This was not significantly different from what was found in the 2013-14 ZDHS, which showed a similar prevalence of LBW of 10% in 3 major provinces (Lusaka, Copperbelt and Southern Province)(90). An adapted conceptual framework for adverse birth outcome is shown in figure 1 below. The factors which were considered in this thesis were those that were part of our study. These factors directly or indirectly influence birth outcomes through maternal and infant characteristics.

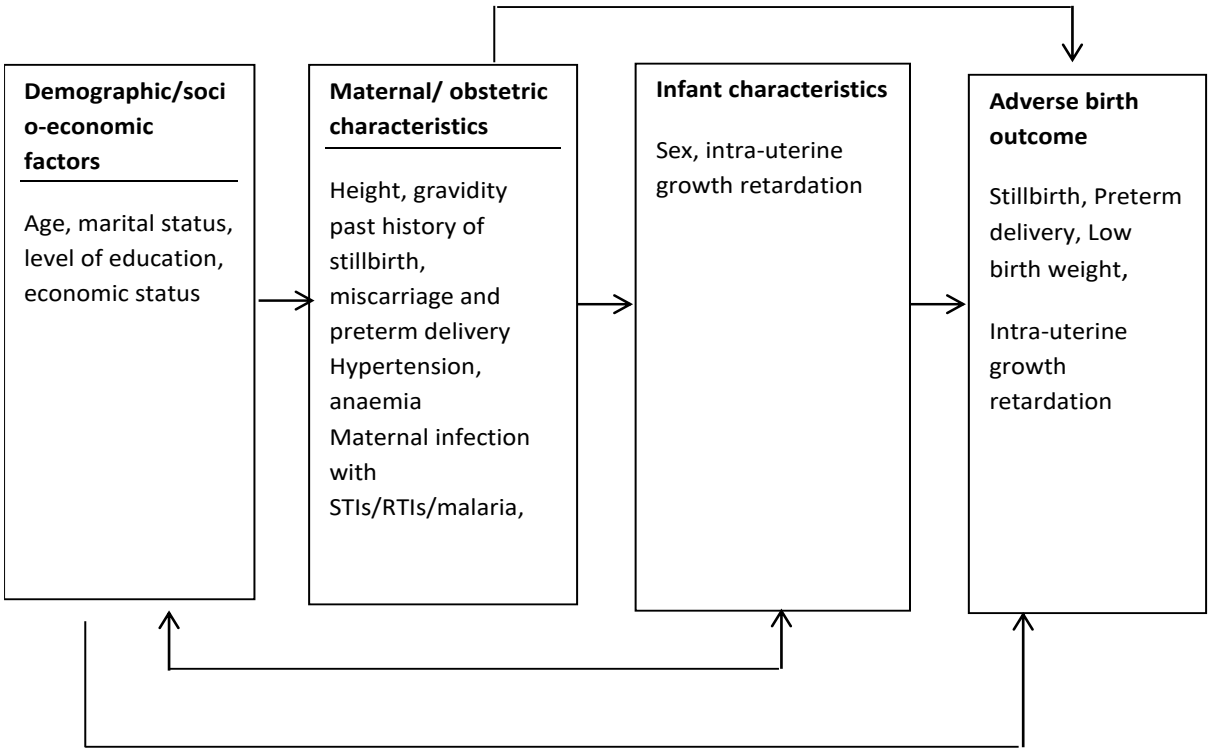


Figure 1. Conceptual framework for adverse birth outcomes

Adopted from Olusanya and Ofovwe, 2010 (91)

1.2.5.4 Antenatal care in Zambia

According to the 2018 ZDHS, 97% women between the age of 15 and 49 who had a live birth in the 5 years preceding the survey received ANC care from a skilled provider during their most recent birth, and 64% had at least four ANC visits. There has been a steady increase in the past 26 years despite a small drop between 1996 and 2001-02 where the rate decreased from 92% to 88%. The percentage increased from 94% to 97% between 2007 and 2018. Of the women who received ANC care from skilled providers, 93% did so from a nurse/midwife, whereas 3% received care from a doctor (92).

Ninety-nine percent of women in urban areas received care from skilled personnel, compared to 96% of women in rural areas. Women receiving ANC from a skilled provider ranged from 91% in Luapula to 99% in Copperbelt and Lusaka. Of women who had a live birth, 65% had at least four ANC visits, while 33% of women had two to three ANC visits. Women living in rural versus urban areas were slightly more likely to have at least four ANC

visits, 65% compared to 61%. First trimester ANC booking was at 37% whilst 48% had their first visit during the fourth or fifth month of their pregnancy and 13% received their first ANC during their sixth and seventh month of pregnancy. The median gestational age at which women made their first ANC visit was 4.4 months (92).

At ANC booking, the Zambian ANC package encompasses the following procedures; blood pressure recording, urinalysis, weight measurement, height measurement, blood sample collection for syphilis, and hemoglobin testing, HIV testing after counselling, provision of iron supplementation , IPTp-SP, discussion of birth preparedness plan, provision of treatment for intestinal parasites and tetanus toxoid vaccination (90, 93). Procedures for subsequent visits include recording the blood pressure, checking the fundal height, checking the fetal heart, provision of folic acid and ferrous sulphate to prevent anaemia and provision of monthly IPTp-SP from the second trimester. In places where available, ultrasound examination is provided at least 3 times during the course of the pregnancy. The syndromic management of curable STIs/RTIs, despite not being part of the core ANC package, is provided to women with symptomatic STIs/RTIs and is national policy (94).

Eighty-four percent of live births in the 5 years preceding the survey which occurred in health facilities (92) . Despite a decline in health facility deliveries from 51% to 44% from 1992 to 2001-02, health facility deliveries increased to 48% in 2007 and 84% in 2018 due to an increase in health centres in rural areas. It is also reported that frequency of births delivered in a health facility decreases with increasing mother's age at birth; the higher the birth order, the less likely a woman would deliver at a health facility. Almost 92% of the first-order births are delivered in a health facility, as compared with 75% of sixth- or higher order births (92).

1.2.6 Interventions to reduce the burden of malaria in pregnancy

Intermittent preventive treatment of malaria in pregnancy is recommended by the WHO for women who are resident in areas of moderate to high malaria transmission (95). All pregnant women should be given IPTp-SP at least three times during pregnancy with at least one month between doses and beginning as early as the 13th gestational week (95). IPTp-SP reduces maternal anaemia by 40% (95% CI 47-75) and LBW by 27% (95% CI, 61-87) as shown in meta-

analyses of studies conducted in pregnancies during the first and second trimester (96, 97). Three or more doses of IPTp-SP improved birth weight and reduced the risk of LBW by 56% in comparison to less than three SP doses (p = 0.009). However, resistance to SP in sub-Saharan Africa poses a major challenge conferred by the successive acquisition of polymorphisms in the parasite genes encoding the targets of sulfadoxine and pyrimethamine *Pfdhps* and *Pfdhfr,* respectively. (98, 99).In areas with greater than 90% prevalence of *Pfdhps*-K540E, the efficacy of SP to clear peripheral parasites and prevent new infections during pregnancy is compromised. However, IPTp-SP still confers increases in birth weight and maternal hemoglobin. Nevertheless, even in areas where parasites routinely harbor up to 5% *Pfdhfr* and *Pfdhps* resistance-conferring mutations, SP has remained effective as IPTp, presumably owing to the partial immunity acquired by pregnant women. These findings support WHO's recommendation to continue using SP for IPTp in these high-resistance areas. However, an important exception is areas where the prevalence sextuple mutants are greater than 37% (100). Therefore, there is a need to monitor continuously the effectiveness of this useful intervention in the light of *P. falciparum* resistance to SP, while investigating alternative drugs for use in IPTp (99).

1.2.7 Alternative antimalarial therapies to sulfadoxine-pyrimethamine

Dihydroartemisinin-piperaquine (DP) has been the leading candidate to replace SP for use in IPTp. DP reduces the prevalence of malaria infection, placental and clinical malaria. It is the current alternative to SP in IPTp (81). In 2022, Figueroa-Romero *et al* published a review of clinical trials conducted over the last decades for IPTp for malaria in pregnancy looking for alternatives to IPTp-SP (81). The trials are summarized in appendix 9.1.

Four of these clinical trials are summarised in Table 3 on page 37 and 38, each involving head-to-head comparisons of IPTp-SP and IPTp-DP. Kajubi *et al*. reported a study conducted in an area of perennial high malaria transmission and high SP resistance in Uganda. The clinical trial was a double-blind, randomised trial conducted in Busia District, southeastern Uganda between September 2016, and May 2017. In total, 782 HIV-uninfected women were enrolled

between 12 weeks and 20 weeks gestation. There was no significant difference in the risk of composite adverse birth outcome between the DP and SP treatment groups (54 [16%] of 337 women vs 60 [18%] of 329 women. The protective efficacy of the IPTp-DP group was 12% [95% CI -23-37, (p=0·45)]. The study showed that monthly IPTp-DP, when compared to IPTp-SP, did not lead to significant improvements in composite pregnancy outcomes. Although the results suggested trends towards a reduced risk of LBW and preterm birth in the IPTp-DP arm, these differences were not statistically significant (101).

Another trial conducted in a similar setting of widespread SP resistance was reported by Kakuru *et al.* in Uganda among 300 HIV-negative women from June to October 2014. This was a double-blind, randomized, three-group controlled trial in pregnancy in which participants were randomly assigned to a standard SP regimen, a three-dose DP regimen, or a monthly DP regimen (106, 94, and 100 participants respectively). The prevalence of placental malaria was significantly higher in the SP group (50.0%) compared to the three-dose DP group (34.1%, p=0.03) or the monthly DP group (27.1%, P=0.001). Adverse pregnancy outcomes were less prevalent in the monthly DP group (9.2%) than the SP group (18.6%, P=0.05) or the three-dose DP group (21.3%, P=0.02). Symptomatic malaria had a higher incidence in the SP group (41 episodes over 43.0 person-years at risk) than in the three-dose DP group (12 episodes over 38.2 person-years at risk, P=0.001) or the monthly DP group (0 episodes over 42.3 person-years at risk, P<0.001). There were no significant differences in the detection of malaria parasites by means of microscopy in placental and maternal blood among the treatment groups. However, detection of malaria parasites by Loop-Mediated Isothermal Amplification (LAMP) in placental and maternal blood was significantly more common in the SP group than in the three-dose DP group or the monthly DP group. (102).

Desai *et al.* (103) published data from an open-label, three-group, randomized controlled superiority trial conducted at four sites in western Kenya with high malaria transmission and SP resistance where they recruited 1,546 participants between August 2012 and June 2014. HIV-negative pregnant women with a gestational age between 16-32 weeks were randomly assigned to receive intermittent IPTp-SP, screening, and treatment (IST) with DP or IPTp-DP. Malaria infection at delivery was less prevalent in the IPTp-DP group than in the IPTp-SP group (15 [3%] of 457 women vs 47 [10%] of 459 women; RR 0.32, 95% CI 0.18-0.56), but not in the

IST with DP group (57 [13%] of 452 women; CI 1.23, 0.86-1.77). The incidence of malaria infection during pregnancy was lower in the IPTp-DP when compared to the IPTp-SP group (192.0 vs 54.4 events per 100 person-years; IRR 0.28, 95% CI 0.22-0.36) and clinical malaria during pregnancy (37·9 vs 6.1 events; CI 0.16, 0.08-0.33), whereas IST with DP was associated with a higher incidence of malaria infection (232.0 events; CI 1.21, 1.03-1.41) and clinical malaria (53.4 events; CI 1.41, 1.00-1.98) (103).

The IMPROVE trial, an individually randomised, double-blind, three-arm, partly placebo-controlled trial in areas of high SP resistance in Kenya, Malawi, and Tanzania recruited 4,680 pregnant women between March 2018 and July 2019. In this trial, HIV-negative women were randomly assigned to receive monthly IPTp- SP, monthly IPTp-DP plus placebo at the first visit, or monthly IPTp-DP plus 2g azithromycin. Kenya recruited 1,490 women, Malawi 1,404, and Tanzania 1,786. The study found that monthly IPTp with DP did not improve adverse pregnancy outcomes, and the addition of 2g azithromycin at the first visit did not enhance the effect of monthly IPTp with DP. Adverse pregnancy outcomes were reported more frequently in the DP group (403 [27.9%] of 1,442; risk ratio 1.20, 95% CI 1.06–1.36; p=0.0040) and in the DP plus azithromycin group (396 [27.6%] of 1,433; 1.16, 1.03–1.32; p=0.017) compared with 335 (23·3%) of 1435 women in the SP group (104).

Table 3. Clinical trials of IPTp comparing SP versus DP

Country(105)	Site & year of study	Gravidae	IPTp-SP			IPTp-DP			Key findings
			LBW	Peripheral malaria	Placental malaria	LBW	Peripheral malaria	Placental malaria	
Uganda (Kajubi *et al,* 2019) (101)	Busia District, 2016-17	NA	9% (29/329) (95% CI:5.98-12.4)	8% (28/336) (95% CI:5.8-11.8)	9% (28/320) (95% CI:5.9-12.4)	7% (24/337) (95% CI: 4.6-10.4)	<1% (1/342) (95% CI:0.0074-1.6)	<1% (1/333) (95% CI: 0.02-4.1)	Monthly intermittent preventive treatment with DP was safe but did not lead to significant improvements in birth outcomes compared with SP.
Uganda (Kakuru *et al,* 2016) (102)	Tororo, 2014	NA	14.1% (14/99) (95% CI:7.9-22.6)	4.9% (5/102) (95% CI:1.6-11.1)	5.2% (5/96) (95% CI: 1.7-11.7)	8.2% (8/98) (95% CI:3.6-15.5)	(0/97)	2.1% (2/96) (95% CI:0.3-7.3)	The burden of malaria in pregnancy was significantly lower among pregnant women who received IPTp with DP than among those who received IPTp with SP, and monthly treatment with DP was superior to three-dose DP regarding several outcomes.
Kenya (Desai *et al*, 2015) (103)	Siaya County, 2012-14	Primigravid or secundigravid: IPTp-SP= 57% (292/514), IPTp-DP= 51% (263/514) Multigravid: IPTp-SP= 43% (222/514), IPTp-DP= 49% (251/514)	4% (18/409) (95% CI:2.6-6.8)	12% (54/459) (95% CI:8.96-15.1)	37% (159/426) (95% CI:32.7-42.1)	5% (22/414) (95% CI:3.4-7.9)	4% (16/457) (95% CI:2.0-5.6)	33% (139/421) (95% CI:28.5-37.7)	Screen-and-treat strategy was not superior to IPTp-SP in areas of high SP resistance and high transmission. The prevalence of malaria was lower in the IPTp-DP group when compared to the IPTp-SP group. However, the prevalence of malaria at delivery was not lower in the screen-and-treat group. IPT-DP had a higher reduction in the risk of malaria during pregnancy compared to IPTp-SP even though birthweight was higher in the IPTp-SP group

LBW = low birth weight; ITN = insecticide treated nets: IPTp-SP =intermittent preventive treatment with Sulfadoxine-Pyrimethamine: IPTp-DP-intermittent preventive treatment with Dihydroartemisinin-piperaquine

Country(105)	Site, year of study	Gravidae	IPTp-SP			IPTp-DP			Key findings
			LBW	Peripheral malaria	Placental malaria	LBW	Peripheral malaria	Placental malaria	
Kenya, Malawi, and Tanzania (Madanitsa *et al* 2023) (104)	southern Malawi, northeastern Tanzania, and western Kenya, 2018-19	NA	(7.2%) 101/1,401 (95% CI:5.9-8.7)	(70.8%) 378/5338(95% CI:6.4-7.8)	(1.7%) 20/1208 (95% CI:1.0-2.5)	10.3% (145/1407) (95% CI:8.8-12.0)	(34.5%) 184/533·8 (95% CI:3.0-4.0)	(0.7%) 8/1204 (95% CI:0.3-1.3)	Monthly IPTp with DP did not improve pregnancy outcomes, and the addition of a single course of azithromycin did not enhance the effect of monthly IPTp with DP. Trials that combine SP and DP for IPTp should be considered

LBW = low birth weight; ITN = insecticide treated nets: IPTp-SP =intermittent preventive treatment with Sulfadoxine-Pyrimethamine: IPTp-DP-intermittent preventive treatment with Dihydroartemisinin-piperaquine: NA- Not applicable

A mediation analysis, which was to assess direct and indirect effects of the treatments was conducted. Data from three of these four studies (101-103) formed the basis of the mediation analysis that showed IPTp-DP to be superior to IPTp-SP when considering malarial specific endpoints (106). However, and importantly, IPTp-SP was not inferior to IPTp-DP when considering birthweight at delivery and the incidence of LBW. Sulfadoxine-pyrimethamine conferred greater protection for LBW attributable to its non-malarial effect compared to its antimalarial effect. More studies are needed to assess monthly DP with SP or with other compounds with non-malarial effects to achieve greater protection against malarial and non-malarial causes of low birthweight. This is why IPTp-SP continues to be useful against LBW despite parasite resistance due to other non-malarial effects. Since DP is a good antimalarial drug, it requires a partner drug with strong non-malarial protective effects if indeed is to replace SP for IPTp.

1.3 Prevalence of curable STIs/RTIs in pregnancy

A systematic review and meta-analysis by Nyemba *et al.* in 2022 contained published and unpublished studies from March 2015 to October 2020, from 14 sub-Saharan African countries, and reported prevalence estimates of *Chlamydia trachomatis* (CT), *Trichomonas vaginalis* (TV), *Neisseria gonorrhoeae* (NG), *Treponema pallidum* (syphilis), *Mycoplasma genitalium* (MG) and bacterial vaginosis among pregnant women in sub-Saharan Africa. Pooled prevalence estimates (with 95% CI and number of women tested) were as follows: CT, 10.8% (6.9-15.5, n=6700); TV 13.8% (10.0-18.0, n=9264); NG, 3.3% (2.1-4.7, n=6019); syphilis, 2.9% (2.0-4.0, n=95308) and BV, 36.6% (27.1-46.6, n=5042). By region, BV was the most prevalent and ranged from 28.5% (24.5-32.8, n=1030) in Eastern Africa to 52.4% (33.5-70.9, n=2305) in Southern Africa; NG had the lowest prevalence, ranging from 1.4% (95% CI: 0.1-3.1, n=367) in Central Africa to 4.4% (2.6-6.4, n=4042) in Southern Africa. This review showed that the prevalence of curable STIs and BV among pregnant women was substantial in sub-Saharan Africa and was most prevalent in Southern Africa where HIV prevalence is highest (107). In the review by Chico *et al*. total of 171 studies were reviewed and provided 307-point prevalence estimates for malaria or STIs/RTIs and included a total of 340 904 women. It was found that not one of the 171 studies report malaria and STIs/RTIs co-infection as there are few studies on co-infection (35).

Partner notification and treatment are key areas of STI control as untreated partners contribute to increased rates of reinfections with STIs. In a prospective cohort study which was conducted in Gaborone, Botswana, which who enrolled 300 pregnant women found that pregnant women were willing to utilize patient-based partner notification (108). In a cohort study of pregnant women conducted in Brazil, from September 2018 to November 2019, STIs were found to be common in sexual partners of pregnant women. STIs in partners of pregnant and should be addressed to prevent reinfection of pregnant women (109). A method that has shown improvement partner management is expedited partner treatment (EPT) which is defined as the treatment of partners before they are assessed by a health care provider(110).

1.3.1 Prevalence of curable STIs/RTIs in pregnancy in Zambia

There is a dearth of data about the prevalence of curable STIs/RTIs among pregnant women in Zambia. The systematic review by Nyemba *et al.* (107) identified only one study in Zambia, a pregnancy cohort study conducted in Nchelenge district in 2013-2014 which reported the prevalence among 1,086 ANC attendees on their first visit. Study participants were screened for curable STIs/RTIs (syphilis, CT, NG, TV, and BV). Among participants with complete results (N = 1,071) the prevalence rates were as follows; BV was 48.3% (95% CI= 45.2–51.2), TV; 24.8% (95% CI =22.3–27.5), CT;5.2% (95% CI=3.9–6.7), NG;3.1% (95% CI= 2.2–4.4), syphilis;7.1% (95% CI= 5.6–8.7), composite STI Positive 34.5% (31.7–37.4), and STI/RTI; 64.8% (95% CI = 61.7–67.4). The proportion of women who had an STI/RTI but did not have malaria tested by PCR was 26.0% (95% CI = 23.5–28.8) (36).

1.3.2 Effects of curable STIs/RTIs on pregnancy outcomes

Sections 1.3.3 to 1.3.7.3 present a summary of individual curable STIs/RTIs, outlining their effects on pregnancy and interventions to reduce the burden.

1.3.3 Syphilis in pregnancy

1.3.3.1 Effects of syphilis on pregnant outcomes

Two studies looked at the impact of syphilis in pregnancy at a population level. One of the studies was conducted in Malawi and another one in Tanzania (111, 112). In Malawi, a prospective study of malaria chemoprophylaxis in pregnant women was conducted between 1987 and 1990 in a rural district. A total of 3,591 pregnant women were enrolled and tested for syphilis. Women with active syphilis were 11 times more likely to experience a stillbirth (aOR 10.89; 95% CI, 6.61-17.93) and 26% of stillbirths were attributed to active syphilis (111). In a retrospective cohort study conducted in Mwanza Tanzania, 380 pregnant women were recruited and tested for syphilis at delivery. Syphilis was associated with increased risk of stillbirth (aRR 18.1; 95% CI, 5.5-59.60), LBW in live born infants (aRR 3.3; 95% CI, 2.0-5.4), preterm delivery (aRR 6.1; 95% CI, 2.5-15.3) and intra-uterine growth retardation (IUGR) (aRR 2.1; 95% CI, 1.0-4.2) (112). These two studies show that syphilis continues to be a cause of adverse pregnancy outcome among pregnant women who do not receive treatment.

1.3.3.2 Interventions to reduce the burden of syphilis in pregnancy

Treatment of syphilis has been shown to be beneficial in preventing adverse pregnancy outcomes, particularly if done in the first trimester compared to the third. Pregnant women who received treatment for active syphilis with the regimen of single-dose benzathine penicillin had the same or lower risks of adverse pregnancy outcomes when compared with women who were seronegative for syphilis (113). A systematic review of treatment guidelines for syphilis in pregnancy by Trinh et al. 2019 showed that 95% of the guidelines recommended benzathine penicillin G (BPG) as the first-line therapy for syphilis in pregnancy, consistent with WHO guidelines (114). Another systematic review and meta-analysis published in 2023 (the search for the studies was conducted in May 2022), which included 22 observational studies conducted in developing countries, showed that treatment of active syphilis in pregnant women reduced the risk of preterm birth by 52% (95% CI = 42%-61%; 11,043 participants); stillbirths by 79% (95% CI = 65%-88%; 14,667 participants); and LBW by 50% (95% CI = 41%-58%; 9,778 participants) (115).

A study in done in Tanzania from September 1997 to November 1999, 19,878 women were screened for syphilis by RPR and 7.7% (1,522) were found to be seropositive. All eligible RPR-positive women were treated with single-dose benzathine penicillin. This study demonstrated that there was no increased risk of adverse pregnancy outcomes for women treated for high titre active syphilis (OR 0.76; 95% CI, 0.4 -1.4) or low titre active syphilis (OR 0.95; 95% CI, 0.6 -1.5) compared with sero-negative women. Women were recruited, tested for syphilis and treated on the same day (mean recruitment age, 25.8 weeks) (113). However, in the Kenya study, despite improved birth outcomes from the treatment of syphilis at ANCs, this did not eliminate the risk completely (116). In our study about 98% of women who tested positive on RPR testing were treated with penicillin. The diagnosis of syphilis was confirmed by the *Treponema pallidum* hemagglutination assay (TPHA).

1.3. 4 *Chlamydia trachomatis* in pregnancy

1.3.4.1 Effect of Chlamydia trachomatis in pregnancy

In a systematic review published in 2023 by Gamberini and team, which included five studies from four sub-Saharan African countries, provided an overall association of non-viral genital

infections with adverse pregnancy outcomes (117). Particularly, one of the studies reported in this review conducted in South Africa by Van Rensburg *et al* investigated the association between CT infection and preterm birth and reported a statistically significant increase in incidence of preterm delivery attributable to CT (OR: 4.29, CI: 1.52–12.07) (118).

In another study conducted in the Netherlands among 3,913 pregnant women, CT infection was associated with preterm delivery occurring before 32 weeks (aOR 4.35; 95% CI, 1.3 - 15.2) and 35 weeks gestation (aOR: 2.66; 95% CI, 1.1- 6.5), but not before 37 weeks (aOR 1.17; 95%CI, 0.6-2.4) and not with LBW (*P* = 0.25) (119). In a study of 343 pregnant women in South Africa, 36 (10.5%) delivered before 37 weeks gestation and CT was found in 8 (22.2%) of women who had preterm delivery in contrast to 32 (10.4%) of women who had full-term deliveries (*P* = 0.037) (120). In another study, which was a case control study in Switzerland, CT was associated with preterm delivery (aOR; 7.93, 95% CI, 1.34-46.76) (121). In an unmatched case-control study conducted in Uganda, results showed no association between CT infection and premature rupture of membranes (PROM) (aOR; 2.05, 95% CI, 0.37-11.49) (122).

In a record-based retrospective cohort study involving 354,217 participants and conducted in Australia, it was found that women with CT had increased risk of preterm delivery (aOR 1.17, 95% CI, 1.01-1.37) and stillbirth (aOR 1.40, 95% CI, 1.00 -1.96) but no association was found between infection with CT and small for gestational age (aOR 0.99, 95% CI, 0.89-1.09) (123). In another retrospective cohort study in the US, electronic medical records from a STI clinic were matched with state birth records for 730 women (124). CT was associated with LBW (aOR 2.07; 95% CI: 1.01 - 4.24). A population based retrospective cohort study in the US found that Chlamydial-infected women were at increased risk of preterm delivery (aRR 1.4; 95% CI: 1.08 -1.99) and PROM (aRR 1.5; 95% CI 1.03 -2.17) compared with uninfected women (125). No increased risk of LBW was observed in this study (aRR 1.12; 0.61-1.68).

In two other prospective studies from the US which were included in the same review, it was shown that CT had significant impact on pregnancy outcomes. In the first study, researchers found that infection with CT was associated with both IUGR (aOR 2.4; 95% CI, 1.3-4.2) and preterm delivery (aOR 1.6; 95% CI, 1.0-4.2) (126). In the second study, it was found that infection with CT was associated with LBW (aOR 2.7; 95% CI, 1.3 to 5.7), PROM (OR 2.4; 95%

CI 1.7 to 5.4) and preterm labour of less than 34 weeks (aOR 4.0; 95% CI, 1.7-9.2) (127). However, a third study showed no such association between infection with CT and adverse pregnancy outcomes (128). The absence of association could be due to some of the women having taken antibiotics during the antenatal period (126).

1.3.4.2 Interventions to reduce the burden of chlamydia in pregnancy

Antenatal infection with CT may lead to an increased risk of preterm delivery (129). Syndromic management is used to treat pregnant women with suspected CT infection based on symptoms of vaginal discharge. A systemic review by Tong *et al*. comprising five observational studies and two randomized control trials were included for detection and treatment of CT infection and was published in 2023. The review showed that treatment of pregnant women with chlamydia infection reduced the risk of preterm birth by 42% (95% CI = 7%-64%; 5468 participants) and possibly reduces the risk of LBW by 40% (95% CI = 0%-64%; 4684 participants) (115). Another review article by Folger showed an association between early detection and successful treatment of CT during antenatal period and the likelihood of preterm birth among pregnant women (129). A retrospective cohort study was conducted in the US between 2006-2011 to evaluate the risk of preterm birth among women with maternal CT detected and eradicated at or before 20 weeks gestation (intervention group) compared to women whose infections were detected after 20 weeks gestation or persistent during the pregnancy (reference group). The study population contained 3,354 pregnant women with documented CT infections. The relative risk for moderate to late spontaneous preterm birth (32-36 weeks gestation) was 0.54 (95 % CI 0.37-0.80) for women in the intervention group who were 19 years of age and younger. Pregnant adolescents benefited the most from early detection and eradication of antenatal CT infections through a reduction in the risk of preterm babies at 32-36 weeks gestation (129).

1.3.5 Neisseria gonorrhoeae in pregnancy

1.3.5.1 Effect of *Neisseria gonorrhoea* in pregnancy

Infection with *Neisseria gonorrhoeae* is associated with several adverse pregnancy outcomes, including spontaneous abortion, stillbirth, preterm labour, miscarriage, growth retardation, and intrauterine death (130) . A systematic review by Vallely *et al.* 2021 to assess associations

between NG infection during pregnancy and the risk of spontaneous abortion, preterm birth, premature rupture of membranes, perinatal mortality, and LBW for studies published between 1948 and 2020. Studies which reported testing for NG during pregnancy and compared pregnancy, perinatal and/or neonatal outcomes between women with and without NG were included. The study included 30 publications in the meta-analyses. It was found that women with NG were more likely to experience preterm birth (OR 1.55, 95% CI 1.21 to 1.99, n=18 studies); PROM (OR 1.41, 95% CI 1.02 to 1.92, n=9); perinatal mortality (OR 2.16, 95% CI 1.35 to 3.46, n=9); and LBW (OR 1.66, 95% CI 1.12 to 2.48, n=8). Summary adjusted ORs were, for preterm birth 1.90 (95% CI 1.14 to 3.19, n=5) and for low birth weight 1.48 (95% CI 0.79 to 2.77, n=4). In addition, low-income and middle-income countries were more associated with preterm birth (OR 2.21, 95% CI 1.40 to 3.48, n=7) than high-income countries (OR 1.38, 95% CI 1.04 to 1.83, n=11) (131).

Neisseria gonorrhoea has been shown to be associate with adverse birth outcomes in several studies conducted in the past. The review of studies by Mullick and his colleagues in their publication in 2005 showed that NG was associated. One study elucidated complications which were found in untreated gonorrhoea noted in case reports. These included preterm delivery, premature rupture of the membranes, LBW, postpartum endometritis, and ophthalmia neonatorum (132). In a case-control study conducted in Nairobi consisting of 166 cases and 175 controls, NG was isolated from 11% of cases and 4% of controls (OR2.9, 95% CI 1.2 to 7.2) and was associate with 14% of cases of LBW in this population (133). In a prospective study conducted in South Africa, 167 pregnant women with NG at ANC booking were followed up until delivery and it was found that 5 of 9 women with NG delivered a premature baby compared with 24 out of 158 uninfected women (RR 6.0, 95% CI 1.5 to 34.0). In addition, women who had NG delivered babies with LBW (mean weight 2252 g v 2,970 g, p,0.005). It was found that women with gonorrhoea delivered significantly smaller babies (mean weight 2252g versus 2970, $P < 0.005$) (134).

1.3.5.2 Interventions to reduce the burden of Neisseria gonorrhoea in pregnancy

Antibiotics such ceftriaxone and cefixime are effective in treating gonorrhoea in pregnancy (135). However, Tong et al could not conduct a meta-analysis due to the limited number of studies on the impact of antibiotics on adverse pregnancy outcomes due to NG infection.

(115). Syndromic management based on vaginal discharge is used in the treatment of NG in pregnancy in poor-resourced countries though many asymptomatic infections are missed due to a great proportion of infections being asymptomatic.

1.3.6 Trichomonas vaginalis in pregnancy

1.3.6.1 Effects of *Trichomonas vaginalis* on pregnancy outcomes

TV in pregnancy has been associated with adverse pregnancy outcomes (136). Several studies have suggested that TV increases the risk of premature birth in sub-Saharan Africa (137, 138) TV can cause damage to host epithelial cells due to its complex interaction with the host and the normal flora. However, infections of the female genital tract by TV can cause a range of symptoms, including vaginitis, cervicitis, and urethritis (136, 139) and has been associated with atypical pelvic inflammatory disease (140). It increases the vaginal pH during infection, leading to elevation of polymorphonuclear leukocytes. TV is also associated with an increased risk of HIV acquisition and mother-to-child transmission in HIV infected women (141).

In a systematic review by Van Gerwen *et al*. published in 2021 to examine the association between trichomoniasis in pregnant women and adverse pregnancy outcomes including preterm delivery, PROM and LBW, 19 studies met the inclusion criteria for a meta-analysis. The selected studies were conducted between 1965 and 2015 and were published between 1974 and 2019 and included 94,335 pregnant women were included. Significant associations were found between TV and preterm delivery (OR 1.27; 95% CI 1.08-1.50), PROM (OR 1.87; 95% CI 1.53-2.29) and LBW (OR 2.12; 95% CI 1.15-3.91). The study showed that TV was associated with preterm delivery, PROM and LBW in pregnant women (142). Among the four studies of TV in pregnancy included in this review, only one study was done in Africa (143). The studies associated infection with TV in pregnancy with preterm delivery and LBW (137, 143-145). In the Democratic Republic of Congo, trichomoniasis infection in pregnancy was associated with LBW in infants born to infected mothers (aOR 2.4; 95% CI, 1.2-4.5) (143). In the three studies in the US, one found that infection with TV was associated with LBW among adolescents (145); in the second study, women infected with TV were significantly more likely to have PROM ($P < 0.03$) (144). Trichomoniasis was associated with

LBW (aOR 1.3; 95% CI, 1.1 to 1.5), preterm delivery (aOR 1.3; 95% CI, 1.1 -1.4) and preterm delivery of a LBW infant (aOR 1.4; 95% CI, 1.1 to 1.6) in the third study (137).

1.3.6.2 Interventions to reduce the burden of *Trichomonas vaginalis* in pregnancy

Treatment of TV involves a single oral dose of metronidazole. However, in cases where the patient is allergic to metronidazole, or where there is drug resistance to metronidazole (33, 146, 147), tinidazole can be utilized. Both MTZ and tinidazole are safe during pregnancy in the second and third trimesters (148). There are several methods by which TV may be diagnosed with varying advantages. The impact of antibiotic treatment of TV during pregnancy has seen mixed outcomes. In a review led by Sangkomkamhang to assess the effectiveness of antenatal lower genital tract infection screening and treatment programs for reducing preterm birth and subsequent morbidity, one study (4,155 women at less than 20 weeks' gestation) met the inclusion criteria. The intervention group (2,058 women) received infection screening and treatment for BV, TV, and candidiasis; the control group (2,097 women) also received screening, but the results of the screening program were not revealed, and women received routine ANC. The rate of preterm birth was significantly lower in the intervention group (3% versus 5% in the control group) with a risk ratio of 0.55 (95% CI 0.41-0.75). The incidence of LBW was significantly lower in the intervention group than in the control group (RR 0.48, 95% CI 0.34 to 0.66 and RR 0.34; 95% CI 0.15 -0.75) (149). Nyemba *et al.* 2022 assessed the impact of diagnosing and treating curable STIs during pregnancy on adverse pregnancy outcomes in South Africa. Data from two prospective studies of pregnant women attending ANC clinics were combined. Pregnant women were enrolled, tested, and treated for STIs. The association between any STI at the first ANC visit and a composite adverse pregnancy outcome (miscarriage, stillbirth, preterm birth, LBW or early neonatal death) was evaluated. The prevalence of TV was 18% (n =120). Overall, treated STIs, including TV, at the first ANC visit, were not associated with adverse pregnancy outcomes (150).

However, some studies have shown that treatment of TV infection in pregnancy has no effect on gestational age and birthweight. A study in South Africa found that gestational age at delivery and birth weight of infants born to women who were treated with benzoyl-metronidazole and in those who did not receive treatment were similar (151). . A review aimed at determining whether antibiotic treatment for BV or TV during pregnancy decreases

the risk of preterm delivery and associated adverse pregnancy outcomes concluded that there is no evidence to support the use of antibiotic treatment for these infections for the purpose of reducing the risk of preterm delivery and associated adverse birth outcomes (152). These conclusions were based on two studies. One US study evaluating treatment of asymptomatic TV (selectively enrolled asymptomatic women) with MTZ suggested that the drug failed to prevent preterm delivery (153). In this study women treated with MTZ were significantly more likely to deliver a preterm infant than untreated women (RR 1.8; 95% CI 1.2-2.7). The second study was a sub-analysis of a randomised trial in Uganda which showed that women who were treated for TV with MTZ were 2.5 times more likely to deliver a LBW infant (RR 2.49; 95% CI 1.12-5.50) (154). Although the original study was not designed to assess the effect of TV treatment, the authors inferred that this might have been due to metronidazole exposure. However, what was observed in this study may be attributable to some other factor rather than what was inferred by the authors since the risk of preterm delivery was not increased in other clinical trials of MTZ treatment of BV during pregnancy (155-158). Furthermore a sub-analysis of a study of pregnant women in four sub-Saharan African sites found that treatment of trichomoniasis with MTZ did not influence the risk of preterm delivery or LBW, women randomised to the antibiotic arm were not more likely to deliver a preterm infant (20.9% versus 19.8%, *P* = 0.84) or to deliver an infant with a lower mean birth weight (2992 versus 2930 *P* = 0.27) (159). The treatment effectively resolved *T. vaginalis* infection.

1.3.6.3 Microscopy for the diagnosis of *trichomonas vaginalis*

Direct observation using microscopy of a wet mount preparation is the most used method to diagnose TV (160). The observation of the pear-shaped organism with the jerky or tumbling motility is specific for TV. However, the sensitivity of microscopy ranges from 44% to 68% even with experienced microscopists when compared to nucleic acid amplification tests (NAATs) (161). Time between specimen collection and microscopic examination, storage conditions and transportations conditions can also further reduce the sensitivity of the test (162).

1.3.6.4 Culture for the diagnosis of *trichomonas vaginalis*

Amplification of TV in liquid culture provides improved sensitivity over direct microscopic observation. Culture techniques include the use of Diamond's modified medium, and the InPouch TV test (Biomed Diagnostics, Oregon, USA) (163). Inoculation of the specimen on the culture medium, should be conducted immediately after collection or in less than 1 hour. Cultures are incubated at 37°C and examined microscopically each day for up to 5 days until motile trichomonads are observed. Compared with highly sensitive NAATs, the sensitivity of culture ranges from 44% to 75% for detection of TV (162).

1.3.6.5 Point of care tests of *trichomonas vaginalis*

These are immunochromatographic strip tests that use specific antibodies to detect trichomonas protein antigens (164). When present, TV antigens bind the antibodies resulting in the formation of a blue line on the test strip. Commercially available antigen detection tests include the OSOM Trichomonas Rapid Test (Sekisui Diagnostics, California, USA) (165) and the TV latex agglutination test (Kalon Biological, Surrey, UK) (166). The RDTs allow for an increased period between the collection of specimens and testing and more flexible sample storage temperatures and requires no instrumentation, and results are available in 30 minutes or less. The sensitivities of the RDTs are like culture and are higher than wet mount microscopy. They range from 40% to 95% depending on the specific test and the reference standard (162).

1.3.6.6 Nucleic acid amplification tests (NAATs) for the diagnosis of *trichomonas vaginalis*

The sensitivity of NAATs has important advantages for diagnosing TV and ranges from 76% to 100% (167). Specimen storage, processing and transportation allow for a wider range of temperatures and time intervals between specimen collection and testing. Thus, NAATs can facilitate use in different settings both clinical and non-clinical. NAATs has the advantage that testing for other STIs can be conducted using one vaginal specimen (141). The failure to preserve viable organisms in specimens processed for NAATs limits the potential for antimicrobial susceptibility testing for TV and other pathogens for which the molecular basis of resistance is not known. Thus, although NAATs may eventually replace sensitive tests for diagnosis of TV infection. Culture will continue to be important in cases of persistent infection

or suspected treatment failure for which antimicrobial susceptibility testing of clinical isolates may be warranted (162, 168).

1.3.7 Bacterial vaginosis in pregnancy

BV is the most prevalent infection of the genital tract among the reproductive age group (169, 170). BV is associated with adverse pregnancy outcomes including preterm birth, premature rupture of membranes, and LBW (171) . BV does not have a single causative organism but is believed to result from the disturbance of *Lactobacillus* species (172, 173). As a unique clinical syndrome, it presents as a recurrent or chronic foul-smelling vaginal discharge due to changes in the normal vaginal flora (172, 174). Often, infection with BV clears spontaneously without treatment. Both women of reproductive age and post-menopausal women can be affected though it is less common in the later (175). In normal conditions, the vaginal flora consists of 95% lactobacilli which produce several by-products including hydrogen peroxide and lactic acid that are less likely to be associated with BV. Bacterial vaginosis is associated with overgrowth of anaerobic bacteria such as *Gardnerella vaginalis*, *Ureaplasma urealyticum, Mycoplasma hominis, Mobiluncus* species, *Prevotella* species, and other anaerobes (176-178). Several risk factors for acquiring the infection include early sexual debut, vaginal douching, intrauterine devices, black race, cigarette smoking, and new or multiple sexual partners (170).

1.3.7.1 Effect of bacterial vaginosis in pregnancy

Overall, asymptomatic pregnant women with BV have a greater than twice the risk of preterm delivery compared with those without BV. This risk is increased to more than 5 times when BV is diagnosed before 20 weeks of gestation and worse when BV is diagnosed before 16 weeks of gestation (> 7 times) (179) . In a meta-analysis of 18 studies with 20,232 participants, by Leitich H *et al,* it was found that BV is an important risk factor for preterm delivery and spontaneous abortion. BV was found to increase the risk of preterm delivery more than 2-fold (odds ratio, 2.19; 95% CI, 1.54-3.12). In pregnant women who were screened for BV at less than 16 weeks and less than 20 weeks of gestation, the risks were higher (odds ratio, 7.55; 95% CI, 1.80-31.65) and (odds ratio, 4.20; 95% CI, 2.11-8.39) respectively. BV also significantly increased the risk of spontaneous abortion (odds ratio, 9.91; 95% CI, 1.99-49.34) (177).

1.3.7.2 Interventions to reduce the burden of bacterial vaginosis in pregnancy

Bacterial vaginosis can be treated using metronidazole or clindamycin both of which can be administered orally or intravaginally and have similar efficacy. Treatment for BV is mainly advised for symptomatic cases, though literature suggests that treatment of asymptomatic BV may reduce the risk of associated complications (33, 172). The diagnosis of BV is most made by using the Nugent scoring. However, treatment trials showed mixed results in preventing preterm delivery (179). Treatment of BV with clindamycin in the treatment phase of a controlled drug trial in the US was associated with reduced PTD (RR 0.5; 95% CI, 0.3-0.8) and PROM (RR 0.5; 95% CI, 0.2-1.4) (180). However, treatment trials showed mixed results in preventing PTD. Findings in another study in the US showed that women with clinically diagnosed BV, and were treated using metronidazole and erythromycin, had a lower incidence of PD (31% with treatment versus 49% with placebo, $P = 0.006$) (156).

Brocklehurst *et al*. 2013 reviewed the impact of treating BV in pregnancy with antibiotics. Selection was based on randomised trials comparing antibiotic treatment with placebo or no treatment or comparing two or more antibiotic regimens in pregnant women with BV and found that antibiotic treatment could eradicate BV in pregnancy, but the overall risk of preterm delivery was not significantly reduced. This review included 21 good quality trials, involving 7,847 women diagnosed with BV or intermediate vaginal flora. Antibiotic therapy was shown to be effective at eradicating BV during pregnancy (average risk ratio (RR) 0.42; 95% CI 0.31-0.56; 10 trials, 4403 women). It also reduced the risk of late miscarriage (RR 0.20; 95% CI 0.05-0.76; two trials, 1270 women). Treatment did not reduce the risk of preterm birth before 37 weeks (average RR 0.88; 95% CI 0.71-1.09; 13 trials, 6491 women), or the risk of preterm prelabour rupture of membranes (RR 0.74; 95% CI 0.30 to 1.84; two trials, 493 women) (181).

Another study in the US designed to evaluate the effect of early BV screening (at < 22 weeks gestation) and treatment followed by re-screening and re-treatment if necessary, found that treatment of BV (with oral metronidazole or intravaginal metronidazole or clindamycin) was significantly associated with a reduction in the risk of preterm delivery (OR 0.5; 95% CI, 0.3-0.8) (182). Similar results were found in another study in the United Kingdom where screening and treatment with intravaginal clindamycin were done early (13-20 weeks gestation)(152).

Treated women experienced a significant reduction in PD compared with the placebo group [4% versus 10%, ($P < 0.03$)] (183).

In a review of studies on screening, treatment, on adverse pregnancy outcome data in women with asymptomatic BV, no benefit was found in the treatment of asymptomatic BV (184).
In the review by Klebanoff *et al* published in 2023, to determine whether BV treatment during pregnancy reduced preterm delivery or prolonged time-to-delivery, asymptomatic pregnant individuals with BV were randomised to antibiotics or control. There were 96 studies with 23 eligible trials (11,979 participants) and 13 studies (6,915 participants) providing individual participant data. Odds ratios for preterm delivery for MTZ and clindamycin versus placebo were 1.00 (95% CI 0.84, 1.17), $I^2 = 62\%$, and 0.59 (95% CI 0.42, 0.82), $I^2 = 0$ before; and 0.95 (95% CI 0.81, 1.11), $I^2 = 59\%$, and 0.90 (95% CI: 0.72, 1.12), $I^2 = 0$, after imputation. Comparing the two treatments, time-to-delivery did not differ from null. There was no evidence that either drug was more effective when administered earlier, or among those with a history of PD. From this analysis, it appeared that treatment of BV during pregnancy did not reduce PTD (179).

1.3.7.3 Diagnosis of bacterial vaginosis by Nugent scoring

There are several methods by which BV may be diagnosed but the Nugent score is the gold standard (185, 186). The other commonly used method for the diagnosis of BV is the Amsel's criteria which is generally preferred in routine clinical care because Nugent scoring requires considerable time and expertise of a microscopist (185). Nugent scoring utilizes gram stain techniques to diagnose BV (187). Based on the presence of different bacterial types, the scoring of ≥ 7 indicates the presence of BV, 4-6 intermediate and 0-3 normal (172, 188). Nugent scoring involves three highly reproduceable bacterial morphotypes including *Lactobacillus* (large Gram-positive rods), *Gardnerella* and *Bacteroides* (small Gram-positive or Gram-variable rods), and *Mobiluncus* (curved Gram-negative or Gram-variable rods) (187). Nugent scoring needs a set of skill set to read slides and the scoring of morphotypes is also subjective and depends on individual skill (185, 187).

1.3.8 Effect of treatment of curable STIs/RTIs in pregnancy

Left untreated, STIs/RTIs in pregnancy can result in adverse birth outcomes for mother and child (189). The benefits of STIs/RTIs treatment on pregnancy outcome have been demonstrated in randomised trials. The study by Nyemba *et al* which is highlighted in the sections above, assessed the impact of diagnosing and treating curable STIs during pregnancy on adverse pregnancy and birth outcomes. Pregnant women were enrolled, tested, and treated for STIs. An evaluation of the association between any STI at the first ANC visit and composite adverse pregnancy outcome (miscarriage, stillbirth, preterm birth, early neonatal death, or LBW) was done. The study highlighted that treated STIs at the first ANC visit were not associated with adverse pregnancy outcome overall (150).

1.3.9 Antenatal care package for curable STIs/RTIs in pregnancy

It is recommended by the WHO, the Centre for Diseases Control (CDC) and by the Zambian ministry of Health (MOH) that screening and treatment of syphilis is offered to pregnant women in the routine ANC (190-192). However, screening for other STIs/RTIs is based on the syndromic management algorithm as recommended by the WHO (33). Despite the diagnosis of STIs/RTIs being accurately made using advanced diagnostic techniques, they are not widely available in resource-poor settings. In addition, the implementation of these superior techniques requires expertly trained laboratory staff and expensive laboratory equipment (34, 193).

1.3.10 Syndromic management of STIs/RTIs

The WHO recommends diagnostic and treatment algorithms for the management of STIs based on syndromic management guidelines for vaginal discharge (33). The syndromic approach was developed to support resource-limited countries, with limited laboratory capacities, using the symptom-based approach to identify and treat STIs/RTIs including NG, CT, TV, and BV (194). However, this approach does not go far enough to detect and combat STIs/RTIs because a large proportion of women with STIs/RTIs are asymptomatic despite having the infection which leads to these infections being missed by the syndromic approach. As an example, the microscopic examination test for BV using the Nugent scoring system, is notably lower in sensitivity. However, it has been shown to perform significantly better than syndromic approach (195, 196). Various reports have shown that a high proportion of women with NG and CT have asymptomatic infections ranging from 70 to 80% (197, 198) and as high as 50 to 70% for BV and TV (199, 200)

1.4 Shortcomings of current interventions

1.4.1 Shortcoming in the interventions for malaria in pregnancy

To decrease the adverse pregnancy outcomes associated with malaria infection in pregnancy, the WHO recommends the use of SP for intermittent preventive treatment in pregnancy (95, 201, 202). The use of IPTp-SP to reduce the risk of LBW, parasitaemia in the placenta and anaemia in pregnant women, is supported by evidence generated from various randomised controlled clinical trials (203-206). Ter Kuile *et al*., in their review, showed that the 2-dose IPTp-SP provided substantial benefit to HIV-negative semi-immune pregnant women (207). Even when one dose of IPTp-SP is given, it has been shown to be beneficial. It was shown to protect against maternal parasitaemia (OR 0.20, 95% CI, 0.12-0.34, $P < 0.001$) (208) and to protect against LBW (209). It has been shown that administration of 3 doses instead of 2 doses of SP reduces the prevalence of placental malaria, LBW, and PTB by 50% in a high malaria transmission area (31, 210, 211). In comparison to a 2-dose regimen, 3 doses of IPTp-SP were found to be superior in reducing the effect of malaria in pregnancy and appear to be effective even in areas with resistance to SP (31). Despite the increasing prevalence of SP-resistant *P. falciparum,* the use of IPTp-SP was not associated with higher parasite densities, greater placental inflammation, or worsened delivery outcomes in a study in Malawi (212). A recent systematic review and meta-analysis reports no evidence of paucigravidae being protected against delivering a LBW baby in areas where the parasite population of Arg-581 is greater than 10.1%. (213). Therefore, an evidence-based policy revision is urgently needed (214).

However, the protective effect of IPTp-SP has been compromised (215) and a decline in IPTp-SP efficacy has been reported in Africa (216). Widespread *P. falciparum* resistance to SP, particularly in East and Southern Africa, compromises the effectiveness of IPTp-SP (100, 217, 218). A large meta-analysis and systematic review from 57 studies from 17 African countries between 1994 and 2014 confirmed the relationship between quintuple mutants of *dhps* K540E and reduced efficacy of IPTP-SP. (100). Despite the high resistance, IPTp-SP still reduces LBW including in places where the prevalence of dhps Lys540Glu

exceeds 90% but where the sextuple-mutant parasite (harbouring the additional dhps Ala581Gly mutation) is uncommon (214, 217).

1.4.2 Shortcomings in the interventions for STIs/RTIs in pregnancy

Trichomonas vaginalis is likely the most prevalent nonviral STI (219). Bacterial vaginosis, in women of reproductive age, is the most common lower genital tract syndrome (220). Accurate detection of curable STIs/RTIs, including TV and BV, requires advanced diagnostic techniques as well as highly trained staff to handle the sophisticated test equipment (34, 221). Many countries in sub-Saharan Africa, however, do not have the capacity to provide these expensive diagnostics as part of routine ANC. As noted previously, to support these countries, WHO developed syndrome-based algorithms for the diagnosis and management of curable STIs/RTIs including TV and BV for symptomatic cases (222). For women, however, most STIs/RTIs are asymptomatic and commonly undetected (223). About half of women infected with TV do not have symptoms (146) but even higher proportions (60-70%) of asymptomatic TV and BV have been reported (199, 200).

An observational study of syndromic management in Nchelenge, Zambia, found that 700 women out of the 1084 participants had a minimum one STI/RTI (64.6%; 95% CI, 61.7, 67.4). However, treatment was provided to just 10.2% of women infected with STIs/RTIs (excluding syphilis). For TV and BV, 7.8% (prevalence 24.8%; 95% CI, 22.3, 27.5) and 7.5% (prevalence 48.7%; 95% CI, 45.2, 51.2) positives received treatment respectively. The odds of TV infection were highest among primigravidae (aOR = 2.40; 95% CI: 1.69, 3.40), and reduced with the following pregnancy. Women who were aged 20 to 29 years were more likely to be diagnosed with BV compared to women ≥30 (aOR = 1.58; 95% CI: 1.19, 2.10) (224).

Metronidazole is the drug of choice for the treatment of both TV and BV (225). There are some concerns about the trichomonas parasite sensitivity to MTZ (226). The efficacy of the 7-day dose of MTZ (500 mg twice daily for 7 days) was compared to the single-dose MTZ standard (2 g one-time dose) for the treatment of TV in a randomized, parallel, multi-

site, open-label, laboratory-blinded trial with a test of cure (TOC) at 4 weeks after completion of treatment measured using NAAT or culture. Women in the 7-day dose arm had significantly lower TOC positive rates than those in the single-dose arm [34/312 (10.9%) vs. 58/311 (18.6%), p=0·001] [R.R. 0.55 (95% C.I. 0.34–0.70)]. In intention-to-treat analysis by the status of BV, women who received 7 day-dose MTZ had lower TOC positive rates irrespective of BV status. In the presence of BV, women on the 7 day-dose treatment arm were less likely to have a TOC positive [RR 0·59, (0·43–0·80), p< 0·001](227, 228). In vitro antibiotic resistance to MTZ in TV remains low (4.3%) but should be monitored (219). It is recommended that symptomatic BV should be treated using oral MTZ 500 mg twice daily for 7 days. Other alternative treatments include vaginal MTZ gel and oral or vaginal clindamycin cream. Longer courses of therapy for BV are recommended for women with documented multiple recurrences (172, 229).

1.5 Co-infection and the need to address dual burden of malaria and curable STIs/RTIs

In the prospective pregnancy cohort study, which was conducted in Nchelenge district, Zambia, 1,086 women enrolled at ANC booking as described in the sections above. Women provided biological samples to test for peripheral malarial infection and curable STIs/RTIs, namely syphilis, chlamydia, gonorrhoea, TV, and BV. Of 1,071 participants for whom data were available, 38.7% (95% CI 35.7–41.6) were found to be infected with malaria and at least one STI/RTI. Malaria infection alone was found in 18.9% (95% CI 16.5–21.2). Those who had no malaria but were infected with at least one STI/RTI were 26.0% (95% CI 23.5–28.8) and 16.4% (95% CI = 14.1–18.6) of the participants were free from both malaria and STIs/RTIs (36). Knowing that more women were co-infected with malaria and at least one curable STI/RTI than malaria alone or a curable STI/RTI alone strongly suggests that combination therapy may be required to improve pregnancy outcomes. Not surprisingly, TV and BV were most implicated in these co-infections (36, 83).

1.6 Possible solutions with combination treatment

As noted earlier, MTZ can be administered as a single dose of 2g for TV or BV in pregnancy. SP and DP need to be combined with such an antibiotic to be effective against malaria and STI/RTI co-infection and this may result in better outcomes than SP given alone. Combination treatment may achieve additive or synergistic effects specific to the inhibition of malaria, TV, or BV with the provision of MTZ. There are old data that suggest MTZ may have some antimalarial properties (230, 231). Consequently, combining MTZ with either SP or DP may increase the antimalarial effects that would otherwise be observed with SP or DP alone. Similarly, SP has been shown to protect women who have TV and/or BV and no concurrent malaria infection from adverse pregnancy outcomes (202). In the cohort study conducted in Nchelenge district in Zambia between November 2013 and April 2014 described in the sections above, there was no significant differences in the prevalence of curable STIs/RTIs at enrollment in the different groups of exposure to IPTp-SP. These groups were classified as 0–1 dose vs ≥2 doses, and 2 doses vs ≥3 doses. When women who received ≥2 doses were compared to those who received 0–1 dose, the odds of adverse pregnancy outcomes were reduced by 45% (OR, 0.55; 95% CI, 0.36, 0.86) and a reduction 57% reduction with ≥3 doses (OR, 0.43; 95% CI, 0.27, 0.68). One of our most interesting findings was that women who received ≥2 doses and had neither malaria nor curable STIs/RTIs were more protected against any adverse pregnancy outcome, LBW, and preterm delivery compared to those who received 0–1 dose (202).

1.7 Rationale for further studies in Nchelenge

The pregnancy cohort study in the Nchelenge district previously described showed that about one-half, 48.3% (95% CI = 45.2–51.2), of women had BV and one-quarter, 24.8% (95% CI = 22.3–27.5) had TV. In addition, 37% (95% CI = 33.9–40.1%) had co-infection (malaria/BV, malaria/TV, malaria/TV/BV) (95% CI = 33.9–40.1%) (36). The study also showed that more than half, 57.8% (95% CI = 54.8–60.7) of the pregnant women had a malaria infection confirmed by PCR. For women who had blood slides paired for day 0 and day 28, the overall parasitological and prophylactic failure (failure to protect malaria infection) was 18.6% (95% CI 15.5, 21.8; 109 of 590)(232). The prevalence of SP resistance

markers among malaria parasites was also very high (36, 84): 92.7% (95% CI 85-96.5) showed *dhfr* triple mutation, 70.8% (95% CI 60.8-79.2) had *dhps* double mutation, 68.8% (95% CI 58.6- 77.3) carried the *dhfr* and *dhps* quintuple mutation, and 9.4% (95% CI 4.2-16.0) had the *dhfr* and *dhps* sextuple mutation (232). Due to this high burden of co-infection, coupled with the shortcomings of current interventions, a new integrated approach in the ANC package is needed to reduce the burden of malaria, TV, and BV.

To examine the potential for combination therapies that will reduce this dual-burden, the ASPIRE trial was conducted in Nchelenge district among 5,436 HIV-negative pregnant women to determine whether the combination of MTZ with IPTp of malaria in pregnancy using IPTp-SP or IPTp-DP is superior to IPTp-SP alone in reducing adverse pregnancy outcomes. Nchelenge district is in north-west Zambia where malaria transmission is high, malaria parasite-resistance to SP is high, and the prevalence of BV and TV in pregnant women is also high. I was the Zambian Principal Investigator and used data collected for the ASPIRE trial to form my thesis.

1.8 Objectives

There are four objectives of this thesis:

Objective 1: *IPTp protective efficacy*

To determine whether intermittent preventive treatment with sulfadoxine-pyrimethamine plus metronidazole or Intermittent preventive treatment with dihydroartemisinin-piperaquine plus metronidazole is superior to intermittent preventive treatment with sulfadoxine-pyrimethamine alone in protecting against adverse pregnancy outcomes among pregnant women in Nchelenge district.

Objective 2: *Sulfadoxine-pyrimethamine treatment efficacy*

To determine the day 28 treatment efficacy against *Plasmodium falciparum* infection among pregnant women in Nchelenge district who receive either intermittent preventive treatment with sulfadoxine-pyrimethamine alone, intermittent preventive treatment with sulfadoxine-pyrimethamine plus metronidazole, or intermittent preventive treatment with dihydroartemisinin-piperaquine plus metronidazole

Objective 3: *Prevalence of 540 and 581 resistance markers*

To determine the prevalence of dhps 540 and 581 markers of resistance responsible for sulfadoxine resistance in *P. falciparum* found among pregnant women in Nchelenge district who receive either intermittent preventive treatment with sulfadoxine-pyrimethamine alone, intermittent preventive treatment with sulfadoxine-pyrimethamine plus metronidazole, or intermittent preventive treatment with dihydroartemisinin-piperaquine plus metronidazole

Objective 4: *Prevalence of curable STIs/RTIs at visit 1 and visit 2 of antenatal care*

To compare the prevalence of STIs/RTIs at the first and second antenatal care visits among pregnant women in Nchelenge district who received either sulfadoxine-pyrimethamine alone, sulfadoxine-pyrimethamine plus metronidazole or dihydroartemisinin-piperaquine plus metronidazole

2 Methods

2.1 Chapter introduction

This chapter describes all the methods that were used in the study including design, sample size calculation, inclusion and exclusion criteria, enrolment procedures, data and sample collection, laboratory procedures and analyses. It details the specific laboratory procedures which were conducted to produce the results.

2.2 Study design and sample size

2.2.1 Study design

We conducted a phase three, partially placebo-controlled individually randomised superiority trial of three different IPTp regimens. Group 1 received IPTp-SP (standard of care) plus MTZ placebo, group 2 received IPTp-SP plus MTZ, and group 3 received IPTp-DP plus MTZ for the reduction of adverse pregnancy outcomes. SP was given as 3 tablets each containing 500mg sulfadoxine and 25mg pyrimethamine (day 0). The dose for MTZ was 2g (given as 4 tablets each containing 500mg) as directly observed therapy at day 0. DP was given 3 tablets of 40mg of dihydroartemisinin and 320mg of piperaquine (Days 0, 1, 2).

To be included in the trial, pregnant women had to meet the following inclusion and exclusion criteria.

i) HIV-negative
ii) between 16-28 gestational weeks (16 weeks 0 days to 28 weeks 0 days measured by sonography)
iii) carrying a single viable pregnancy
iv) resident in the study area
v) express willingness to adhere to scheduled and unscheduled study visit and procedures
vi) willing to deliver at a trial facility

Women were excluded if they had:

i) a known cardiac ailment,
ii) severe malformations or nonviable pregnancy observed by ultrasound,
iii) history of receiving IPTp-SP during the current pregnancy,
iv) known allergy or contraindication to any of the study drugs,
v) been unable to give consent,
vi) concurrently participating in any other trial, including prior enrolment in this trial.

2.2.2 Sample size

The sample size calculation was based on data from several sources. The national incidence of LBW in Zambia was 13% among mothers aged less than 20 years and 8-9% among mothers aged 20-49 years (233). A multicentre trial in sub-Saharan Africa, conducted from October 2010 to November 2013, reported the incidence of adverse pregnancy outcomes among women who received IPTp-SP to be 23.7% (95% CI: 21.5, 25.9) (234). In the pregnancy cohort study conducted between 2013-14 in Nchelenge, Zambia, as previously described, 35% (251/717) of pregnant women who received IPTp-SP still went on to have adverse pregnancy outcomes. This study found that 48.3% of pregnant women had BV, and 24.8% had TV; 29.2% were co-diagnosed with malaria and BV, whereas 15.2% had malaria and TV co-infection (36). These estimates align with results from a meta-analysis of pregnant women attending ANC in East and Southern Africa where 50.1% (95% CI: 43.3, 58.4%) had BV and 29.1% (95% CI: 20.9, 37.2) had TV (35). Thus, we made a conservative assumption that 15% of women would experience an adverse pregnancy outcome in Group 1 (IPTp-SP) under an intention-to-treat analysis. Thus, it was reasonable to expect that one-half of women in Group 2 and Group 3 would have their risk of preterm birth reduced by 50% relative to Group 1, and this will result in an overall 25% reduction in adverse pregnancy outcomes (preterm delivery, still births, low birth weight and neonatal deaths). To detect a 25% relative reduction in adverse pregnancy outcomes, from 15% in the IPTp-SP arm to 11.25%, in either of the two intervention arms with 80% power and a significance level of 2.5% to allow for the

comparison of both intervention arms with control, the trial needed a sample size of 4,620. We conservatively assumed 15% loss to follow-up and therefore recruited 5,436 pregnant women to the trial. The recruitment of the participants for the trial began on 17th December 2019 and ended on 18th March 2022. The last participant last visit, which was the day 28 post-delivery, was held on 21st October 2022.

2.3 Study site

Nchelenge district is a rural district in the northwest of Zambia, in Luapula Province, in the marshlands of the Luapula River and bordering Lake Mweru, sharing an international border with the Democratic Republic of Congo (figure 2). It covers an area of 4,090 km2 and the district is at an altitude of approximately 800 meters above sea level. The habitat is characterized as marsh, with a single rainy season from November through April, followed by a dry season from May to October. The local geography consists of miombo woodland and wetlands fed by the Luapula River and its tributaries. Residents reside mainly in sun-dried brick and thatch houses and subsist on farming and fishing. The district is heterogeneous, with a densely populated urban area along the lake and farmland located further inland. However, the population is mobile, traveling between the lake for the fishing season and inland for farming, with a fishing ban in effect from 1st of December to the end of February. It has a population of about 233,696 people according to latest census estimates of 2022 (not officially published).

Despite the rollout of malaria control interventions and seasonality of rains, Nchelenge district experiences intense perennial transmission of malaria (235, 236). The main malaria vector is pyrethroid-resistant *An. funestus* that peaks during the dry season (237, 238). *Anopheles gambiae* is also present but in lower numbers, mainly along the lakeside (237-239). Nchelenge has among the highest parasite prevalence ranging between 30-50% and high malaria case fatality rates countrywide, and perhaps globally. The incidence rate for malaria in the general population was 471.4 in 2022 and 502.6 in 2023. The incidence is higher in under 5 children at 813.9 in 2022 and 772.7 in 2023. This is despite once having achieved near-universal Insecticide-treated Nets (ITN) coverage in 2006 during

the implementation of the first National Malaria Control Strategic Plan, and conducting annual targeted indoor residual spray (IRS) campaigns since 2008 (240) . The indoor residual spraying is conducted just before the onset of rains from October to November of each year. Nchelenge District has 11 health centres, three health posts and one first level hospital (241).

The study was conducted in four health centres, Kabuta, Kafutuma, Kashikishi and Nchelenge. The community in the catchment area of the 4 health centres, which served as recruitment sites, was selected due to the close location of the 4 health centres to St. Paul's Mission Hospital, the only hospital in the district. According to the 2018 Zambia Demographic and Health Survey, 91% of women aged between 15 and 49 in Luapula Province reported having received ANC from a skilled health care provider during pregnancy for their most recent birth in the preceding five-year period. Health facility deliveries were at 88% in Luapula province (192). In the Malaria indicator survey of 2021, the coverage of IPTp-SP was reported that 93.8% of pregnant women received at least one dose, 86.1% received 2 doses and 72.9% received three doses or more (242). The high IPTp uptake is due to the increased access to health services and reducing the demographic and socioeconomic disparities by the national malaria control program. All the four health centres offered ANC, laboratory services and maternity services.

In 2022, Nchelenge recorded a total of 8,637 deliveries of which 7,997 were normal deliveries and 491 were by caesarean section. There were 8,349 live births of which 723 (8.7%) were LBW babies and 136 were still births. Preterm deliveries were not recorded as they are not part of the routine data capture system (243). Figure 2 shows the location of Nchelenge district in Zambia.

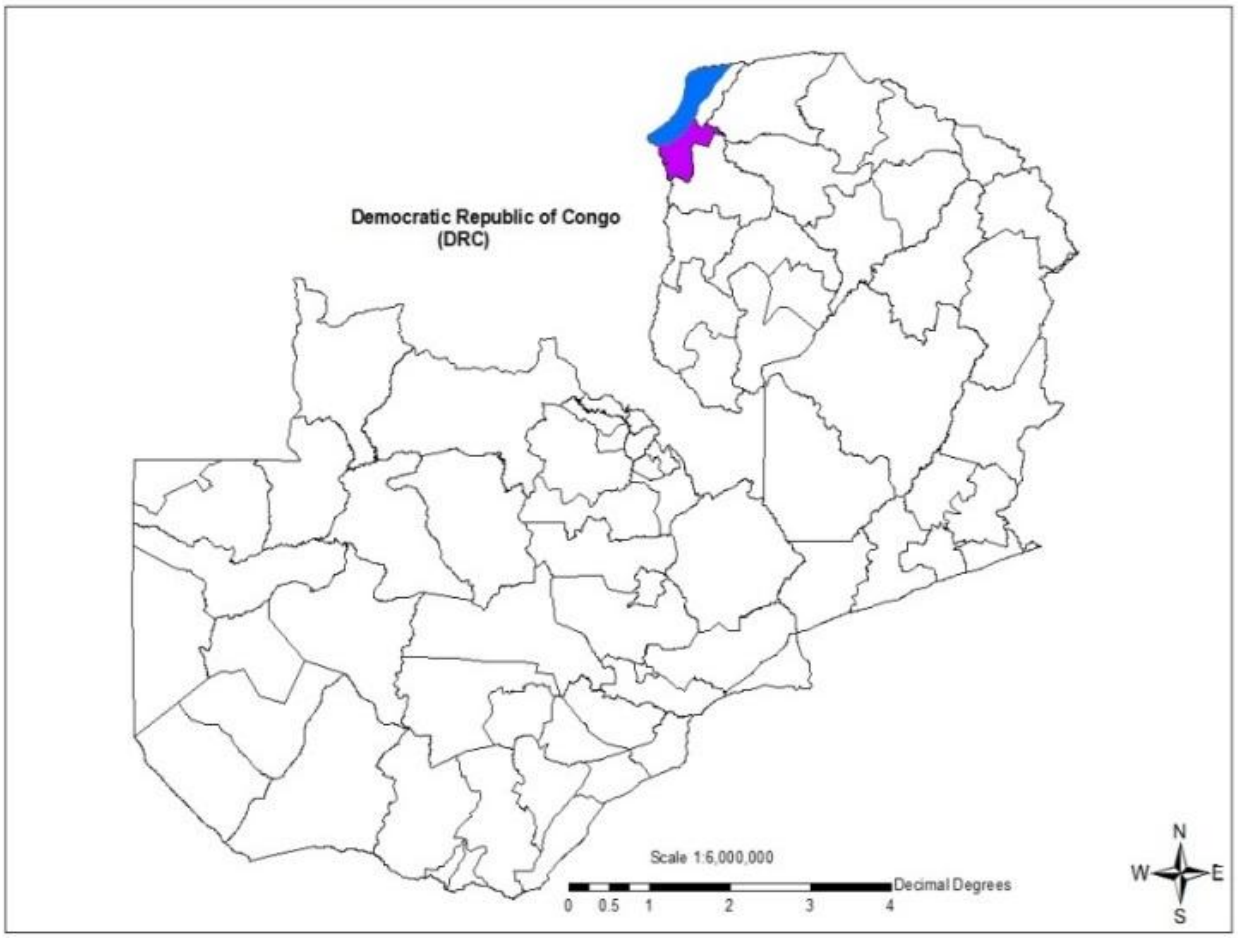


Figure 2. Map showing the position of Nchelenge district in Zambia (Map courtesy of Ministry of Lands, Lusaka)

Key: Blue area indicates the area within the borders of Zambia covered by Lake Mweru. Purple area indicates the location of Nchelenge District.

2.4 Study Procedures

2. 4.1 Recruitment Strategy

Messages about the trial were broadcasted on a local radio station after approval from the TDRC ethics committee. The study also used the Community Advisory Board (CAB) established in Nchelenge by the TDRC. The CAB comprised of village leaders and community health workers and provided a village-level interactive forum for study participants and community members to learn more about the trial. It also spearheaded community mobilization activities by holding meetings with the community and disseminating messages through the community radio station.

2.4.2 Consenting, enrollment, and follow-up

Written consent was obtained from the pregnant women by the nursing staff using the local language (Bemba). The consenting process took place at the health facility and was administered individually to each participant before any screening procedures were conducted. The participants were given time to understand the study procedures and to ask questions bore signing the informed consent form. Details of the informed consent are provided in appendix 9.2. After informed consent had been signed, participants were randomly allocated to receive either IPTp-SP plus MTZ placebo, IPTp-SP plus MTZ, or IPTp-DP plus MTZ and issued with their unique study identification number. A sample of the case report form for screening and enrolment is provided in appendix 9.10. Baseline information was recorded for each participant including age, area of residence, maternal height and weight, use of insecticide-treated bed-net, number of previous known pregnancies, number of previous births and corresponding pregnancy outcomes reported and estimated date of last menstrual period. Relevant information already collected during the screening process or at previous antenatal appointments for the same pregnancy was documented. At each subsequent visit, a clinical assessment was conducted and maternal weight, and fundal height were recorded. The last menstrual period was recorded and ultrasound was performed on the enrolment day to assess the gestational age of the foetus.

All participants were asked about any symptoms or illnesses they had in the previous month, and any medication taken. Cases were recorded in the adverse events section of the case report form. After administration of the study drugs, all adverse events were recorded and also evaluated for seriousness and reported accordingly. Subsequent clinic visits were scheduled monthly after the first visit. IPTp was administered to asymptomatic women at scheduled visits. Dried blood spots were collected on the enrolment day and on every other visit throughout the trial for retrospective PCR analysis. Rapid diagnostic testing (RDT) was performed on symptomatic women during the trial and women who had malaria were treated with Artemether-lumefantrine. The next dose of IPTp was administered after 4 weeks. Routine antenatal care and treatment of any illness identified at each visit were provided. Participants were followed to delivery and placental blood and tissue were collected by trained nurses and midwives. The nurses at all four health centres in Nchelenge and Saint Paul's Mission Hospital were informed about the study and trained on how to identify study participants and collect delivery data. Identification of the participants on delivery day was done by using the study identity cards that study participants had been given.

2.4.3 Randomization and masking

Pregnant women were allocated to treatment groups randomly in a 1:1:1 ratio between groups with a computer-generated random allocation sequence. All laboratory staff were masked to the treatment assignment of individual women. The trial statistician was also masked regarding the treatment code while developing the statistical analysis plan and writing the statistical programmes. The actual allocation was only provided to the study team after locking of the database and approval of the statistical analysis plan by the independent Data Monitoring and Ethics Committee (DMEC) before they review any trial results. The study statistician conducting the interim analysis also remained blinded throughout the analysis.

After consenting and determining eligibility, the next sequential pre-pack study medication envelop with a QR code on the front was scanned using a computer tablet.

The QR code reader automatically assigned the patient ID following the random allocation sequence. We obtained SP and MTZ from Baxy Pharmaceuticals, a Zambian manufacturer based in Ndola Zambia. Baxy also produced the MTZ placebo for the trial as well. We purchased DP directly from the WHO-prequalified manufacturer, Alfasigma SpA.

2.4.4 Study drug administration

After written informed consent had been provided, women were randomly allocated to receive one of the three IPTp regimens at enrolment and at each subsequent monthly ANC visit during the second and third trimesters. The groups were as follows:

- Group 1: IPTp-SP plus MTZ placebo (control).
- Group 2: IPTp-SP plus MTZ
- Group 3: IPTp-DP plus MTZ.

MTZ placebo and MTZ were administered at visit 1 and visit 2 only.

A pharmacologist at the London School of Hygiene & Tropical Medicine conducted content and solubility analysis of the SP and MTZ using high-pressure liquid chromatography at the beginning and mid-point of the trial. Both SP and MTZ tablets satisfied the pharmacopeia tolerance limits for content and dissolution and were of acceptable quality. The drug product characteristics for SP, MTZ and DP are presented in appendix 9.3. The first dose of each course of IPTp was given under supervision of study staff on day 0. Thus, all dosing for women in groups 1 and 2 were entirely directly observed; dosing for women in group 3 was observed on day 0 and, thereafter, women self-administered doses on the next two consecutive days at home. A sub-sample of 300 women allocated to receive DP were visited at their homes unannounced on day 3 to assess adherence to the regimen on day 1 and day 2. The adherence was checked by asking the woman to show the pack of DP and seeing if it contained any remaining tablets. This was checked on the third day of taking the medication.

2.4.5 Assessment on the newborn

Newborns were weighed within 24 hours of delivery using digital scales (± 2 grams) and LBW defined as < 2,500g. The weighing scales used were the CONCORD supplied by Jan & Bros LLC of Dubai United Arab Emirates. Gestational age was assessed using ultrasound dating at enrolment and preterm defined as <37 weeks' gestation. Foetal loss was assessed monthly at scheduled ANC visits. If women did not return, they were contacted by mobile phone and/or visited at home to document this. Intrauterine fetal death was defined as a fetal death at $\leq$ 28 weeks gestation, stillbirth as fetal death at >28 weeks gestation, and preterm birth as delivery at <37 weeks.

The presence of congenital abnormalities and jaundice were assessed at delivery and 28 days later. The vital status of participants was recorded at each scheduled and unscheduled visit, and through home visits if women did not attend the scheduled visit.

2.5 Laboratory procedures

2.5.1 Overview of laboratory sample collections

Blood spots were collected at each visit for retrospective PCR analysis for detection of *Plasmodium falciparum.* Placental tissue and cord blood were collected at delivery for analysis postpartum. If a woman complained of vaginal discharge at any time during the trial, clinical staff collected a cervical and vaginal swab, and provided treatment as per syndromic management guidelines. One vaginal swab was used as part of retrospective batch analysis of BV by Nugent scoring and TV by PCR methods. A sub-group of women provided a second vaginal swab for future analyses of the vaginal microbiome and maternal cytokine profile. Cervical swabs were collected from women who experienced vaginal discharge; these samples were used for future testing of microbial sensitivity to sulfadoxine, pyrimethamine, their combination, and other antimicrobial agents. Haemoglobin levels were tested at each visit in all women. Women not previously tested for syphilis in this pregnancy were tested and treated if positive. The HIV testing was conducted in the routine antenatal clinic and the results were recorded on the antenatal

card. Only HIV negative women were eligible to be enrolled into the trial. This was because HIV positive women could be taking cotrimoxazole which is part of the HIV care package. HIV testing was not repeated during the trial. Stool samples were collected from a smaller group (n=60 total) at visit 1 and visit 2 (pre-dosing). Samples were stored for characterise the changes in the gut microbiome.

2.5.2 Detection of *Plasmodium falciparum*

Apart from collecting DBS samples at each ANC and delivery visits, samples were also collected for retrospective PCR, microscopy, and placental histological analyses at delivery. Most of the laboratory analyses were conducted at TDRC laboratories in Ndola, Zambia. DNA was isolated from DBS using the chelex method (244). The detection of *P. falciparum* was a real-time (RT) PCR method using the QuantiNova SYBR green PCR kit. In brief, the reactions were carried out in a total volume of 20µl composed of 10ul of 1x SYBR green master mix, 0.1µl, QN Rox reference dye. A pair of forward and reverse *P. falciparum* specific complimentary primers to a final concentration of 0.7 µM were added. A 2 µl template genomic DNA and an appropriate amount of nuclease free water was added.

The reaction was conducted on the Applied Biosystems® 500 fast real time thermocycler platform using following procedures: an initial heat activation stage of 95^0C for 2 minutes, followed by 40 cycles of denaturation at 95^0C for 15 seconds, an annealing/ extension at 60^0C for 30 seconds. This was superseded by a melting curve stage composed of 4 sub-stages of 95^0C for 15 seconds, 60^0C a minute, another 95^0C for 30 seconds and a final data collection stage of 60^0C for 15 seconds. Appendix 9.4 shows the SOPs for malaria detection.

2.5.3 Detection of 540 and 581 resistance markers

At two time points in the trial, a sub-group of samples that were PCR-positive for *P. falciparum malaria* at enrolment were analysed to measure the frequency of 540 and 581 markers of resistance for SP. In the first instance, 200 samples from the first *P. falciparum*

PCR-positive women and collected between 17th December 2019 and 9th November 2021 were analysed at TDRC laboratories. The second set of samples consisted of 176 *P. falciparum* PCR-positive samples collected between 11th February 2020 and 14th October 2021 that were analysed at the University of Copenhagen. Both sets of samples were selected based on their strong positivity as indicated by critical threshold (CT) values ranging from 24 to 29 cycles. The codons for *dhfr* were 16, 51, 59, and 108, whereas from the *dhps* gene codons 431, 436, 437, 540, 581 and 613 were analyzed.

For the analysis at TDRC, the following methods were applied. Antifolate resistance polymorphisms in *dhfr* and *dhps* genes were detected by nested PCR and allele-specific restriction enzyme digestion as described in (245-247). Briefly, reaction volumes of 25 µl containing the following were used: 2µl of sample DNA, 0.25µM of primer, 200 µM dNTPs, and 1.5 mM $MgCl_2$/1U of Taq polymerase. Reaction conditions of a 40-cycle PCR programme were as follows: initial denaturation at 94˚C for 2 min; 94˚C for 45 seconds, annealing at 43.4˚C for 45 seconds and extension at 65˚C for 1 minute, and final extension at 65˚C for 2 minutes. Restriction digestion was performed following manufacturer instructions. 4 µl of PCR product was used in the reaction mix, however, for samples showing faint bands (low concentration of the amplicon) the amount of nested PCR product was adjusted to 6-8 µl. Restriction fragments and PCR amplicons were resolved on an ethidium bromide-stained 1.5% agarose gel and visualised under ultra-violet light. Names and sequences of the primers that were used are shown in Appendix 9.5.

The second set of samples, which were first sent to the London School of Hygiene & Tropical Medicine for DNA extraction and qPCR. The samples were extracted by the Qiagen Qiasymphony methodology. The extracted DNA was analysed by a Cytb qPCR protocol (248) to test for *p. falciparum* DNA. All samples were then sent to the University of Copenhagen for the following analysis of antimalarial drug resistance markers. In brief, a series of nested PCR fragments was performed as described by Nag *et al.* (248) and modified to be run in simplex. Non-annealing overhangs were incorporated during the nested PCR, to which specific 8-base pair sequences were attached in the following index

PCR, allowing each sample to be dual-indexed with unique barcodes. The samples were then bead purified and diluted to a final concentration of 4 nM, before being spiked with 5% PhiX library (Illumina, California, United States) and prepared for sequencing.

Quality control (MultiQC (249) and trimming with a phred score of 20 Trimmomatic (250)) were performed using tools at (http://usegalaxy.org.au) before the R1 and R2 files were combined into one fastq file per sample.

The fastq files were aligned to a 3D7 reference sequence using the Python-based program *Assimpler*. For the analysis of SNPs, a cut-off of 50 reads was used to acknowledge the presence of a nucleotide in each position. If more than two nucleotides were present in one position with more than 25% of the total reads, the sample was considered a mixed infection.

2.5.4 Diagnosis of syphilis

Diagnosis of syphilis was done among all women at enrolment using syphilis point of care test, one step syphilis anti-TP test (SD Bioline Syphilis 3.0, SD Diagnostics Inc. Republic of Korea) at the trial site and women who were reactive were offered treatment with benzathine penicillin. Similarly, rapid plasma reagin (RPR) (Omega Diagnostics Limited, Alva, Scotland, UK) was also conducted at the St. Paul's Mission Hospital laboratory on samples from all women. Mothers who were RPR reactive were offered treatment with injectable benzathine penicillin (2.4 million units IM weekly x 3 doses) according to the national STI treatment policy. Plasma aliquots of RPR positive samples were made and stored at -80℃ pending transportation to National STI Reference Laboratory at TDRC. Transportation of RPR seropositive samples was done using a dry shipper (temperature of about -190℃). Testing of RPR seropositive samples at National Reference laboratory was done with *Treponema pallidum* haemagglutination assay (TPHA) (Atlas Medical, Blankenfelde-Mahlow, Germany) for confirmation of syphilis and RPR positivity. Appendix 9.6 shows the SOP for TPHA testing (251-254).

2.5.5 Diagnosis of chlamydia, gonorrhoea, and trichomoniasis by molecular methods

The extraction of DNA from cervico-vaginal swabs was conducted within 24 hours of collection and the extracts from each sample were stored at -20^{0}C pending molecular detection by PCR. The collected specimens were transported to the TDRC laboratories for the molecular detection of *Chlamydia trachomatis*, *Neisseria gonorrhoea* and *Trichomonas vaginalis*. DNA was extracted from swabs that had been collected from study participants using the Zymo research Quick-DNA ^{TM} Miniprep Kit Cat D3025. In brief, 100 µl of each patient sample was mixed with 400 µl genomic buffer, separated in a Zymo spin^{TM} column at 10,000g for a minute. The extract was then washed with 200ul DNA Pre-wash buffer before the and 500ul of g-DNA wash buffer. DNA was eluted in 50 µl elution buffer, of which 5 µl was used for the 25 µl PCR reactions.

In brief, 100 µl of each patient sample was mixed with 400 µl genomic buffer, separated in a Zymo spin^{TM} column at 10,000g for a minute. The extract was then washed with 200ul DNA Pre-wash buffer before the 500ul of g-DNA wash buffer. DNA was eluted in 50 µl elution buffer, of which 5 µl was used for the 25 µl PCR reactions. Realtime PCR was conducted using internal control and primer sequences were supplied by Inqaba Biotechnical Industries (Pty) Ltd (Pretoria South Africa), as published by Tayoun AN *et al*.(255) For patient samples, we prepared 25 µl PCR reactions consisting of ^{TM} SYBR Green master mix (Thermo Fisher Scientific, V.A Graiciuno Vihius Lithuania) 1 × Sybr green, 10 µl extracted DNA 5 µl, 100 nM forward and re-verse CT primers, 175 nM forward and reverse NG primers, 125 nM forward and reverse TV primers and 150 nM IC2 M forward and reverse primers. PCR amplification was performed on the Applied Biosystems ® (Abs 7500 fast) using a touchdown PCR protocol with the following conditions: a 5 minute hot start (95 °C) followed by 2 cycles of 5 second denaturation (95 °C) and 30 second annealing/extension at 70 °C, 2 cycles of 5 at 95 °C and 30 s at 67 °C, 2 cycles of 5 s at 95 °C and 30 s at 65 °C, 2 cycles of 5 s at 95 °C and 30 s at 63 °C, and finally 32 cycles of 5 s at 95 °C and 30 s at 60 °C. A melt protocol was also included from 60–95 °C at 0.2 °C/s. All the PCR parameters employed to detect STIs in the trial are indicated in Appendix 9.8.

2.5.6 Diagnosis of bacterial vaginosis

The vaginal smear samples for the diagnosis of BV were air dried and Gram stained using crystal violet as the primary stain and safranin as a counter stain. Gram staining of slides was conducted at the St. Paul's Mission Hospital laboratory where first reading was conducted. Thereafter, slides were transported to TDRC laboratory for second reading. BV diagnosis was based on the Nugent criteria (7-10) (256) and recorded in the appropriate result record form. For quality control, slides underwent repeat reading by a second observer. Any disagreements were resolved by a third observer. The SOP for Nugent criteria is shown in appendix 9.7.

2.5.7 Quality control of molecular STIs analysis

Five vaginal swabs from five different symptomatic patients for NG were cultured by Thayer-Martin agar and the NG organisms isolated. DNA was extracted from these samples. They were then serially diluted and then run in duplicate on using the method by Tayoun *et al.* (255). From the five samples, three were positive. These were then used as positive controls for NG. Similarly, five samples were collected from patients with symptoms for TV. The samples were cultured and isolated in Inpouch for culturing TV. They were also serially diluted and then run in duplicate on using the above method (255). Two samples were positive from the five samples. The positive samples were then used as positive controls for TV. As for CT, positive isolations were obtained from another reference laboratory. These samples underwent validation.

On each 96 well plate, there were two negative controls (1. Extraction negative control and 2. No template control). The plate also included three positive controls (1. CT, 2. NG and 3. TV). The plate also had a maximum of 91 study samples. A run was considered successful if there was no amplification and melting in both negative controls and if all the three positive controls successfully amplified and melted at specific

temperatures. Furthermore, each plate was run by two people and results verified by the lab supervisor.

2.5.8 Diagnosis of placental malaria by histology

Placental histological diagnoses were conducted at the University Teaching Hospital in Lusaka Zambia by a consultant pathologist. Quality assurance testing was conducted in a blinded manner with the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia, United States of America. Of the first 1500 histopathology analysis slides that were read, 128 (8.5%) were randomly selected based on the various subgroups of slides as follows: slides with malaria parasites 50 slides (approximately 50% with formalin pigment and 50% without formalin pigment); past infections, 25 with formalin pigment and 25 without and uninfected, 25 with formalin pigment and 25 without pigment. In total, 127 slides were shipped. The grading performance scale of parasite detection method contained in the WHO Quality Assurance Manual, Version 2 on malaria microscopy (257) was adapted to histology reading for interpretation of results (258, 259). The grading performance has the following categories of agreement ≥ 95%, excellent, 85 ≤ 95%, very good, 75 ≤ 85%, good and ≤ 75%, poor. There was 94.5% (120/127) agreement in the case of diagnosis of active infection (presence of parasites) and 79.4% (101/127) agreement in the case of past infections (pigment in fibrin) between the two readers. Details of the placental histology procedures are described in appendix 9.9.

2.5.9 Sample collection at delivery

Instructions on sample collection were provided to the maternity ward staff and training conducted. Maternal and cord blood haemoglobin were measured using 301 Haemocue analyzer. The cord blood haemoglobin was measured from cord blood collected in 4ml EDTA tube. The cord blood was collected after delivery of the new-born and before delivery of the placenta. The end of the cord was wiped clean with gauze and cord blood was collected using a syringe from the umbilical vein into an EDTA vacutainer tube. The blood was gently mixed with anticoagulant by inverting the tube about 5 times. Haemoglobin was measured by placing a tip of a cuvette on a tilted EDTA tube to access

the blood. Maternal blood estimation was done from a finger-prick or venous blood collected in the 4 ml EDTA tube. A 301 haemocue cuvette tip was placed in the middle of the drop or EDTA blood and allow the blood to be drawn into the cuvette by capillary action.

Placental tissue samples were collected as follows: A 2cm x 2cm x 1cm specimen was taken from the maternal side and placed in 10% formalin filled bottles labelled with the patient ID number and date of collection. Delivery characteristics and drug history were collected at delivery. The schedule of sample collection and scheduled procedures in the trial is summarized below in table 4.

Table 4. The schedule of sample procedures and sample collection

Field procedures and activities	Visit 1 Wk 16-19	Visit 2 Wk 20-24	Visit 3 Wk 25-29	Visit 4 Wk 30-34	Visit 5 Delivery	Visit 6 Wk 4
Secure informed consent						
Review obstetric history/gestational age						
Provide complete exam						
Conduct test and treat for HIV/syphilis						
Measure haemoglobin levels						
Measure glucose/protein levels						
Collect pin-prick blood spots						
Collect vaginal and cervical swabs						
Collect stool samples						
Provide treatment per group randomisation						
Monitor adverse events						
Provide syndromic management for STIs						
Record pregnancy outcome						
Prepare placental smear/cord blood smear						
Collect placental tissue						
Conduct neonate exam						

Activities w/ no samples= Blood= Urine= Placental tissue= Vaginal and cervical swabs= Stool=

2.5.10 Quality assurance of data and sample collection

To ensure that good quality data was collected, nurses, laboratory staff were trained in data collection. Nightly data checks were conducted by the study coordinator and by the investigators before being uploaded to the server. The collection of cervico-vaginal samples was restricted to midwives after training on how samples were to be collected for diagnosis of BV and other STIs/RTIs. Other biological samples were collected by trained laboratory technologists who routinely carry out procedures such as venous and peripheral blood collection and slide preparation.

2.6 Collection of global position system coordinates

Landmarks near the homes of participating women and home addresses of all the participants were collected at enrolment. Members of the research team visited the homes of participants guided by the information collected at recruitment. Permission to visit a participant home was obtained at recruitment. The purpose of the visit was to collect global positioning system coordinates of participant homes for the purpose of locating women at the time of delivery if they required transportation to the hospital.

2.7 Ethical consideration

The trial protocol was approved by the Ethics Committee of the London School of Hygiene & Tropical Medicine (Ref: 17149), Tropical Diseases Research Centre Ethics Review Committee (Ref: TRC/C4/07/2019) in Zambia, the Zambia Medicines Regulatory Authority (Ref: DMS/7/9/22/CT/091), and the National Health Research Authority of the Ministry of Health (MOH) Zambia. The letters of ethical and regulatory approval are shown in appendices 9.11, 9.12 and 9.13. The trial was also registered at ClinicalTrials.gov: NCT04189744.

2.8 Data analysis

At each site, electronic tablets were used for electronic data capture. The study generated quantitative data from electronic case record forms at the study facilities. These data were collected during the ANC visits, facility delivery, and one visit 28 days post-partum. Trial data

was electronically entered on tablets into a GCP-compliant offline trial-specific mobile application [Research Electronic Data Capture (REDCap), Nashville, TN, US. Data from tablet PCs were synced once daily over a secure 3G connection with a web-based REDCap database hosted by the London School of Hygiene and Tropical Medicine (LSHTM).

The primary outcome in this trial was adverse pregnancy outcome and was based on the intention-to-treat (ITT) population. The adverse birth outcome was defined as the composite of foetal loss (spontaneous abortion or stillbirth), or singleton live births born with low birthweight (LBW), or preterm, or subsequent neonatal death by day 28. All eligible women enrolled in the study were included in the ITT population regardless of whether they prematurely discontinued treatment or where protocol deviation occurred. However, any woman lost to follow up where there was no data on the primary endpoint was excluded in the analyses for efficacy by the statistician. Participants were excluded if they were randomised but subsequently found to be ineligible or were known to have used prohibited medication.

Spontaneous abortion was defined as spontaneous foetal loss in the first 27 weeks of gestation. Stillbirth is when the baby was born with no signs of life at or after 28 weeks' gestation. Low birth weight was defined as birthweight <2500g. Preterm birth was spontaneous birth before 259 days (37 weeks) gestation. The gestational age at delivery was determined as number of days between delivery and registration, plus gestation age (days) at registration defined by ultrasound scan.

Clinical malaria at any point from enrollment including delivery and 28 days postpartum, was defined as documented fever (≥37.5°C), or recent history of fever in the past 24 hours, or other symptoms of acute illness that resulted in a woman seeking care with an unscheduled visit or maternal malaria infection detectable by Rapid Diagnostic Test (RDT) for women with malaria symptoms only. Maternal malaria infection was defined as detected by PCR during pregnancy in the peripheral blood at any point during pregnancy (scheduled and unscheduled visits) from study registration, including delivery. Any malaria at delivery was defined as malaria infection detected by microscopy in the peripheral or placental blood or by histology (active infection) at delivery. Placental malaria infection was defined as placental malaria

detected by microscopy, molecular methods, or by histology (past and active infection) including any *Plasmodium* species detected in the placental blood or biopsy tissue by either, placental incision, smear microscopy (standard microscopy), placental malaria by histology (active infection), or by PCR on placental blood (maternal).

Concerning placental histology, past Infection was defined as pigment in fibrin detected in the absence of asexual parasites whilst active infection was defined as either chronic or acute. Chronic infection was when both the pigment and asexual parasites were present and acute infection was when the pigment was absent in the presence of asexual parasites. Maternal anaemia was measured at enrollment and delivery. This was categorised as: No anaemia (Hb $\geq$11 g/dL), mild anaemia (Hb $\geq$10 to <11 g/dL), moderate anaemia (Hb $\geq$7 to <10 g/dL, and severe anaemia (Hb <7 g/dL).

Data were analysed using Stata version 18. Baseline characteristics were summarised by treatment arm. Descriptive statistics for continuous variables included mean, Standard deviation, median, range, and number of observations. Categorical variables were summarised as counts and proportions. All analyses accounted for the nature of the distribution of the outcome and results are presented as appropriate effect sizes with a measure of precision (95% CIs). Both unadjusted analyses and analyses adjusted for stratification factors based on a comprehensive analysis plan were carried out. In addition, sub-group analyses to explore between-group differences in the primary outcome, malaria status, and degree of SP parasite resistance were also conducted. All sub-group analyses had been performed by including a variable (or variables, as appropriate) for the sub-group and its interaction with the treatment effect in the model.

3 Results (1) The protective efficacy of IPTp in protecting against adverse pregnancy outcomes

3.1 Chapter introduction

This chapter presents the results of the protective efficacy of IPTp-SP compared to IPTp-SP with MTZ or IPTp-DP with MTZ. This is for both the composite adverse outcome and for individual adverse birth outcomes which are spontaneous abortions, stillbirths, preterm deliveries, low birthweight, and neonatal deaths. The occurrence of adverse pregnancy outcomes in each arm is described. Thereafter, the individual occurrence of adverse pregnancy outcomes in the SP arm were compared with adverse pregnancy outcomes in the SP plus MTZ arm and the DP plus MTZ arm.

3.2 Baseline characteristics

Baseline characteristics were similar between the three treatment groups. The median age at enrollment was 27 years (IQR 24–230). Of the 5,436 women, 24.1% (n=1,309) were less than 20 years old, 36.9% (n=2,004) were aged 20 -24 years, 17.9% (n= 975) were between 25-29, and 21.1% (n=1,148) were above 30 years of age. Specific to gravidity, 47.1% (n=2,563) were multigravidae, whilst 30.8% (n= 1,675) were primigravidae, and 21.2% (n=1,155) were secundigravidae. At enrollment, 41.2% (n= 2,240) of women had a gestation of less than 20 weeks, and 34.2% (n= 1,857) had gestational age between 20 to 24 weeks, whilst the rest 24.6% (n=1,339) had a gestational age greater than 24 weeks. Over 98% of the women reported using an insecticide treated net the previous night. Only 0.3% (n=15) of the women had severe anaemia with HB <7 g/dL. It is important to note that the protocol was amended during the trial at the suggestion of the data monitoring and ethics committee to exclude women with haemoglobin less than 7g/dl to prevent SAEs related to anaemia. Training in emergency obstetric care was conducted for all nurses in the four clinics where the trial was conducted to improve care for the pregnant women in Nchelenge. However, 23.8% (n= 1,293) participants had moderate anaemia with the HB ≥ 7 to <10g/dL and 23.6% (1,284) had mild anaemia. The rest, 52.3% (n=2,840), were not anaemic. The baseline characteristics are shown in Table 5.

Table 5. Baseline characteristics according to treatment arms

Characteristics	SP	SP plus MTZ	DP plus MTZ
	% (n)	% (n)	% (n)
Total women	1812	1812	1812
Age (years)			
<20	24.3 (441)	25.4(461)	22.5(407)
20 to 24	36.5(662)	35.4(644)	38.5(698)
25 to 29	18.1(327)	18.0(326)	17.8(322)
≥ 30	21.1(382)	21.0(381)	21.3(38.5)
Use of bed net previous night	98.4(662)	98.1(704)	98.3(696)
Gravidity			
Primigravidae	31.1(563)	30.3(548)	31.1(564)
Secundigravidae	21.4(388)	22.5(407)	22.1(410)
Multigravidae	47.5(861)	47.3(856)	46.7(846)
Gestational age at enrolment (weeks)			
≤ 20	40.0 (725)	41.1(745)	42.5(770)
> 20 to ≤ 24	35.2(638)	33.8(612)	33.5(607)
≥ 24	24.8(449)	25.1(455)	24.0(435)
Anaemia			
Severe (<7 g/dL)	0.2(4)	0.2(4)	0.4(7)
Moderate (≥ 7 to <10g/dL)	24.8(449)	24.2(438)	22.4(406)
Mild (≥ 10 to < 11g/dL)	22.5(407)	23.9(432)	24.6(445)
No Anaemia (≥ 11.0g/dL)	52.5(950)	51.5(938)	52.6(952)

3.3 Participant distribution

We screened 6,953 pregnant women and enrolled 5,436 of them (mean age 24.2 years [SD 6.1]) from 17 December 2019 to 18 March 2022. Group 1, recipients of SP plus MTZ placebo at visits 1 and 2, had a mean age of 24.3 years [SD 6.1]). Group 2, recipients of SP plus MTZ at visits 1 and 2, had a mean age of 24.2 years [SD 6.2]) and group 3, recipients of DP plus MTZ at visits 1 and 2, had a mean age of 24.3 years [SD 6.0]. In total, 96.2% of women (n=5230 women) contributed to the intention to treat analysis (1,753 in the SP group, 1,735 in the SP plus MTZ and 1,742 in the DP plus MTZ group) and 93.7% (n= 5,091) (93.7%) delivered live babies. Of the participants who were screened, 21.8% (1,517/6,953) were excluded largely because they did not meet the inclusion criteria (n=1,463) and 54 women declined to take part in the trial. Details of the distribution of participants at recruitment and follow-up to delivery are presented in the CONSORT diagram in Figure 3.

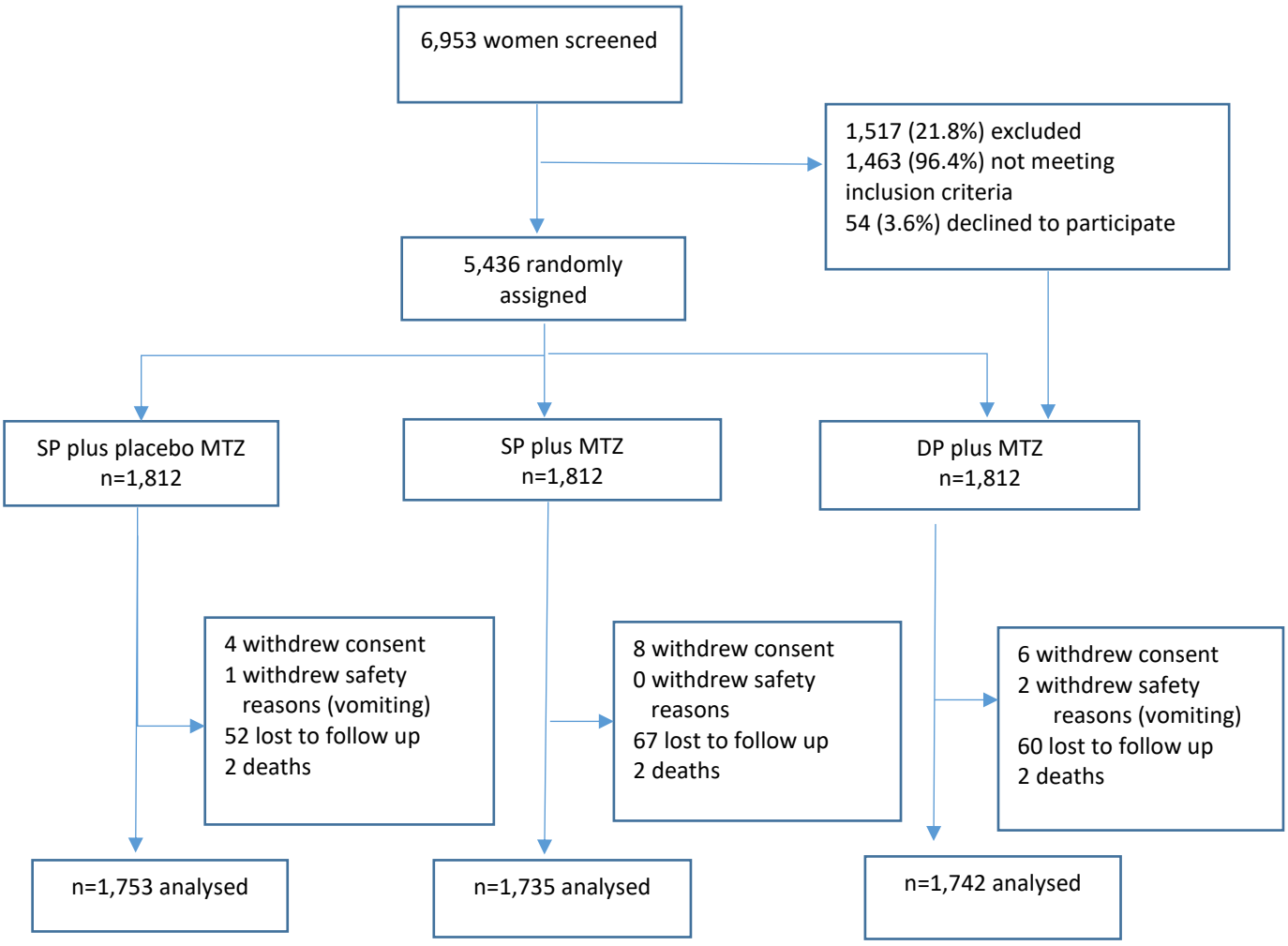


Figure 3. Trial Profile: Intention to Treat Analysis

3.4 Primary endpoint: Adverse pregnancy outcome and components

The prevalence of adverse pregnancy outcome as a composite measure was no different across treatment groups. When SP was compared to SP plus MTZ or DP plus MTZ, the frequency of adverse pregnancy outcomes was 18.4%, 18.8% (p=0.7), and 18.0% (p=0.88), respectively. SP remained non-inferior to SP plus MTZ and DP plus MTZ for each component of the composite endpoint. For spontaneous abortions, the risk was 0.6%, 0.5% (p=0.84) and 0.6% (p=0.99) for SP, SP plus MTZ and DP plus MTZ, respectively. For stillbirth, there was no difference in the risk of adverse pregnancy outcomes when the SP arm was compared to the SP plus MTZ or the DP plus MTZ arms. The incidence of stillbirth was 2.3%, 2.1% (p=0.68) and 1.8% (p=0.30), respectively. For preterm deliveries, there were, again, no significant differences in the three arms. SP had 7.2%, SP plus MTZ had 8.0% (p=0.37) and DP plus MTZ had 6.6% (p=0.51). For LBW (≤ 2500g), the incidence was 10.0% in the SP arm, 10.7% in the SP-MTZ arm (p=0.51) and 9.5% in the DP plus MTZ arm (p=0.61). When neonatal deaths (death within 28 days of delivery) were compared, there were no significant differences among the three arms. The risk of neonatal death was 1.9% for the SP arm, 1.5% for the SP plus MTZ arm (p=0.39) and 2.4% for the DP plus MTZ arm (p=0.30). Table 6 shows the adverse events and their components.

Table 6. Primary endpoint: Adverse pregnancy outcome and components

	SP % (n)	SP-MTZ % (n)	P-value	DP-MTZ % (n)	P-value
Total women	1,524	1,501		1,504	
Adverse pregnancy outcome	18.8 (287)	19.4 (292)	0.70	18.6 (280)	0.88
Spontaneous abortion	0.6 (10)	0.5 (9)	0.84	0.6 (10)	0.99
Stillbirth	2.3 (41)	2.1 (37)	0.68	1.8 (32)	0.30
Preterm birth	7.2 (124)	8.0 (138)	0.37	6.6 (115)	0.51
Low birthweight	10.0 (162)	10.7 (174)	0.51	9.5 (156)	0.61
Neonatal death	1.9 (34)	1.5 (27)	0.39	2.4 (43)	0.30

3.5 Efficacy of SP-MTZ and DP-MTZ compared to SP: risk of adverse pregnancy outcomes

The efficacy of SP-MTZ and DP-MTZ was not different from the efficacy of SP alone. In the SP group, 18.8% (287 of 1,524) women had an adverse pregnancy outcome, compared to 19.5% (292 of 1,501) women in the SP plus MTZ group [RR 1.04, 95% CI 0.90 to 1.20], p=0.62), and 18.6% (280 of 1,504) women in the DP-MTZ group [RR 0.99, 95% CI 0.85-1.14, p=0.76], (Table 6).

There was no difference in the risks of individual adverse pregnancy outcomes including spontaneous abortions, stillbirths, preterm delivery, LBW and neonatal death among the treatment groups. There was a total of 29 spontaneous abortions, 110 stillbirths, and 104 neonatal deaths, with no differences individually or as a composite between the treatment groups.

Concerning spontaneous abortion, the differences were not significant from the standard treatment of SP [0.6% (10 of 1,753)] compared to those who received SP plus MTZ [(0.5%) 9 of 1753, RR 0.91, CI 0.37 to 2.24, p=0.85] and when compared to those who received DP plus MTZ [(0.6%), 10 of 1,742, RR 1.00, CI 0.42 to 2.40, p=0.99]. When stillbirths were considered, women in the SP plus MTZ arm [(2.1%), 37 of 1,735, RR 0.90, CI 0.58 to 1.40, p=0.66] and DP plus MTZ arm [(1.8%), 32 of 1,742, RR 0.79, CI 0.49 to 1.23, p=0.28] did not show any difference with the women who received SP [(2.3%), 41 of 1,753]. For preterm delivery, there was again no difference between the SP arm [(7.2%), 124 of 1,731] and the SP plus MTZ arm [(8.0%), 138 of 1,731, RR 1.11, CI 0.88 to 1.41, p=0.36] and the DP plus MTZ arm [(6.6%), 115 of 1,731, RR 0.92, CI 0.72 to 1.17, p=0.50].

There was no difference in the risk of LBW in women given SP [(10%), 162 of 1620] compared to those given SP plus MTZ [(10.7%) 174 of 1,625], RR 1.07, CI 0.88 -1.31, p=0.48]. There was also no difference between the SP arm when compared to DP plus MTZ arm [(9.5%), 156 of 1647, RR 0.93, CI 0.76 to 1.14, p=0.5]. In terms of neonatal death, the interventional arms showed no significant improvement in the neonatal death outcome. Women in the SP plus MTZ arm [(1.5%), 27 of 1,449, RR 0.80, CI 0.49 to 1.32, p=0.39] and DP plus MTZ arm [(2.4%), 43 of 1,468, RR 1.23, CI 0.79 to 1.91, p=0.36] did not show any difference with the women in

the SP arm [(1.5%), 27 of 1,449]. Table 7 shows the composite outcome and the outcomes of spontaneous abortion, stillbirth, preterm delivery, LBW, and neonatal death.

Table 7. Efficacy outcomes. Adverse pregnancy outcome= composite spontaneous abortion, stillbirth, preterm birth, low birthweight, or neonatal death

	% (n/N)	% (n/N)	Crude RR (95% CI)	P-value	Adjusted RR (95% CI)	P-value
SP plus MTZ vs SP	**SP plus MTZ**	**SP**				
Adverse pregnancy outcome	19.5 (292/1501)	18.8 (287/1524)	1.03 (0.89,1.19)	0.7	1.04 (0.90, 1.20)	0.62
Spontaneous abortion	0.5 (9/1753)	0.6 (10/1753)	0.91 (0.37,2.23)	0.84	0.91 (0.37,2.24)	0.85
Stillbirth	2.1 (37/1735)	2.3 (41/1753)	0.91 (0.59,1.41)	0.68	0.90 (0.58,1.40)	0.66
Preterm birth	8.0 (138/1731)	7.2 (124)	1.11 (0.88,1,40)	0.37	1.11 (0.88,1,41)	0.36
Low birthweight	10.7 (174/1625)	10.0 (162/1620)	1.10 (0.87,1.31)	0.51	1.07 0.88,1.31)	0.48
Neonatal death	1.5 (27/1449)	1.9 (34/1465)	0.80 (0.49,1.32)	0.39	0.80 (0.49,1.32)	0.39
DP plus MTZ vs SP	**DP MTZ**	**SP**				
Adverse pregnancy outcome	18.6 (280/1504)	18.8 (287/1524)	0.99 (0.85,1.15)	0.88	0.99 (0.85,1.14)	0.76
Spontaneous abortion	0.6 (10/1742)	0.6 (10/1753)	1.00 (0.42,2.41)	0.84	1.00 (0.42,2.40)	0.99
Stillbirth	1.8 (32/1742)	2.3 (41/1753)	0.79 (0.50,1.24))	0.3	0.79 (0.49,1.23))	0.28
Preterm birth	6.6 (115/1731)	7.2 (124)	0.92 (0.72,1.18)	0.51	0.92 (0.72,1.17)	0.5
Low birthweight	9.5 (156/1647)	10.0 (162/1620)	0.95 (0.77,1.17)	0.61	0.93 (0.76,1.14)	0.5
Neonatal death	2.4 (43/1468)	1.9 (34/1465)	1.26 (0.81,1.97)	0.3	1.23 (0.79,1.91)	0.36

4 Results (2) Efficacy of IPTp options against malaria related outcomes

4.1 Chapter introduction

This chapter presents factors associated with the efficacy against malaria related outcomes of IPTp-SP compared with IPTp-SP plus MTZ or IPTp-DP plus MTZ. For this analysis, a WHO protocol (260) was used to test efficacy of IPTp regimens in clearing peripheral parasitaemia by PCR at day-28 post-dosing This protocol was aimed to be a guide for various chemoprevention efficacy studies. Different protocols from ongoing studies were used to come up with this protocol for therapeutic efficacy studies and some of the treatment efficacy evaluations which are needed to ensure standard means of monitoring and assessing efficacy of drugs used in malaria chemoprevention were adapted in coming up with this protocol.

Blood samples were collected on day 0 (enrolment), and on day 28 (visit 2) post-treatment to test for malaria parasites, and to estimate parasitological efficacy in the treatment and prevention of parasitaemia (261).

4.2 Day-28 efficacy of sulfadoxine-pyrimethamine against Plasmodium falciparum

Dried-blood samples for PCR testing were collected at every visit during the trial. The prevalence of malaria infections at enrollment was equivalent in all the three arms. This was 46.7%, 46.5% and 47.0% for SP, SP plus MTZ and DP plus MTZ respectively. There was a substantial reduction in the prevalence of malaria infection in the DP plus MTZ arm between visit 1, 47.0% (850/1,806) compared to visit 2, 5.3% (87/1,642), p=<0.001. There was also a significant reduction in malaria prevalence in the SP group between visit 1, 46.7% (844/1,847) compared to visit 2, 20.5% (336/1,640), p=<0.001. This was the same in the SP plus MTZ group where there was a significant reduction in the prevalence of malaria between visit 1, 46.5% (840/1,810), compared to visit 2, 19.1% (311/1,628), p=<0.001. This is shown in table 9.

Table 8. Day 28 efficacy of sulfadoxine-pyrimethamine against *Plasmodium falciparum* according to treatment group

	Visit number	Number of women (% of group)	Malaria (PCR)		
			% (n)	95% CIs	P-value
SP	Visit 1	1,807 (33.3)	46.7 (844)	44.4-49.0	
	Visit 2	1,640 (33.4)	20.5 (336)	18.6-22.5	
					<0.001
SP and MTZ	Visit 1	1,810 (33.4)	46.4 (840)	44.1-48.7	
	Visit 2	1,628 (33.2)	19.1 (311)	17.2-21.1	
					<0.001
DP and MTZ	Visit 1	1,806 (33.3)	47.1 (850)	44.7-49.4	
	Visit 2	1,642 (33.4)	5.3 (87)	4.3-6.5	
					<0.001

SP- Sulfadoxine-pyrimethamine, MTZ= Metronidazole, DP= Dihydroartemisinin-piperaquine, 95% CI= 95% Confidence Intervals

PCR= Polymerase Chain Reaction

4.3 Outcomes related to malaria infection during pregnancy and at delivery

4.3.1 Maternal anaemia at delivery

Broadly, prevalence of maternal anaemia at delivery, in the DP plus MTZ arm, was significantly lower when compared to the SP arm (p=0.03). However, the difference between the SP arm and the SP plus MTZ arm was not significantly different (p=0.94). For severe anaemia (<7g/dl), the prevalence in the SP arm was 0.4% (7/1,574). This was 0.3% (5/1,586) in the SP plus MTZ group and 0.2% (3/1,589). For moderate anaemia (≥7 to <10g/dL), there was 13.6% (214/1,574) in the SP arm, 13.4% (213/1,586) in the SP plus MTZ group, and 10.8% (171/1,589) in the DP plus MTZ group. In terms of mild anaemia (>10 to <10g/dL), the SP arm had 21.4% (336/1,574) compared to the SP plus MTZ arm which had 21.6% (342/1,586) and 20.7% (329/1,589) in the DP plus MTZ arm. Participants with no anaemia (Hb>11.1g/dl) also had a similar distribution among the three groups. About 64.6% (1017/1,574) had no anaemia in the SP group, 64.7% (1026/1,586) in the SP plus MTZ group, and 68.3% (1086/1,589) in the DP plus MTZ group.

For participants who were febrile during the follow-up visits, RDT were done to test for malaria and treatment offered to women who tested positive. There was no significant difference in the RDT positivity rate between the SP and the SP plus MTZ group [7.89% (143) vs 9.11% (165), p=0.19]. However, there was a significant difference in the positivity rate of the RDT with the DP plus MTZ group [4.3% (78), p=<0.001]. These results are summarized in table 10.

4.3.2 Detection of placental malaria

For placental histology, there was a significant reduction in the histological prevalence of malaria between the SP group and the DP plus MTZ group (p=0.001). However, the difference was not significant when the SP group was compared to the SP plus MTZ group (p=0.1000). The prevalence of acute infection, chronic infection, and past infection with malaria were all lower in the DP plus MTZ group when compared to the SP group or the SP plus MTZ group. Acute infection was 0.9% (13/1,574) in the SP group, 1.2% (19/1,586) in the SP plus MTZ group and 0.8% (13/1,589) in the DP plus

MTZ group. For chronic infection with malaria, the frequency was 2.3% (35/1,574) in the SP group, 1.4% (21/1,586) in the SP plus MTZ group and 0.8% (12/1,589) in the DP plus MTZ group. The incidence of past infection was 11.9%, 13.5% and 9.6% for the SP, SP plus MTZ and DP plus MTZ groups respectively. Eighty-five percent in the SP group had no placental malaria infection like 84% in the SP-MTZ group and 88.8% in the DP plus MTZ group. For any placental malaria at delivery, there was a significant difference in the DP-SP arm when compared to the standard of care SP (11.2% vs 15.0%, p=0.002). There was no significant difference with the SP plus MTZ arm (16.0%, p=0.43). The prevalence of peripheral malaria at delivery measured by PCR was significantly lower in the DP plus MTZ group at 6.9% (p=<0.001) when compared to the SP (14.6%). The prevalence in the SP plus MTZ group was 14.6% and not significantly different from the SP group (p=0.1). Table 10 describes the malaria outcomes during pregnancy and at delivery

Table 9. Outcomes related to malaria infection during pregnancy and at delivery

	SP % (n)	SP plus MTZ % (n)	P-value	DP plus MTZ % (n)	P-value
Number of women	1574	1586		1589	
Maternal anaemia at delivery			0.94		0.03
Severe (<7g/dL)	0.4 (7)	0.3 (5)		0.2 (3)	
Moderate (≥7 to <10g/dL)	13.6 (214)	13.4 (213)		10.8 (171)	
Mild (>10 to <10g/dL)	21.4 (336)	21.6 (342)		20.7 (329)	
No anaemia (≥11.1g/dL)	64.6 (1017)	64.7 (1026)		68.3 (1086)	
Maternal Malaria (positive RDT at any ANC visit)	7.89 (143)	9.11 (165)	0.19	4.3 (78)	<0.001
Placental malaria (histology)			0.1		0.001
Acute infection	0.9 (13)	1.2 (19)		0.8 (13)	
Chronic infection	2.3 (35)	1.4 (21)		0.8 (12)	
Past infection	11.9 (182)	13.5 (209)		9.6 (150)	
No infection	85.0 (1304)	84.0 (1305)		88.8 (1383)	
Any placental malaria	14.99 (230)	16.02 (249)	0.43	11.23 (175)	0.002
Peripheral malaria (PCR at delivery)	16.9 (212)	14.6 (183)	0.1	6.9 (88)	<0.001

5 Results (3) The prevalence of 540 and 581 markers of resistance in *P. falciparum*

5.1 Chapter introduction

This chapter concerns the results of the prevalence of SP resistance markers. High-levels of malaria parasite resistance compromise the therapeutic efficacy of SP, underscoring the importance of monitoring SP-resistance markers in the *Plasmodium falciparum* population. The chapter presents results of the first 200 falciparum-positive samples collected at enrollment from asymptomatic women were analysed for the frequency of the *dihydropteroate synthase (dhps) 540 and 581* mutation and *dihydrofolate reductase (dhfr)* mutations in Nchelenge district, Zambia. Another 176 falciparum positive samples were analysed in Copenhagen using advance genotyping methods

5.2 The prevalence of *DHFR* and *DHPS* mutations associated with SP resistance

Samples positive for *P. falciparum* malaria identified at enrolment were analysed for the frequency of 540 and 581 markers of resistance for SP at two time points during the trial. The first group of samples were 200 *falciparum* PCR positive samples collected in 2020 analysed at the TDRC lab. The second set of samples consisted of 176 *falciparum* PCR-positive samples collected in 2022 that were analysed at the University of Copenhagen. The codons for *dhfr* were 16, 51, 59, and 108 while the *dhps* that were analysed were 436, 437, 540, 581 and 613.

In our trial, the prevalence of the *dhps*540 mutation among the 200 pf PCR-positive samples collected in 2020 was 66.8% (133/199, 95% CI: 59.8, 73.3) and the prevalence of the *dhps*581 mutation was 5.0% (10/199, 95% CI: 2.4, 9.0).

The bar chart (figure 4) below shows the frequency of 540 and 581 mutations over last decade.

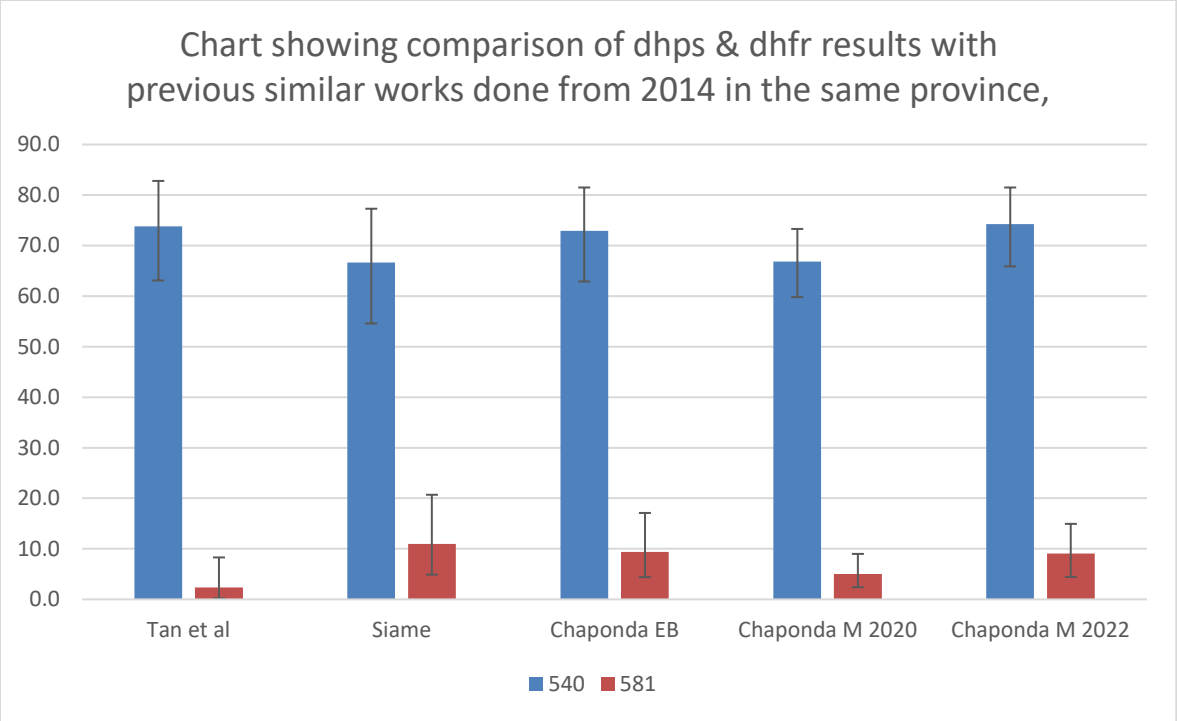


Figure 4. Frequency of *dhps* 540 and *dhps* 581 mutation

In total 176 samples from 2022 were successfully sequenced on the Illumina Miseq platform. The *Pfdhfr* SNPs in codons 51,59, 108 were observed at very high prevalence, whereas no SNPs were found in codon 164. For the *Pfdhps,* no SNPs were found in codon 431. The 437G mutant was found in 114/135 samples (84.4%), and the 540E mutation in 98/132 samples (74.2%), whereas the 581G mutation was found in 9.4% (12/127 samples). The 613S mutation was only found in 1/136 samples (0.7%). The quintuple haplotype was found in 66% whereas as 11% of the samples had the sextuple haplotypes. Table 11 shows the prevalence of SNPs in *dhfr* and *dhps* markers.

Table 10. Prevalence of SNPs in Pfdhfr, Pfdhps in 2022 (This is a presentation of results of samples that were collected in 2022, and were analysed in Copenhagen using a Illumina Miseq sequence platform)

***Pfdhfr* codons**		**N (%)**	**95% CI**	***Pfdhps* codons**		**N (%)**	**95% CI**
51	N51	2 (1.4)	**0.16-4.8**	**431**	I431	136 (100)	
	51I	143 (96.6)	**92.3-98.9**		431V	0 (0)	
	N51I	3 (2.0)	**0.4-5.8**		I431V	0 (0)	
	N	**148**			**N**	136	
59	C59	3 (2.0)	**0.4-5.8**	**436**	S436	132 (97.8)	
	59R	140 (94.6)	**89.6-97.6**		436A	0 (0)	
	C59R	5 (3.4)	**1.1-7.7**		S436A	3 (2.2)	0.4-6.4
	N	**148**			**N**	**135**	
108	S108	0 (0)		**437**	A437	9 (6.7)	3.1-12.3
	108N	167 (100)			437G	114 (84.4)	77.2-90.1
	S108N	0 (0)			A437G	12 (8.9)	4.7-15.0
	N	**167**			**N**	**135**	
164	I164	145 (100)		**540**	K540	23 (17.4)	11.4-25.0
	164L	0 (0)			540E	98 (74.2)	65.9-81.5
	I164L	0 (0)			K540E	11 (8.3)	4.2-14.4
	N	**145**			**N**	**132**	
				581	A581	115 (90.6)	80.2-92.3
					581G	12 (9.4)	4.8-15.3
					A581G	0	
					N	**127**	
				613	A613	135 (99.3)	96.0-99.9
					613S	1 (0.7)	0.01-4.0
					A613S	0	
					N	**136**	

6 Results (4) Treatment efficacy of MTZ against STIs/RTIs

6.1 Chapter introduction

This chapter describes the comparison of the efficacy of MTZ against STIs/RTIs at first and second antenatal care visits in pregnant women in Nchelenge district comparing the three treatment arms SP alone, SP plus MTZ and DP plus MTZ. Nucleic acid amplification was used on visit 1 (enrolment) and visit 2 samples to diagnose the STIs and, separately, Nugent scoring for BV to provide a test of cure (262). A single dose of MTZ 2g was administered to women in the SP plus MTZ ground and the DP plus MTZ group (263), and compared to recipients of SP plus placebo MTZ.

6.2 Prevalence of STIs/RTIs within study arms at visit 1 and visit 2

There was a significant reduction in the prevalence of all STIs/RTIs between visit 1 and visit 2 except for chlamydia in the DP plus MTZ arm. Trichomoniasis was significantly reduced in all study arms. For the SP group, between visit 1 and visit 2, the prevalence reduced from 13.7% (248/1,807) to 10.5% (100/955, p=0.014). This was the same in the SP plus MTZ group in which the prevalence TV reduced from 13.7% (248/1,810) to 7.6% (72/945, p=<0.001). For the DP-MTZ, the prevalence was 14.3% (258/1,806) at visit 1 and reduced to 8.4% (80/957, p=<0.001) at visit 2.

The prevalence of BV in the SP arm at visit 1 was 35.5% (644/1,807) and significantly reduced to 29.7% (488/1,643, p=<0.001) at visit 2. In the SP plus MTZ group the frequency of BV at visit 1 was 34.6% (626/1,810) and reduced significantly to 23.1% (377/1,632, p=<0.001) at visit 2. For the DP plus MTZ group, there was a significant reduction of the prevalence of BV between visit 1 and visit 2 from 33.4% (606/1,86) to 19.2% (315/1,640, p=<0.001). For NG, the prevalence at visit 1 in the SP group was 13.1% (237/1,807) and significantly reduced to 6.8% (65/955, p=<0.001). The prevalence of NG in the SP plus MTZ group was 12.7% (229/1,807) at visit 1 and significantly went down to 7.4% (70/945, p=<0.001). In the DP plus MTZ group the frequency of NG at visit 1 was 11.7% (211/1,806) and reduce to 6.8% (65/957, p=<0.0001) at visit 2.

Chlamydia trachomatis infection was not significantly reduced in the DP plus MTZ arm between visit 1 and visit 2 from 5.7% (103/1,806) to 4.4% (42/957, p=0.14). However, it was

significantly reduced between visit 1 and visit 2 in the SP group from 7.1% (129/1,807) to 2.1% (20/955, p=<0.001) and the SP plus MTZ group from 6.0% (109/1,080) to 3.0% (28/945, p= <0.0001) respectively. Table 12 shows the Chi square test comparing visit 1 and visit 2 prevalence of STIs/RTIs within study arms.

Table 11. Comparison of prevalence of STI/RTIs at the first and second visit within study arms

	Overall	SP			SP plus MTZ			DP plus MTZ		
STIs/RTIs	**Visit 1**	**Visit 1**	**Visit 2**	**P-value**	**Visit 1**	**Visit 2**	**P-value**	**Visit 1**	**Visit 2**	**P-value**
	5423	1807	955		1810	945		1806	957	
	% (n), 95% CI	% (n)	% (n)		% (n)	% (n)		% (n)	% (n)	
Syphilis	11.2(608), 10.4-12.1	10.5 (190)	N/A		11.2 (203)	N/A	N/A	11.9 (215)	N/A	N/A
Gonorrhoea	12.5(677), 11.6-13.4	13.1 (237)	6.8 (65)	<0.001	12.7 (229)	7.4 (70)	<0.001	11.7 (211)	6.8 (65)	<0.001
Chlamydia	6.3(341), 5.7-7.0	7.1 (129)	2.1 (20)	<0.001	6.0 (109)	3.0 (28)	<0.001	5.7 (103)	4.4 (42)	0.14
Trichomoniasis	13.9(754), 13.0-14.9	13.7 (248)	10.5 (100)	0.014	13.7 (248)	7.6 (72)	<0.001	14.3 (258)	8.4 (80)	<0.001
Bacterial vaginosis*	34.6(1876), 33.3-35.9	35.5 (644)	29.7 (488)	<0.001	34.6 (626)	23.1 (377)	<0.001	33.4 (606)	19.2 (315)	<0.001

* for BV more tests were done at visit 2 compared to other STIs (N= 1,596, 1,583, 1,599, for SP, SP-MTZ & DP-MTZ respectively

6.3 Comparison of prevalence of STIs/RTIs at visit 1 versus visit 2 among women who tested positive at visit 1

We analysed day-28 reduction of STIs/RTIs in women who tested positive at visit 1 by comparing visit 1 versus visit 2 (28 days later) results. In the SP arm, 79.4% [(81/102), (95% CI 70.3,86.0)] of women were PCR-positive for TV at visit 1 and PCR-negative at visit 2 whilst in the SP plus MTZ group, 87.4% [(83/95) (95% CI 79.0,93.3)] were PCR-positive for TV at visit 1 and PCR-negative at visit 2. In the DP plus MTZ arm, 90.0% [(90/100) 95% CI 82.4,95.1)] of women were PCR-positive for TV at visit 1 and PCR-negative at visit 2.

For BV, in the SP arm, 33.5% [(193/475), (95% CI 29.2,37.9)] were PCR-positive at visit 1 and PCR-negative at visit 2. In the SP plus MTZ arm, 53.6% [(289/457), (95% CI 48.9,58.3)] were PCR-positive for BV at visit 1 and PCR-negative at visit 2. In the DP plus MTZ arm 62.2% [(335/439), (95%CI 57.5,66.7)] were PCR-positive at visit 1 and PCR negative at visit 2. For Gonorrhoea, in the SP arm, 82.0% [(73/89) (95% CI 72.5,89.3)] were PCR-positive at visit 1 and PCR-negative at visit 2. In the SP-MTZ arm, 85.7% [(84/98) (95%CI 77.2,92.0)] were PCR-positive for NG at visit 1 and PCR-negative at visit 2. In the DP plus MTZ arm, 83.1% [(69/83) (95% CI 73.3,90.5)] were PCR-positive for NG at visit 1 and PCR negative at visit 2.

Concerning infection with *chlamydia trachomatis*, 98.0% [(50/51) (95%CI 89.6,99.9)] were PCR-positive for CT at visit 1 and PCR-negative at visit 2 in the SP arm. For the SP plus MTZ, 90.0% [(45/50) (95%CI 78.2,96.7)] were PCR-positive for CT at visit 1 and PCR-negative at visit 2. In the DP plus MTZ arm, 79.1% [(34/43) (95%CI 64.0,90.0)] were PCR-positive for CT at visit 1 and PCR-negative at visit 2. Table 13 shows the proportion of women who were PCR- or Nugent-score-positive for STIs/RTIs at visit 1 and negative at visit 2, on day 28 post-treatment.

Table 12. Proportion of women becoming test negative for specific RTI/STI at visit 2 among women who tested positive at visit 1

STIs/RTIs	SP			SP plus MTZ			DP plus MTZ		
	N	N	Day 28 test negative % (95% Cl)	N	n	Day 28 test negative % (95% Cl)	N	n	Day 28 test negative % (95% Cl)
Gonorrhoea	89	73	82.0 (72.5,89.3)	98	84	85.7 (77.2,92.0)	83	69	83.1(73.3,90.5)
Chlamydia	51	50	98.0 (89.6,99.9)	50	45	90.0 (78.2,96.7)	43	34	79.1(64.0,90.0)
Trichomoniasis	102	81	79.4 (70.3,86.8)	95	83	87.4 (79.0,93.3)	100	90	90.0(82.4,95.1)
Bacterial vaginosis	475	193	33.5 (29.2,37.9)	457	289	53.6 (48.9,58.3)	439	335	62.2(57.5,66.7)

6.4 Prevalence of malaria and STI/RTI co-infection at visit 1 and visit 2 according to study arms

There was a significant reduction of STIs/RTIs and malaria-STIs/RTIs co-infection in all the study arm between visit 1 and visit 2. The prevalence of any STI/RTI without malaria in the SP arm was 52.5% (952) at visit 1 and 30.8% [(506), p=<0.001] at visit 2. In the SP plus MTZ arm, visit 1 had 51.1% (962) compared to 24.6% [(401), p=<0.001] on visit 2 When it comes to the DP plus MTZ arm, at visit 1, 49.7% (901) had STIs/RTIs and 21.8% [(358), p=<0.001] at visit 2.

The prevalence of malaria plus any STI/RTI co-infection was significantly reduced in all study arms. In the SP arm, there was 25.4% (459) at visit 1 and 6.7% [(110), p=<0.001] at visit 2. For the SP plus MTZ arm, there was 24.6% (444) for visit 1 compared to 4.8% [(78), p=<0.001] at visit 2. In the DP plus MTZ group, the prevalence of malaria with any STI/RTI was 23.8% [(431) at visit 1 and 1.1% [(18), p=<0.001]. In terms of malaria with syphilis co-infection, the prevalence was similar in the 3 treatment arms at visit 1. This was 5.4% (97), 5.4% (98) and 5.4% (98) for the SP, SP plus MTZ and DP plus MTZ arms respectively. Syphilis was only tested at enrolment.

Malaria and gonorrhoea co-infection reduced significantly after 28 days in all the three study arms. In the SP arm co-infection was 6.0% (108) for visit 1 and 1.2% [(11), p=<0.001] at visit 2. In the SP plus MTZ arm, the prevalence at visit 1 was 5.8% [(104) and 1.0% (9), p=<0.001] at visit 2 whilst the prevalence was 5.0% (91) and 0.3% [(3), p=<0.001] in the DP plus MTZ arm at visit 1 and visit 2 respectively. Chlamydia and malaria co-infection was also significantly reduced in the three study arms. In the SP arm, the reduction was from 4.1% (73) to 0.3% [(3), p=<0.001] between visit 1 and visit 2. For the SP plus MTZ arm, the prevalence dropped from 2.8% (50) to 0.4% [(4), p=<0.001] and 2.9% (52) to 0.2% [(2), p=<0.001] in the DP plus MTZ group. There was a reduction of malaria and TV co-infection in all study arms between visit 1 and visit 2. The reductions were as follows; 7.0% (127) to 1.5% [(14), p=<0.001], 7.3% (131) to 1.1% [(10), p=<0.001] and 7.3% (132) to 0.4% [(4), p=<0.001] for SP, SP plus MTZ and DP plus MTZ arms respectively.

The prevalence of co-infection of malaria with BV was also measured between visit 1 and 2. The prevalence was significantly reduced 17.1% (308) to 6.2% [(99), p=<0.001] for the SP arm, 16.2% (292) to 4.3% [(68), p=<0.001] in the SP plus MTZ arm, and 16.9% (305) to [(0.9% [(14), p=<0.001] in the DP plus MTZ arm. Table 14 shows the prevalence of malaria and STI/RTI co-infection at visit 1 and visit 2.

Table 13. Prevalence of malaria and STI/RTI co-infection at visit 1 and visit 2

		SP			SP plus MTZ			DP plus MTZ		
	Overall Visit 1	**Visit 1**	**Visit 2**	**P-value**	**Visit 1**	**Visit 2**	**P-value**	**Visit 1**	**Visit 2**	**P-value**
Number of women	5423	1807	955		1810	945		1806	957	
	% (n)	% (n)	% (n)		% (n)	% (n)		% (n)	% (n)	
Any STI/RTI	51.9 (2815)	52.5 (952)	30.8 (506)	<0.001	51.1 (962)	24.6 (401)	<0.001	49.7 (901)	21.8 (358)	<0.001
Malaria (PCR)	46.7 (2534)	46.7 (844)	20.5 (336)	<0.001	46.5 (840)	19.1 (311)	<0.001	47.0 (850)	5.3 (87)	<0.001
Malaria + any STI/RTI	24.6 (1334)	25.4 (459)	6.7 (110)	<0.001	24.6 (444)	4.8 (78)	<0.001	23.8 (431)	1.1 (18)	<0.001
Malaria + syphilis	5.4 (293)	5.4 (97)	-		5.4 (98)	-		5.4 (98)	-	
Malaria + gonorrhea	5.6 (303)	6.0 (108)	1.2 (11)	<0.001	5.8 (104)	1.0 (9)	<0.001	5.0 (91)	0.3 (3)	<0.001
Malaria + chlamydia	3.2 (175)	4.1 (73)	0.3 (3)	<0.001	2.8 (50)	0.4 (4)	<0.001	2.9 (52)	0.2 (2)	<0.001
Malaria + trichomoniasis	7.2 (390)	7.0 (127)	1.5 (14)	<0.001	7.3 (131)	1.1 (10)	<0.001	7.3 (132)	0.4 (4)	<0.001
Malaria + bacterial vaginosis	16.7 (905)	17.1 (308)	6.2 (99)	<0.001	16.2 (292)	4.3(68)	<0.001	16.9 (305)	0.9 (14)	<0.001

7 Discussion

7.1 Protective efficacy of IPTp-SP compared to IPTp-SP plus MTZ or IPTp-DP plus against adverse pregnancy outcomes among pregnant women in Nchelenge district

Results from the ASPIRE trial showed that IPTp-SP was non-inferior to IPTp-SP plus MTZ or IPTp-DP plus MTZ for protection against adverse pregnancy outcomes [(18.4% (287) vs 18.8% (292) vs 18.0% (280) respectively] in this setting of high malaria and curable STIs/RTIs. In the pregnancy cohort study conducted in the same facilities between 2013-2014, malaria and STIs/RTIs co-infection was high: 38.7% of women had malaria and at least one STI/RTI, 18.9% were infected with malaria parasites only, and 26.0% were infected with at least one STI/RTI but no malaria parasites (36) .

Prior to ASPIRE, three trials in East Africa compared IPTp-DP with the standard of care IPTp-SP (101-103). According to these studies, DP is considered a promising alternative to SP for IPTp because it is well tolerated and associated with greater reductions in the incidence maternal anaemia, clinical malaria, and the prevalence of peripheral malaria infection at delivery than SP (101-103). The IMPROVE trial that compared monthly intermittent IPTp-SP versus monthly IPTp-DP or monthly IPTp-DP plus azithromycin also showed that use of DP was associated with reduced incidence of clinical malaria, malaria infection detected by microscopy, and placental malaria. However, compared with the ASPIRE trial, IMPROVE was conducted in areas of lower malaria transmission, where the incidence of LBW attributable to malaria was low and this could have affected the effect of DP on adverse birth outcomes. (104), which may partially explain why SP was superior to DP and DP plus azithromycin in terms of reducing the risk of adverse pregnancy outcomes. In contrast as ASPIRE trial was done an area with very high transmission, the effect SP alone and SP plus metronidazole was not different from DP plus metronidazole in protection against adverse pregnancy outcomes.

In a nested study of the IMPROVE trial, serial prenatal ultrasound measurements were conducted and demonstrated a significant negative association between STIs/RTIs and fetal growth trajectories. Having infection with both malaria and STIs/RTIs was associated with adverse outcomes particularly in paucigravidae pregnancies. Fetal growth was

inhibited among women who tested positive for STIs/RTIs at enrolment, compared to women who only tested positive in late pregnancy (32-36 weeks). This suggests that the deleterious effect of STIs/RTIs on fetal growth is determined early in pregnancy before the third trimester when growth peaks (264).

A meta-analysis by ter Kuile, *et. al.* published in 2007 found that even in areas where SP treatment failures rates in children were high, IPTp-SP maintained its effectiveness in preventing LBW, maternal anaemia, maternal parasitaemia at delivery, and placental malaria (207). A study conducted in a low malaria setting in Lusaka Zambia between February 2006, and December 2012 analysed data from 48,246 HIV pregnant with singleton births. Results from this study suggested that IPTp-SP could have been having an effect of genital tract infections or interacting with the immune system of the pregnant women leading to the reduced risk of LBW. The study showed that the risk of LBW reduced when the doses of IPTp-SP increased and was lowest among women who received three doses (aRR = 0.78; 95% CI = 0.64–0.95). Sulfadoxine-pyrimethamine was also associated with a reduced risk of LBW in HIV positive women, including those receiving antiretroviral therapy (265).

In spite of high parasite resistance to SP, as expressed both by parasite molecular markers, this did not affect the associations between IPTp-SP and birth outcomes: Irrespective of resistance, IPTp-SP is associated with improved birth outcomes, particularly, reduction in LBW and increase in maternal Hb. This finding may be explained by several factors. There may be a difference in the degree to which resistance affects the ability of IPTp-SP to treat or prevent infections vs the ability to prevent malaria-associated morbidity if partial suppression of parasitaemia may be sufficient to reduce some of the adverse effect on fetal growth and maternal anaemia. SP may also have secondary, off-target effects on bacterial or fungal infections that promote fetal growth and maternal health.

It appears there is a non-malarial pathway in which IPTp-SP still protects against adverse birth outcomes(106). The mechanism by which SP does this is not well understood, and it

appears to have an effect which overcomes the effect of DP on birthweight. This may be due to the antibacterial properties of sulfadoxine or pyrimethamine, or both in combination, leading to the inhibition of bacteria in the genital tract. (266, 267). As agued in the IMPROVE trial, the impact of SP on adverse pregnancy outcomes and birthweight is made possible by its impact on intrauterine fetal growth instead of the effect by the age of the pregnancy. The effect of SP on birthweight was independent of the degree of malaria transmission and SP resistance in an area. However, there is little knowledge on the pathways in which fetal growth is promoted by SP. One of the postulations is that it could be that there is an effective non-malarial SP pathway of SP whose effect outstrips the effect of DP on birthweight enabled by the better protection from malaria associated with DP. It is also possible that sulfadoxine changes vaginal microbiome indirectly or directly suppresses bacterial infections (104).

It also appears that birth outcomes depend more on the pathology of the placenta. Since IPTp-DP reduces detectable peripheral malaria without affecting the pathology of the placenta sufficiently, it does not lead to a significant reduction in adverse pregnancy outcomes (268). Furthermore, a mediation analysis of several trials showed that the mere detection of malaria parasites in the periphery did not result in LBW in the absence of placental malaria infection (106). However, there was no difference in adverse pregnancy outcomes among livebirths (preterm delivery or LBW).

7.2 Day 28 efficacy of sulfadoxine-pyrimethamine against Plasmodium falciparum

We assessed the burden of infection with *Plasmodium falciparum* at visit 1 (enrolment) and visit 2 (day 28). We found that IPTp with DP-MTZ was superior to IPTp with SP alone or IPTp with SP-MTZ for protection against maternal malaria. The prevalence of malaria infections at enrollment was uniform in all the three arms, 46.7%, 46.5% and 47.0% for SP, SP-MTZ and DP-MTZ respectively. There was a substantial reduction in the prevalence of malaria infection in the DP-MTZ arm between visit 1 and visit 2 (47.0%, 850/1,806 at visit 1 compared to 5.3%, 87/1,642 at visit 2, p=<0.001). There was also a significant reduction in malaria prevalence in the SP group between visit 1 and visit 2 (46.7%,

844/1,847 at visit 1 compared to 20.5%.,336/1,640, p=<0.001 at visit 2). This was the same in the SP-MTZ group where there was a significant reduction in the prevalence of malaria (46.5%, 840/1,810 at visit 1, compared to 19.1%, 311/1,628, p=<0.001 at visit 2). These findings were not unexpected because it has been shown in several studies conducted over the past decade that DP was superior at reducing the burden of maternal malaria in pregnancy and this is what we found in our study conducted in a high transmission setting with high-grade resistance to SP by *Plasmodium falciparum*. However, this reduction in maternal malaria did not translate into reduced adverse birth outcomes when compared to IPTp-SP. This was also shown previously by Kajubi *et al*. (101) and recently in the IMPROVE trial (104) in a study conducted in Kenya, Tanzania, and Malawi with a sample size of 4,680 women. In this trial, it was shown the IPTp-SP was superior to IPTp-DP with azithromycin or IPTp-DP alone. Monthly IPTp-DP did not improve pregnancy outcomes, and the addition of a single course of azithromycin did not enhance the effect of monthly IPTp-DP. However, IPTp-SP performed better than both IPT-DP or IPTp-DP plus azithromycin as the malaria transmission was lower in the areas in which the trial was conducted in the three countries. Despite DP being a better antimalarial, its effect of birth outcomes were not clear to see probably because of the lower prevalence of malaria compared to our study which was in a higher transmission area with a higher prevalence on malaria of above 50% (235). In a higher transmission area, IPTp-DP would be expected to perform better than IPTp-SP in reducing the incidence of malaria. In our study, IPTp-DP was associated with more than 50% reduction in the detection of malaria parasites using RDT during pregnancy and a significant reduction of peripheral malaria using PCR at delivery compared with IPTp-SP or IPTp-SP plus MTZ. These findings were also similar with histopathologically confirmed placental malaria in which IPTp-DP cleared placental malaria better than IPTP-SP and IPTp-SP plus MTZ. However, despite notable differences in malaria specific outcomes, there were no significant differences in the risks of adverse pregnancy outcomes among the three treatment groups.

Malaria has been known to cause adverse pregnancy outcomes and it was expected that preventing the incidence of malaria episodes during pregnancy by DP in the IPTp-DP-MTZ

arm would lead to better birth outcomes as DP was a better antimalarial drug than SP. In another study conducted at four sites in Kenya involving 1546 HIV-negative women, between 2012 and 2014, three IPTp regimens were assessed. Monthly IPTp-SP (n=515) or IPTp-DP (n=516) or intermittent screening and treatment with DP (IST-DP) (n=515). In the screening arm, RDTs were used to screen women for parasitaemia and treated with DP when the RDT was positive to malaria. The results showed that IPTp-DP showed a significant reduction in malaria when compared to IPTp-SP. Prevalence of malaria infection at delivery was lower in the IPTp-DP group than in the IPTp-SP group (3% [15 of 457] vs 10% [47 of 459]; RR 0.32, 95% CI 0.18–0.56; p<0.0001). However, there was no significant reduction in the IST-DP group (13% [57 of 452]; 95% CI 1.23, 0.86–1.77; p=0.26). The incidence of malaria infection during pregnancy was lower in the IPTp-DP group when compared with IPTp-SP group (192.0 vs 54.4 events per 100 person-years; IRR 0.28, 95% CI 0.22–0.36; p<0.0001). However, IST-DP was associated with a higher incidence of malaria infection (232.0 events; 1.21, 1.03–1.41; p=0.0177) and clinical malaria (53.4 events; 95% CI 1.41, 1.00–1.98; p=0.0475). In addition, the risks of preterm delivery or LBW did not differ between these treatment groups (103).

Different strategies involving DP have been tried before without showing effect. A two-arm, open-label trial to compare the effect of the intermittent screening and treatment in pregnancy (ISTp) with DP against IPTp-SP in 1,873 pregnant women was conducted in southern Malawi between July 2011 and March 2013 in an area of high *Plasmodium falciparum* resistance to SP. This comprised of treating pregnant women who tested positive after screening for malaria using RDTs at regular intervals, with DP to reduce the risk of malaria infection and the adverse pregnancy outcomes. The study showed that ISTp-DP was not any better than IPTp-SP. The rate of malaria infections was high in both groups and that ISTp-DP did not improve pregnancy outcomes (269). Our findings in the ASPIRE trial are also in agreement with Olaleye *et al*. who conducted a systematic review and meta-analysis of 3 clinical trials comparing DP and SP for malaria prevention during pregnancy. This analysis showed that women who received IPT-DP had significantly lower

risks of clinical malaria during pregnancy. However, the impact of DP on LBW and adverse pregnancy outcomes was minimal (270).

7.2.1 Detection of placental malaria

Like other trials have shown in the past, DP was superior in clearing placental malaria compared to SP. Pregnant women are highly susceptible to *Plasmodium falciparum* malaria in malaria transmission areas, and this is characterized by the accumulation of parasitized red blood cells in the placenta (271). Detection of parasites is challenging due to the sequestration of the parasites in the placenta (272). Changes in the histology of the placenta may lead to serious consequences. And therefore, histopathological examination is an important method to understand the possible cause of adverse birth outcomes (273). In our study, the histological prevalence of malaria was significantly lower in the DP-MTZ group when compared to the SP group (p=0.001). However, the difference was not significant when the SP group was compared to the SP-MTZ group (p=0.1000). Acute infection, chronic infection, and past infection with malaria were all lower in the DP-MTZ group. A systematic review and meta-analysis of three clinical trials with 3,719 participants, conducted in Malawi, Kenya, and Uganda between 2011 and 2018, compared DP and SP in preventing malaria during pregnancy. When three dose IPTp-DP was compared to IPTp-SP, there was a significant reduction in clinical malaria during pregnancy (OR 0.17; 95% CI, 0.10–0.29; 1171 participants). The analysis also showed that monthly IPTp-DP resulted in almost no difference in peripheral parasitaemia at delivery when compared to IPTp-SP (OR 0.09; 95% CI, 0.01–1.68; 1 study, 206 participants). The analysis of the studies demonstrated lower odds of placental malaria in those who were given IPT-SP when compared to those who were given IST-DP. Intermittent screening and treatment with DP did not reduce placental malaria (OR 1.29; 95% CI, 1.10–1.50; 2 studies, 2903 participants). Overall, evidence from this analysis suggested that IPT-DP may reduce maternal and placental malaria compared with IPT-SP, and monthly DP is more effective than SP in reducing placental malaria (270). This was also confirmed in the study by mlungu *et al.* who found that the prevalence of histopathologically confirmed placental

malaria was significantly lower in the IPTp-DP arm when compared to the IPTp-SP arm (274).

7.2.2 Maternal anaemia at delivery

In the ASPIRE trial, the prevalence of maternal anaemia at delivery was significantly lower in the DP-MTZ arm when compared to the SP arm (p=0.03). However, the difference between the SP arm and the SP-MTZ arm was not significantly different (p=0.94). Anaemia in the pregnant women was confirmed when blood Hb level was <11.1 g/dL. Severe anaemia was HB <7g/dL, moderate was ≥7 to <10g/dL, and mild anaemia was >10 to <10g/dL. During pregnancy, anaemia has been associated with adverse pregnancy outcomes which include premature birth, LBW, and maternal death (275). In Africa, most Plasmodium infections are usually asymptomatic in pregnancy despite being associated with maternal anaemia (269). In the ASPIRE trial, maternal anaemia at delivery was lower in the DP arm compared to the SP arm. Since DP is superior at reducing the incidence of malaria infections, it is expected that it would reduce the prevalence of anaemia at delivery compared to the SP only arm. Our findings are in contrast with the findings of the trial conducted between January 2017 and May 2019, in which 956 HIV-uninfected, malaria-free pregnant women from moderate malaria transmission areas in Tanzania were enrolled and randomised to receive monthly IPTp-DP (n = 478) or IPTp-SP (n = 478). They found no significant difference in the prevalence of anaemia between the IPTp-SP and IPTp-DP groups at enrolment , during the ANC follow-up period, and at delivery (274). However, since anaemia is multifactorial, it is important to consider the different prevention programs for anaemia in different countries. The introduction of DP as first line treatment in 2006 in Indonesia led to a reduction in the proportion of women with severe anaemia declining from 10.5% before DP deployment to 7.2% after the change of policy. Before that, SP and chloroquine were recommended for the treatment of uncomplicated malaria but due to poor efficacy, the policy for the treatment of malaria was changed (276).

7.3 The prevalence of DHFR and DHPS mutations associated with SP resistance

Previously, studies have shown that clearing of malaria infection has been hampered by the presence of mutations which are responsible for reduced efficacy of SP especially in countries in eastern and southern Africa. The important mutations are three mutations in the *dhfr* gene and two mutations in the *dhps* gene. Therefore, surveillance of resistance markers is important. In addition, it is imperative that new alternative regiments for IPTp are evaluated. At the moment there seems to be no better alternative choices as they have failed to demonstrate beneficial effects over SP due to poor tolerability or not having superior efficacy when compared to IPTp-SP. (28, 29, 31).

In the Nchelenge pregnancy cohort study, 96 malaria-positive samples were analysed of which 70.8% (95% CI: 60.8, 79.2) contained the *dhps* double (Gly-437+Glu-540) mutation and 92.7% (95% CI: 85.3, 96.5) had the *dhfr* triple (Asn-108+Ile-51+Arg-59) mutation. The quintuple mutation (dhfr triple+DHPS double) and the sextuple mutant (*dhfr* triple+*dhps* double+Arg-581) were found among 68.8% (95% CI: 58.6, 77.3) and 9.4% (95% CI: 4.2, 16.0) of samples (232, 277). These results are comparable to a cross-sectional study conducted in 2013 by Siame *et al.* in pregnant women in Nchelenge, which showed a high number of mutations in the *dhfr* and *dhps* genes. Of the 72 PCR-positive samples examined, there was a high prevalence of *dhfr* triple (Asn-108 + Arg-59 + Ile-59) mutant (68%) and *dhps* double (Gly -437 + Glu-540) mutant (21%). The quintuple haplotype was found in 17% with 2 samples bearing an additional Gly-581mutation (84).

Compared with our study, in a sub-group of 200 women who had malaria parasitaemia at enrollment, the prevalence of *dhps*540 mutations and *dhps*581 mutation was 66.8% (133/199, 95% CI: 59.8, 73.3) and 5.0% (10/199, 95% CI: 2.4, 9.0), respectively. Unlike the first set of 200 samples which were run in Zambia on PCR-PFLP platform, the second sub-group of 176 samples were genotyped in Copenhagen on the more sensitive and versatile Illumina Miseq sequencer. In the *pfdhps* gene, only 3 codons exhibited point mutations at positions 437, 540 and 581. The prevalence of *Pfdhps* A-437-G, *pfdhps* 540E and *pfdhps* A-581G was, 76.7%, 74.2% and 9.49.4 % respectively. While 62.5% [95% CI: 52.9-71.5]

were the total number of haplotypes, as many as 37.5% [95% CI: 28.5-47.1] of the samples were found to have mixed infections.

These results are consistent with the earlier finding in the two other studies showing further spread of mutations in the study area. With the prevalence of the *dhps* K540E mutation above 50%, these studies have shown an urgent need to investigate other IPTp options. The absence of reference alleles in the second sub-group of 176 samples parasitaemia could be explained by a study model conducted by Michelle *et al.* (278) which predicted rapid decline of the *dhfr* genotype because of long half-life of SP by providing strong selective pressure against parasites carrying wild-type *dhfr*. Complete absence of the reference alleles poses a danger to complete treatment failure even after reducing drug pressure. Presence of triple *dhfr* and double *dhps* mutations on their own have exhibited less chances of treatment failure with SP. However, combination of these mutations leading to quintuple mutations doubles the chances of treatment failure with SP. In a study conducted in Mansa (A city near Nchelenge) by Tan et al, 2014, they found 26% failure rate of SP giving the moderate prevalence of the quintuple mutant haplotype. They recommended that, despite the presence of resistance, SP retained some efficacy in clearing parasites in pregnant women, and may remain a viable option for IPTp in Zambia. The multiplicity of single *dhps* haplotypes observed speculatively suggests an increase of sextuple mutations over time.

7.4 Prevalence of STIs/RTIs at the first and second antenatal care visits

There was a high prevalence of STIs/RTIs at enrollment which poses a high risk of adverse pregnancy outcomes. However, there was a significant reduction in the prevalence of STIs/RTIs between visit 1 (enrollment) and visit 2 (day 28) after dosing except for CT in the DP-MTZ arm. Trichomoniasis was significantly reduced between visits 1 and 2 in all study arms, as was the case with BV and NG. *Chlamydia trachomatis* infection was not significantly reduced in the DP-MTZ arm between visit 1 and visit 2. However, it was significantly reduced between SP group and the SP-MTZ group. The reductions in the prevalence of STIs (NG, CT, and TV) ranged from 79.1% and 98.0% across treatment arms

while the day 28 reductions of BV ranged from 33.5% to 62.2% across treatment arms. In the Nchelenge pregnancy cohort study of 2013-14, the prevalence of BV and TV was 48.3% (95% CI: 45.2, 51.2) and 24.8% (95% CI: 22.2, 27.8), respectively. One-third of women, 29.2% (95% CI: 26.5, 32.0), had malaria and BV, and 15.2% (95% CI: 12.9%, 17.5%) were diagnosed with malaria and TV. These high measures of co-infection warranted evaluation of new integrated approaches in the ANC package. Intermittent preventive treatment with SP and IPTp-SP-MTZ significantly reduced the prevalence of NG, CT, TV, and BV between visit 1 and 2. The dose of MTZ for the treatment of TV and BV was 2g as recommended by the WHO. Whilst it was expected that the addition of MTZ will clear TV and BV, the reduction of NG and CT in the IPTp-SP arm was surprising. In addition, IPTp-DP-MTZ also significantly reduced the prevalence of NG, TV, and BV between visit 1 and 2.

Despite the fact that TV was reduced significantly after treatment with MTZ, the prevalence was still high. This was not surprising. Despite the optimal timing for the test of cure for TV after completion of treatment is 4 weeks for those receiving single-dose MTZ, though sexual re-exposure and false negatives should be considered (262). Women in our trial were not advised to abstain from sexual activity which explains the possibility of re-infections. The high prevalence could also be due to TV resistance to MTZ which is a growing concern (279).

Our study also showed that SP and SP-MTZ significantly reduced the prevalence of NG, CT, TV, and BV between visit 1 and 2. This was a remarkable finding as neither SP nor MTZ is recommended as treatment for CT or NG. It shows that wide coverage of SP against infections could explain its capability in reducing adverse pregnancy outcomes despite high prevalence of resistance makers in sub-Sahara Africa. The DP-MTZ arm significantly reduced the prevalence of NG, TV, and BV between visit 1 and 2. The significant reduction of NG in this arm is remarkable because neither DP nor MTZ are recommended as treatment for NG. And this is amidst well documented antimicrobial resistance of NG to some antibiotics including MTZ (280). However, Ralph and Amatnieks reported relative

susceptibility of NG to MTZ and its metabolites in their paper in 1980 which could explain the reduction of NG prevalence in our study (281). We also found that DP-MTZ had a significantly higher reduction of BV compared to SP and SP-MTZ. This is a mixed finding. Even though MTZ is used for the treatment of BV, it appears that there was a mixed response in the DP-MT vs the SP-MTZ groups. It is possible that this could be due to an inhibitory effect of SP on MTZ or an enhancement of the MTZ effect by DP.

However, it is important to note that the reductions in the day-28 prevalence of the STs/RTIs do not necessarily represent the clearance of the infection because these were not the same women who were tested at visit 1. There were concerning numbers of new STIs/RTIs at visit 2 among women who were negative at visit 1 across treatment groups. This is possibly because their sexual partners were not screened and treated for STIs, and they were infected before visit 2. In the IMPROVE trial, it was remarkable that despite the addition of azithromycin, to IPTp-DP plus azithromycin, IPTp-SP had a greater reduction of CT more than IPTp-DP with azithromycin. While this was observed, it is arguable that the effect of IPTp-SP was seen to be greater than that of IPTp-DP plus azithromycin between visit 1 and 2 was due to reinfection rather than treatment failure.

7.5 Study limitations, biases, and strengths

The ASPIRE trial and nested sub-studies had some limitations. The results may not be universally generalizable as not all areas in sub-Saharan Africa have the same epidemiology of malaria and RTIs/STIs. The ASPIRE trial was conducted in a high transmission area of malaria with a prevalence of over 50% and high malaria STIs/RTIs co-infection. In addition, we excluded HIV positive women from the trial. This was to avoid the confounding effect of cotrimoxazole which many on the HIV positive women could be taking. This is a limitation as we cannot generalise the finding of the trial to include HIV positive women. The other limitation was that study participants were enrolled between 16 and 28 weeks of gestation. From the results of the study, we are not able to know the birth outcomes of women who would receive fewer IPTp doses when they book for ANC in later weeks and receive few numbers of IPTp doses. In addition, for the IPTp with DP-

MTZ arm, only the first dose of study medications (all MTZ and the first day of DP) were directly observed, with the two other doses self-administered at home. Failure to take the two doses administered at home could have differentially affected the results. However, we observed high compliance among the first 300 participants of the DP-MTZ arm who were visited at home without advance notice to check if they had taken study drugs outside of the facility. A potential source of bias is if a great proportion of eligible women with different profiles did not come for ANC. This is unlikely to be the case in this setting as ANC attendance has been shown to be above 95%. Another study limitation is in the responses to questions on the utilisation of malaria interventions which may have been influenced by social acceptability as close to 98% of women reported using an ITN the previous night.

The other limitation was that we did not analyse the data for small-for-gestational age (SGA) using the Intergrowth-21st standards growth charts. These were published in 2014 and assume that foetal growth is universally equal under ideal conditions. The value of using Intergrowth-21st standards is to make trial results as comparable as possible to other studies. However, in October 2020, the International Federation of Gynaecology and Obstetrics (FIGO) published a position paper2 that challenges the core assumption of Intergrowth-21st standards growth charts. FIGO concluded that foetal growth should not be considered equal across countries, even under optimal conditions, and that Intergrowth-21st may greatly over-estimate SGA.

Furthermore, we did not advise women to refrain from sexual activity between ANC visits nor was partner treatment included. And therefore, we cannot be sure how many cases at day 28 were attributable to reinfection. This is an important limitation. Despite the high prevalence of syphilis in our trial (greater than 5%), testing for syphilis was only done once at enrolment and there was no measurement of the effectiveness of the treatment. Testing for syphilis was confirmed by TPHA which was conducted on all RPR reactive samples. Of those who tested positive by RPR, 95.9% (791/825) were treated with 2.4 million units of intramuscular benzathine penicillin according to the WHO

recommendation. Screening using RPR testing is recommended at antennal booking in Zambia and treatment is offered to all pregnant women who test positive. However, repeat testing is not recommended during the same pregnancy and to test of cure is conducted.

The trial was also negatively affected by the covid pandemic. The first case of Covid-19 in Zambia was reported on 18 February 2020 which was three months after the commencement of the trial. This prompted the Zambian government to put up measures to combat the spread of Covid-19. Despite the Covid-19 cases increasing especially during the first and second waves, recruitment and follow up of participants continued as guidelines were put in place to protect the participants and study staffs. The infection prevention measures slowed down the recruitment of study participants and also increased the cost of running the study. In addition, the study supplies such as investigational products and laboratory supplies could not be accessed easily due closures of borders and lockdown of most countries.

The strengths of this trial are that it was powered to detect differences in the composite primary endpoint of adverse pregnancy outcomes and used ultrasound to accurately determine gestational age. The trial had a large sample size of 5,436 (1,812 per arm) pregnant women and was powered to detect a 25% relative reduction in adverse birth outcomes, from 15% in the IPTp-SP arm to 11.25%, in either of the two intervention arms with 80% power and a significance level of 2.5%. The sample size allowed for 15% loss to follow up. Furthermore, adherence to the follow-up schedule was good, with less than 15% of loss to follow-up. There is sustained malaria transmission throughout the year in this area with no real seasonality though there is an observable increase in transmission in the few months after the rainy season which runs from November to April. However, our recruitment included 2 rainy seasons from December 2019 to March 2022. And therefore, the observed prevalence of malaria, STIs/RTIs, and malaria and STI/RTI coinfection are more likely to be an accurate estimation. On the other hand, misclassification of some of the infected participants as uninfected was reduced by

diagnosis of malaria and some curable STIs/RTIs by PCR methods which generally have higher sensitivities than traditional methods.

7.6 Conclusions

Despite the high parasite resistance to SP, and despite DP being better at reducing maternal malaria, SP was not inferior in protecting against adverse pregnancy outcomes. DP-MTZ was superior to SP and SP-MTZ against maternal and placental malaria, whereas SP-containing regimens were better at reducing the prevalence of curable STIs/RTIs. The prevalence of *dhps* and *dhfr* resistance markers remains high in this area but still the effect of IPTp-SP is not inferior to IPTp-DP-MTZ. This effect of SP suggests specific non-malarial pathways to reducing adverse pregnancy outcomes. Alternative regimens to address the dual burden of malaria and STIs/RTIs are still needed in this setting.

7.7 Recommendations

The results of our study have programmatic implications. Countries with a high burden of malaria, should continue using IPTp-SP because of lack of a superior alternative regimen. Point of care tests are urgently needed to screen and treat pregnant women for curable STIs/RTIs. Research to understand the mechanism of the non-malarial pathways by which IPTp-SP protects against adverse pregnancy outcomes, despite not being superior to IPTp-DP in reducing the incidence of malaria, need to be conducted. In view of the high prevalence on NG seen at enrolment, it is recommended that a policy of administering antibiotic eye ointment to newborn babies be implemented to prevent the occurrence of ophthalmia neonatorum and its complications.

Community messages concerning safe sex practices like condom use should be enhanced to reduce the burden of STIs. Local radio stations can be used for this purpose. In addition, safe sex messages should also be targeted at truck drivers, who are a high-risk group for

STIs, and spend nights in Nchelenge district on their way to crossing the borders into the Democratic Republic Congo.

In addition, this calls for interventions to improve pregnancy outcomes to be implemented in the first trimester to prevent early infections with malaria and STIs/RTIs. Screening for infections in early pregnancy should be given priority in a test-and-treat strategy.

7.8 Future work

Continued research to look for alternative regimen to IPTp-SP needs to continue. Trials to test the efficacy of the combination of IPTp-SP and DP need to be encouraged in order to understand the impact on adverse pregnancy outcomes. We also need to conduct studies to characterise the effect of treatment on across trial arms on the vaginal and intestinal microbiome and maternal cytokines. In addition, there is need to conduct *in vitro* testing of sulfadoxine and other antimicrobial agents to measure the drug sensitivity of several pathogens implicated with WHO syndromes of vaginal discharge, lower abdominal pain, or genital ulcers in the presence of sulfadoxine and other antimicrobial agents. Another consideration is to provide preconception malaria vaccination with RTSS or R21 vaccines to prevent malaria in the early pregnancy. The vaccination strategy can be combined with the test-and-treat strategy which can focus on screening for genital infections.

8 References

Uncategorized References

1. Meeusen EN, Bischof RJ, Lee CS. Comparative T-cell responses during pregnancy in large animals and humans. Am J Reprod Immunol. 2001;46(2):169-79.
2. Abu-Raya B, Michalski C, Sadarangani M, Lavoie PM. Maternal Immunological Adaptation During Normal Pregnancy. Front Immunol. 2020;11:575197.
3. Fakonti G, Pantazi P, Bokun V, Holder B. Placental Macrophage (Hofbauer Cell) Responses to Infection During Pregnancy: A Systematic Scoping Review. Front Immunol. 2021;12:756035.
4. Chua CLL, Hasang W, Rogerson SJ, Teo A. Poor Birth Outcomes in Malaria in Pregnancy: Recent Insights Into Mechanisms and Prevention Approaches. Front Immunol. 2021;12:621382.
5. Sacks GP, Studena K, Sargent K, Redman CW. Normal pregnancy and preeclampsia both produce inflammatory changes in peripheral blood leukocytes akin to those of sepsis. Am J Obstet Gynecol. 1998;179(1):80-6.
6. Gomez-Lopez N, Romero R, Hassan SS, Bhatti G, Berry SM, Kusanovic JP, et al. The Cellular Transcriptome in the Maternal Circulation During Normal Pregnancy: A Longitudinal Study. Front Immunol. 2019;10:2863.
7. Nkumama IN, O'Meara WP, Osier FHA. Changes in Malaria Epidemiology in Africa and New Challenges for Elimination. Trends Parasitol. 2017;33(2):128-40.
8. Barboza R, Lima FA, Reis AS, Murillo OJ, Peixoto EPM, Bandeira CL, et al. TLR4-Mediated Placental Pathology and Pregnancy Outcome in Experimental Malaria. Sci Rep. 2017;7(1):8623.
9. Fried M, Duffy PE. Malaria during Pregnancy. Cold Spring Harb Perspect Med. 2017;7(6).
10. Fried M, Duffy PE. Adherence of Plasmodium falciparum to chondroitin sulfate A in the human placenta. Science (New York, NY). 1996;272(5267):1502-4.
11. Garnham PCC. The placenta in malaria with special reference to reticuloendothelial immunity. Trans R Soc Trop Med Hyg 1938;32(1):13–22.
12. Zakama AK, Ozarslan N, Gaw SL. Placental Malaria. Curr Trop Med Rep. 2020:1-10.
13. Poespoprodjo JR, Fobia W, Kenangalem E, Lampah DA, Warikar N, Seal A, et al. Adverse pregnancy outcomes in an area where multidrug-resistant plasmodium vivax and Plasmodium falciparum infections are endemic. Clin Infect Dis. 2008;46(9):1374-81.
14. Zango SH, Lingani M, Valea I, Samadoulougou OS, Bihoun B, Lankoande D, et al. Association of malaria and curable sexually transmitted infections with pregnancy outcomes in rural Burkina Faso. BMC Pregnancy Childbirth. 2021;21(1):722.
15. Spiegel CA. Bacterial vaginosis. Clin Microbiol Rev. 1991;4(4):485-502.
16. Swidsinski A, Verstraelen H, Loening-Baucke V, Swidsinski S, Mendling W, Halwani Z. Presence of a polymicrobial endometrial biofilm in patients with bacterial vaginosis. PLoS One. 2013;8(1):e53997.
17. Rosca AS, Castro J, Sousa LGV, Cerca N. Gardnerella and vaginal health: the truth is out there. FEMS Microbiol Rev. 2020;44(1):73-105.
18. Brotman RM, Klebanoff MA, Nansel TR, Yu KF, Andrews WW, Zhang J, et al. Bacterial vaginosis assessed by gram stain and diminished colonization resistance to incident gonococcal, chlamydial, and trichomonal genital infection. J Infect Dis. 2010;202(12):1907-15.
19. Lokken EM, Balkus JE, Kiarie J, Hughes JP, Jaoko W, Totten PA, et al. Association of Recent Bacterial Vaginosis With Acquisition of Mycoplasma genitalium. Am J Epidemiol. 2017;186(2):194-201.
20. Atashili J, Poole C, Ndumbe PM, Adimora AA, Smith JS. Bacterial vaginosis and HIV acquisition: a meta-analysis of published studies. Aids. 2008;22(12):1493-501.
21. Donnes A. Animalcules observés dans les matières purulentes et le produit des sécrétions des organes génitaux de l'homme et da la femme. Comptes Rendus Hebdomadaires des Séances de l'Académie des Sciences 1836;12(3):385-6.
22. Hernández HM, Marcet R, Sarracent J. Biological roles of cysteine proteinases in the pathogenesis of Trichomonas vaginalis. Parasite. 2014;21:54.
23. Provenzano D, Khoshnan A, Alderete JF. Involvement of dsRNA virus in the protein composition and growth kinetics of host Trichomonas vaginalis. Arch Virol. 1997;142(5):939-52.
24. Engbring JA, Alderete JF. Characterization of Trichomonas vaginalis AP33 adhesin and cell surface interactive domains. Microbiology (Reading). 1998;144 (Pt 11):3011-8.

25. Kucknoor A, Mundodi V, Alderete JF. Trichomonas vaginalis adherence mediates differential gene expression in human vaginal epithelial cells. Cell Microbiol. 2005;7(6):887-97.
26. Kucknoor AS, Mundodi V, Alderete JF. Adherence to human vaginal epithelial cells signals for increased expression of Trichomonas vaginalis genes. Infection and immunity. 2005;73(10):6472-8.
27. WHO. Meeting report of the Evidence Review Group on intermittent preventive treatment (IPT) of malaria in pregnancy. 2013.
28. Feng G, Simpson JA, Chaluluka E, Molyneux ME, Rogerson SJ. Decreasing burden of malaria in pregnancy in Malawian women and its relationship to use of intermittent preventive therapy or bed nets. PLoS One. 2010;5(8):e12012.
29. Harrington WE, Mutabingwa TK, Kabyemela E, Fried M, Duffy PE. Intermittent treatment to prevent pregnancy malaria does not confer benefit in an area of widespread drug resistance. Clin Infect Dis. 2011;53(3):224-30.
30. Mama A, Ahiabor C, Tornyigah B, Frempong NA, Kusi KA, Adu B, et al. Intermittent preventive treatment in pregnancy with sulfadoxine-pyrimethamine and parasite resistance: cross-sectional surveys from antenatal care visit and delivery in rural Ghana. Malar J. 2022;21(1):107.
31. Kayentao K, Garner P, van Eijk AM, Naidoo I, Roper C, Mulokozi A, et al. Intermittent preventive therapy for malaria during pregnancy using 2 vs 3 or more doses of sulfadoxine-pyrimethamine and risk of low birth weight in Africa: systematic review and meta-analysis. Jama. 2013;309(6):594-604.
32. World Health Organization. Management of patients with sexually transmitted diseases. Report of a WHO Study Group. 1991. Report No.: 0512-3054 (Print)

0512-3054 (Linking).
33. WHO. Guidelines for the management of symptomatic sexually transmitted infections. 2021.
34. Pettifor A, Walsh J, Wilkins V, Raghunathan P. How effective is syndromic management of STDs?: A review of current studies. Sexually transmitted diseases. 2000;27(7):371-85.
35. Chico RM, Mayaud P, Ariti C, Mabey D, Ronsmans C, Chandramohan D. Prevalence of malaria and sexually transmitted and reproductive tract infections in pregnancy in sub-Saharan Africa: a systematic review. Jama. 2012;307(19):2079-86.
36. Chaponda EB, Chico RM, Bruce J, Michelo C, Vwalika B, Mharakurwa S, et al. Malarial Infection and Curable Sexually Transmitted and Reproductive Tract Infections Among Pregnant Women in a Rural District of Zambia. Am J Trop Med Hyg. 2016;95(5):1069-76.
37. Mikomangwa WP, Oms M, Aklillu E, Kamuhabwa AAR. Adverse birth outcomes among mothers who received intermittent preventive treatment with Sulphadoxine-Pyrimethamine in the low malaria transmission region. BMC Pregnancy Childbirth. 2019;19(1):236.
38. Organization WH. World malaria report 2019. Report. 2019.
39. WHO. World malaria Report. 2022.
40. Guyatt HL, Snow RW. Impact of malaria during pregnancy on low birth weight in sub-Saharan Africa. Clin Microbiol Rev. 2004;17(4):760-9, table of contents.
41. Azizi SC, Chongwe G, Chipukuma H, Jacobs C, Zgambo J, Michelo C. Uptake of intermittent preventive treatment for malaria during pregnancy with Sulphadoxine-Pyrimethamine (IPTp-SP) among postpartum women in Zomba District, Malawi: a cross-sectional study. BMC Pregnancy Childbirth. 2018;18(1):108.
42. van Eijk AM, Stepniewska K, Hill J, Taylor SM, Rogerson SJ, Cottrell G, et al. Prevalence of and risk factors for microscopic and submicroscopic malaria infections in pregnancy: a systematic review and meta-analysis. Lancet Glob Health. 2023;11(7):e1061-e74.
43. Mohammed AH, Salih MM, Elhassan EM, Mohmmed AA, Elzaki SE, El-Sayed BB, et al. Submicroscopic Plasmodium falciparum malaria and low birth weight in an area of unstable malaria transmission in Central Sudan. Malar J. 2013;12:172.
44. Moore KA, Fowkes FJI, Wiladphaingern J, Wai NS, Paw MK, Pimanpanarak M, et al. Mediation of the effect of malaria in pregnancy on stillbirth and neonatal death in an area of low transmission: observational data analysis. BMC Med. 2017;15(1):98.
45. Desai M, ter Kuile FO, Nosten F, McGready R, Asamoa K, Brabin B, et al. Epidemiology and burden of malaria in pregnancy. Lancet Infect Dis. 2007;7(2):93-104.
46. Cates JE, Unger HW, Briand V, Fievet N, Valea I, Tinto H, et al. Malaria, malnutrition, and birthweight: A meta-analysis using individual participant data. PLoS Med. 2017;14(8):e1002373.

47. Darling AM, McDonald CR, Urassa WS, Kain KC, Mwiru RS, Fawzi WW. Maternal Dietary L-Arginine and Adverse Birth Outcomes in Dar es Salaam, Tanzania. Am J Epidemiol. 2017;186(5):603-11.
48. McDonald CR, Cahill LS, Gamble JL, Elphinstone R, Gazdzinski LM, Zhong KJY, et al. Malaria in pregnancy alters l-arginine bioavailability and placental vascular development. Sci Transl Med. 2018;10(431).
49. Brabin BJ. An analysis of malaria in pregnancy in Africa. Bull World Health Organ. 1983;61(6):1005-16.
50. McGregor IA. Epidemiology, malaria and pregnancy. Am J Trop Med Hyg. 1984;33(4):517-25.
51. Watson-Jones D, Weiss HA, Changalucha JM, Todd J, Gumodoka B, Bulmer J, et al. Adverse birth outcomes in United Republic of Tanzania--impact and prevention of maternal risk factors. Bulletin of the World Health Organization. 2007;85(1):9-18.
52. McGregor IA, Wilson ME, Billewicz WZ. Malaria infection of the placenta in The Gambia, West Africa; its incidence and relationship to stillbirth, birthweight and placental weight. Trans R Soc Trop Med Hyg. 1983;77(2):232-44.
53. Bader E, Alhaj AM, Hussan AA, Adam I. Malaria and stillbirth in Omdurman Maternity Hospital, Sudan. International journal of gynaecology and obstetrics: the official organ of the International Federation of Gynaecology and Obstetrics. 2010;109(2):144-6.
54. Yatich NJ, Funkhouser E, Ehiri JE, Agbenyega T, Stiles JK, Rayner JC, et al. Malaria, intestinal helminths and other risk factors for stillbirth in Ghana. Infect Dis Obstet Gynecol. 2010;2010:350763.
55. Greenwood AM, Armstrong JR, Byass P, Snow RW, Greenwood BM. Malaria chemoprophylaxis, birth weight and child survival. Trans R Soc Trop Med Hyg. 1992;86(5):483-5.
56. Guyatt HL, Snow RW. Malaria in pregnancy as an indirect cause of infant mortality in sub-Saharan Africa. Trans R Soc Trop Med Hyg. 2001;95(6):569-76.
57. Murphy SC, Breman JG. Gaps in the childhood malaria burden in Africa: cerebral malaria, neurological sequelae, anemia, respiratory distress, hypoglycemia, and complications of pregnancy. Am J Trop Med Hyg. 2001;64(1-2 Suppl):57-67.
58. Steketee RW, Nahlen BL, Parise ME, Menendez C. The burden of malaria in pregnancy in malaria-endemic areas. Am J Trop Med Hyg. 2001;64(1-2 Suppl):28-35.
59. Lawn JE, Blencowe H, Waiswa P, Amouzou A, Mathers C, Hogan D, et al. Stillbirths: rates, risk factors, and acceleration towards 2030. Lancet. 2016.
60. Steketee RW, Wirima JJ, Hightower AW, Slutsker L, Heymann DL, Breman JG. The effect of malaria and malaria prevention in pregnancy on offspring birthweight, prematurity, and intrauterine growth retardation in rural Malawi. Am J Trop Med Hyg. 1996;55(1 Suppl):33-41.
61. Sullivan AD, Nyirenda T, Cullinan T, Taylor T, Harlow SD, James SA, et al. Malaria infection during pregnancy: intrauterine growth retardation and preterm delivery in Malawi. J Infect Dis. 1999;179(6):1580-3.
62. Blencowe H, Krasevec J, de Onis M, Black RE, An X, Stevens GA, et al. National, regional, and worldwide estimates of low birthweight in 2015, with trends from 2000: a systematic analysis. Lancet Glob Health. 2019;7(7):e849-e60.
63. Adam I, Elhassan EM, Haggaz AE, Ali AA, Adam GK. A perspective of the epidemiology of malaria and anaemia and their impact on maternal and perinatal outcomes in Sudan. J Infect Dev Ctries. 2011;5(2):83-7.
64. Campos PA, Valente B, Campos RB, Gonçalves L, Rosário VE, Varandas L, et al. Plasmodium falciparum infection in pregnant women attending antenatal care in Luanda, Angola. Revista da Sociedade Brasileira de Medicina Tropical. 2012;45(3):369-74.
65. Moore KA, Simpson JA, Scoullar MJL, McGready R, Fowkes FJI. Quantification of the association between malaria in pregnancy and stillbirth: a systematic review and meta-analysis. Lancet Glob Health. 2017;5(11):e1101-e12.
66. Kovacs SD, Rijken MJ, Stergachis A. Treating severe malaria in pregnancy: a review of the evidence. Drug Saf. 2015;38(2):165-81.
67. Umbers AJ, Aitken EH, Rogerson SJ. Malaria in pregnancy: small babies, big problem. Trends Parasitol. 2011;27(4):168-75.
68. Verhoeff FH, Le Cessie S, Kalanda BF, Kazembe PN, Broadhead RL, Brabin BJ. Post-neonatal infant mortality in Malawi: the importance of maternal health. Ann Trop Paediatr. 2004;24(2):161-9.

69. Thompson JM, Eick SM, Dailey C, Dale AP, Mehta M, Nair A, et al. Relationship Between Pregnancy-Associated Malaria and Adverse Pregnancy Outcomes: a Systematic Review and Meta-Analysis. J Trop Pediatr. 2020;66(3):327-38.
70. van den Broek NR, Jean-Baptiste R, Neilson JP. Factors associated with preterm, early preterm and late preterm birth in Malawi. PLoS One. 2014;9(3):e90128.
71. Centre NME. Zambia National Malaria Indicator Survey 2018. 2019.
72. NMEC. Guidelines for the Diagnosis and Treatment of Malaria in Zambia. 2017.
73. Sitali L, Chipeta J, Miller JM, Moonga HB, Kumar N, Moss WJ, et al. Patterns of mixed Plasmodium species infections among children six years and under in selected malaria hyper-endemic communities of Zambia: population-based survey observations. BMC Infect Dis. 2015;15:204.
74. Laban NM, Kobayashi T, Hamapumbu H, Sullivan D, Mharakurwa S, Thuma PE, et al. Comparison of a PfHRP2-based rapid diagnostic test and PCR for malaria in a low prevalence setting in rural southern Zambia: implications for elimination. Malar J. 2015;14:25.
75. Thu AM, Phyo AP, Landier J, Parker DM, Nosten FH. Combating multidrug-resistant Plasmodium falciparum malaria. Febs j. 2017;284(16):2569-78.
76. Thanh NV, Thuy-Nhien N, Tuyen NT, Tong NT, Nha-Ca NT, Dong LT, et al. Rapid decline in the susceptibility of Plasmodium falciparum to dihydroartemisinin-piperaquine in the south of Vietnam. Malar J. 2017;16(1):27.
77. Imwong M, Jindakhad T, Kunasol C, Sutawong K, Vejakama P, Dondorp AM. An outbreak of artemisinin resistant falciparum malaria in Eastern Thailand. Sci Rep. 2015;5:17412.
78. Anon. A Six-year Strategic plan: a roadmap for impact on malaria in Zambia 2006-2011. Ministry of Health, Government of the Republic of Zambia; 2006.
79. Ministry of Health. Guidelines for the diagnosis and treatment of Malaria in Zambia. Third ed. Lusaka: Ministry of Health; 2010. p. 41-2.
80. Gamble C, Ekwaru JP, ter Kuile FO. Insecticide-treated nets for preventing malaria in pregnancy. Cochrane Database Syst Rev. 2006;2006(2):Cd003755.
81. Figueroa-Romero A, Pons-Duran C, Gonzalez R. Drugs for Intermittent Preventive Treatment of Malaria in Pregnancy: Current Knowledge and Way Forward. Trop Med Infect Dis. 2022;7(8).
82. Health Mo. Zambia National Malaria Indicator Survey 2021. 2021.
83. Chaponda EB, Chandramohan D, Michelo C, Mharakurwa S, Chipeta J, Chico RM. High burden of malaria infection in pregnant women in a rural district of Zambia: a cross-sectional study. Malar J. 2015;14:380.
84. Siame MN, Mharakurwa S, Chipeta J, Thuma P, Michelo C. High prevalence of dhfr and dhps molecular markers in Plasmodium falciparum in pregnant women of Nchelenge district, Northern Zambia. Malaria journal. 2015;14:190.
85. van Dongen PW, van't Hof MA. Sickle cell trait, malaria and anaemia in pregnant Zambian women. Trans R Soc Trop Med Hyg. 1983;77(3):402-4.
86. Larkin GL, Thuma PE. Congenital malaria in a hyperendemic area. Am J Trop Med Hyg. 1991;45(5):587-92.
87. Depoortere E, Guthmann JP, Pressé J, Sipilanyambe N, Nkandu E, Balkan S, et al. Efficacy and effectiveness of the combination of sulfadoxine/pyrimethamine and a 3-day course of artesunate for the treatment of uncomplicated falciparum malaria in a refugee settlement in Zambia. Trop Med Int Health. 2005;10(2):139-45.
88. Tan KR, Katalenich BL, Mace KE, Nambozi M, Taylor SM, Meshnick SR, et al. Efficacy of sulphadoxine-pyrimethamine for intermittent preventive treatment of malaria in pregnancy, Mansa, Zambia. Malar J. 2014;13:227.
89. ICF CsaCoEa. Ethiopia demographic and health survey 2011. 2017.
90. Central Statistical Office (CSO) [Zambia], Ministry of Health (MOH) [Zambia], ICF International. Zambia Demographic and Health Survey 2013-14. Rockville, Maryland, USA: Central Statistical Office, Ministry of Health, and ICF International 2014.
91. Olusanya BO, Ofovwe GE. Predictors of preterm births and low birthweight in an inner-city hospital in sub-Saharan Africa. Maternal and child health journal. 2010;14(6):978-86.
92. CSO. Zambia Demographic and Health Survey 2018. 2020.
93. Central Statistical Office (CSO) MoHM TDRCT, University of Zambia, Macro International Inc: . Zambia Demographic and Health Survey 2007. Calverton, Maryland, USA: CSO and Macro International Inc.; 2009.

94. Minstry of Health. NATIONAL HIV/AIDS/STI/TB POLICY. Lusaka2002.
95. WHO. WHO Guidelines for malaria. 2022.
96. Radeva-Petrova D, Kayentao K, ter Kuile FO, Sinclair D, Garner P. Drugs for preventing malaria in pregnant women in endemic areas: any drug regimen versus placebo or no treatment. Cochrane Database Syst Rev. 2014;2014(10):Cd000169.
97. Chico RM, Dellicour S, Roman E, Mangiaterra V, Coleman J, Menendez C, et al. Global Call to Action: maximize the public health impact of intermittent preventive treatment of malaria in pregnancy in sub-Saharan Africa. Malar J. 2015;14:207.
98. Amimo F, Lambert B, Magit A, Sacarlal J, Hashizume M, Shibuya K. Plasmodium falciparum resistance to sulfadoxine-pyrimethamine in Africa: a systematic analysis of national trends. BMJ Glob Health. 2020;5(11).
99. Mlugu EM, Minzi O, Asghar M, Färnert A, Kamuhabwa AAR, Aklillu E. Effectiveness of Sulfadoxine-Pyrimethamine for Intermittent Preventive Treatment of Malaria and Adverse Birth Outcomes in Pregnant Women. Pathogens. 2020;9(3).
100. van Eijk AM, Larsen DA, Kayentao K, Koshy G, Slaughter DEC, Roper C, et al. Effect of Plasmodium falciparum sulfadoxine-pyrimethamine resistance on the effectiveness of intermittent preventive therapy for malaria in pregnancy in Africa: a systematic review and meta-analysis. Lancet Infect Dis. 2019;19(5):546-56.
101. Kajubi R, Ochieng T, Kakuru A, Jagannathan P, Nakalembe M, Ruel T, et al. Monthly sulfadoxine-pyrimethamine versus dihydroartemisinin-piperaquine for intermittent preventive treatment of malaria in pregnancy: a double-blind, randomised, controlled, superiority trial. Lancet. 2019;393(10179):1428-39.
102. Kakuru A, Jagannathan P, Muhindo MK, Natureeba P, Awori P, Nakalembe M, et al. Dihydroartemisinin-Piperaquine for the Prevention of Malaria in Pregnancy. N Engl J Med. 2016;374(10):928-39.
103. Desai M, Gutman J, L'Lanziva A, Otieno K, Juma E, Kariuki S, et al. Intermittent screening and treatment or intermittent preventive treatment with dihydroartemisinin-piperaquine versus intermittent preventive treatment with sulfadoxine-pyrimethamine for the control of malaria during pregnancy in western Kenya: an open-label, three-group, randomised controlled superiority trial. Lancet. 2015;386(10012):2507-19.
104. Madanitsa M, Barsosio HC, Minja DTR, Mtove G, Kavishe RA, Dodd J, et al. Effect of monthly intermittent preventive treatment with dihydroartemisinin-piperaquine with and without azithromycin versus monthly sulfadoxine-pyrimethamine on adverse pregnancy outcomes in Africa: a double-blind randomised, partly placebo-controlled trial. Lancet. 2023;401(10381):1020-36.
105. Kozuki N, Lee AC, Silveira MF, Sania A, Vogel JP, Adair L, et al. The associations of parity and maternal age with small-for-gestational-age, preterm, and neonatal and infant mortality: a meta-analysis. BMC public health. 2013;13 Suppl 3:S2.
106. Roh ME, Kuile FOT, Rerolle F, Glymour MM, Shiboski S, Gosling R, et al. Overall, anti-malarial, and non-malarial effect of intermittent preventive treatment during pregnancy with sulfadoxine-pyrimethamine on birthweight: a mediation analysis. Lancet Glob Health. 2020;8(7):e942-e53.
107. Nyemba DC, Haddison EC, Wang C, Johnson LF, Myer L, Davey DJ. Prevalence of curable STIs and bacterial vaginosis during pregnancy in sub-Saharan Africa: a systematic review and meta-analysis. Sex Transm Infect. 2022;98(7):484-91.
108. Offorjebe OA, Wynn A, Moshashane N, Joseph Davey D, Arena K, Ramogola-Masire D, et al. Partner notification and treatment for sexually transmitted infections among pregnant women in Gaborone, Botswana. Int J STD AIDS. 2017;28(12):1184-9.
109. Yeganeh N, Kreitchmann R, Leng M, Nielsen-Saines K, Gorbach PM, Klausner JD. Diagnosis and treatment of sexually transmitted infections in male partners of pregnant women in Brazil. Int J STD AIDS. 2021;32(13):1242-9.
110. Kissinger P, Hogben M. Expedited partner treatment for sexually transmitted infections: an update. Curr Infect Dis Rep. 2011;13(2):188-95.
111. McDermott J, Steketee R, Larsen S, Wirima J. Syphilis-associated perinatal and infant mortality in rural Malawi. Bulletin of the World Health Organization. 1993;71(6):773-80.
112. Watson-Jones D CJ, Balthazar G, Weiss H, Rusizoka M, Ndeki L,Whitehouse A, Balira R, Todd J, Ngeleja D, Ross D, Buvé A, Hayes R, Mabey D Syphilis in pregnancy in Tanzania. I. Impact of maternal syphilis on outcome of pregnancy. J Infect Dis. 2002;186:940-7.

113. Watson-Jones D, Gumodoka B, Weiss H, Changalucha J, Todd J, Mugeye K, et al. Syphilis in pregnancy in Tanzania. II. The effectiveness of antenatal syphilis screening and single-dose benzathine penicillin treatment for the prevention of adverse pregnancy outcomes. J Infect Dis. 2002;186(7):948-57.
114. Trinh T, Leal AF, Mello MB, Taylor MM, Barrow R, Wi TE, et al. Syphilis management in pregnancy: a review of guideline recommendations from countries around the world. Sex Reprod Health Matters. 2019;27(1):69-82.
115. Tong H, Heuer A, Walker N. The impact of antibiotic treatment for syphilis, chlamydia, and gonorrhoea during pregnancy on birth outcomes: A systematic review and meta-analysis. J Glob Health. 2023;13:04058.
116. Temmerman M, Gichangi P, Fonck K, Apers L, Claeys P, Van Renterghem L, et al. Effect of a syphilis control programme on pregnancy outcome in Nairobi, Kenya. Sex Transm Infect. 2000;76(2):117-21.
117. Gamberini C, Juliana NCA, de Brouwer L, Vogelsang D, Al-Nasiry S, Morré SA, et al. The association between adverse pregnancy outcomes and non-viral genital pathogens among women living in sub-Saharan Africa: a systematic review. Front Reprod Health. 2023;5:1107931.
118. van Rensburg HJ, Odendaal HJ. The prevalence of potential pathogenic micro-organisms in the endocervix of pregnant women at Tygerberg Hospital. South African medical journal = Suid-Afrikaanse tydskrif vir geneeskunde. 1992;81(3):156-7.
119. Rours GI, Duijts L, Moll HA, Arends LR, de Groot R, Jaddoe VW, et al. Chlamydia trachomatis infection during pregnancy associated with preterm delivery: a population-based prospective cohort study. European journal of epidemiology. 2011;26(6):493-502.
120. Odendaal HJ, Schoeman J, Grove D, de Jager M, Theron GB, Orth H, et al. The association between Chlamydia trachomatis genital infection and spontaneous preterm labour. South African Journal of Obstetrics and Gynaecology. 2006;12(3):146-9.
121. Baud D, Goy G, Vasilevsky S, Osterheld MC, Roth-Kleiner M, Croxatto A, et al. Roles of bovine Waddlia chondrophila and Chlamydia trachomatis in human preterm birth. New microbes and new infections. 2015;3:41-5.
122. Nakubulwa S, Kaye DK, Bwanga F, Tumwesigye NM, Mirembe FM. Genital infections and risk of premature rupture of membranes in Mulago Hospital, Uganda: a case control study. BMC research notes. 2015;8:573.
123. Liu B, Roberts CL, Clarke M, Jorm L, Hunt J, Ward J. Chlamydia and gonorrhoea infections and the risk of adverse obstetric outcomes: a retrospective cohort study. Sex Transm Infect. 2013;89(8):672-8.
124. Johnson HL, Ghanem KG, Zenilman JM, Erbelding EJ. Sexually transmitted infections and adverse pregnancy outcomes among women attending inner city public sexually transmitted diseases clinics. Sexually transmitted diseases. 2011;38(3):167-71.
125. Blas MM, Canchihuaman FA, Alva IE, Hawes SE. Pregnancy outcomes in women infected with Chlamydia trachomatis: A population-based cohort study in Washington State. Sexually Transmitted Infections. 2007;83(4):314-8.
126. Investigators of the Johns Hopkins Study of Cervicitis and Adverse Pregnancy Outcome. Association of Chlamydia trachomatis and Mycoplasma hominis with intrauterine growth retardation and preterm delivery. Am J Epidemiol 1989;129:1247–57.
127. Gravett MG, Nelson HP, DeRouen T, Critchlow C, Eschenbach DA, Holmes KK. Independent associations of bacterial vaginosis and Chlamydia trachomatis infection with adverse pregnancy outcome. JAMA. 1986;256(14):1899-903.
128. Harrison HR, Alexander ER, Weinstein L, Lewis M, Nash M, Sim DA. Cervical Chlamydia trachomatis and mycoplasmal infections in pregnancy. Epidemiology and outcomes. JAMA. 1983;250(13):1721-7.
129. Folger AT. Maternal Chlamydia trachomatis infections and preterm birth:the impact of early detection and eradication during pregnancy. Maternal and child health journal. 2014;18(8):1795-802.
130. Vaezzadeh K, Sepidarkish M, Mollalo A, As'adi N, Rouholamin S, Rezaeinejad M, et al. Global prevalence of Neisseria gonorrhoeae infection in pregnant women: a systematic review and meta-analysis. Clin Microbiol Infect. 2023;29(1):22-31.

131. Vallely LM, Egli-Gany D, Wand H, Pomat WS, Homer CSE, Guy R, et al. Adverse pregnancy and neonatal outcomes associated with Neisseria gonorrhoeae: systematic review and meta-analysis. Sex Transm Infect. 2021;97(2):104-11.
132. Lacey CJ, Milne JD. Preterm labour in association with Neisseria gonorrhoeae: case reports. The British journal of venereal diseases. 1984;60(2):123-4.
133. Elliott B, Brunham RC, Laga M, Piot P, Ndinya-Achola JO, Maitha G, et al. Maternal gonococcal infection as a preventable risk factor for low birth weight. J Infect Dis. 1990;161(3):531-6.
134. Donders GG, Desmyter J, De Wet DH, Van Assche FA. The association of gonorrhoea and syphilis with premature birth and low birthweight. Genitourin Med. 1993;69(2):98-101.
135. Olaleye AO, Babah OA, Osuagwu CS, Ogunsola FT, Afolabi BB. Sexually transmitted infections in pregnancy - An update on Chlamydia trachomatis and Neisseria gonorrhoeae. Eur J Obstet Gynecol Reprod Biol. 2020;255:1-12.
136. Crucitti T, Van Dyck E, Tehe A, Abdellati S, Vuylsteke B, Buve A, et al. Comparison of culture and different PCR assays for detection of Trichomonas vaginalis in self collected vaginal swab specimens. Sex Transm Infect. 2003;79(5):393-8.
137. Cotch MF, Pastorek JG, 2nd, Nugent RP, Hillier SL, Gibbs RS, Martin DH, et al. Trichomonas vaginalis associated with low birth weight and preterm delivery. The Vaginal Infections and Prematurity Study Group. Sexually transmitted diseases. 1997;24(6):353-60.
138. Gülmezoglu AM, Azhar M. Interventions for trichomoniasis in pregnancy. Cochrane Database Syst Rev. 2011;2011(5):Cd000220.
139. Oyeyemi OT, Fadipe O, Oyeyemi IT. Trichomonas vaginalis infection in Nigerian pregnant women and risk factors associated with sexually transmitted infections. Int J STD AIDS. 2016;27(13):1187-93.
140. Edwards T, Burke P, Smalley H, Hobbs G. Trichomonas vaginalis: Clinical relevance, pathogenicity and diagnosis. Crit Rev Microbiol. 2016;42(3):406-17.
141. Adamson PC, Loeffelholz MJ, Klausner JD. Point-of-Care Testing for Sexually Transmitted Infections: A Review of Recent Developments. Arch Pathol Lab Med. 2020;144(11):1344-51.
142. Van Gerwen OT, Craig-Kuhn MC, Jones AT, Schroeder JA, Deaver J, Buekens P, et al. Trichomoniasis and adverse birth outcomes: a systematic review and meta-analysis. Bjog. 2021;128(12):1907-15.
143. Sutton MY, Sternberg M, Nsuami M, Behets F, Nelson AM, St Louis ME. Trichomoniasis in pregnant human immunodeficiency virus-infected and human immunodeficiency virus-uninfected congolese women: prevalence, risk factors, and association with low birth weight. Am J Obstet Gynecol. 1999;181(3):656-62.
144. Minkoff H, Grunebaum AN, Schwarz RH, Feldman J, Cummings M, Crombleholme W, et al. Risk factors for prematurity and premature rupture of membranes: a prospective study of the vaginal flora in pregnancy. Am J Obstet Gynecol. 1984;150(8):965-72.
145. Hardy PH, Hardy JB, Nell EE, Graham DA, Spence MR, Rosenbaum RC. Prevalence of six sexually transmitted disease agents among pregnant inner-city adolescents and pregnancy outcome. Lancet. 1984;2(8398):333-7.
146. Schwebke JR, Burgess D. Trichomoniasis. Clin Microbiol Rev. 2004;17(4):794-803, table of contents.
147. Kissinger P. Trichomonas vaginalis: a review of epidemiologic, clinical and treatment issues. BMC Infect Dis. 2015;15:307.
148. Seña AC, Bachmann LH, Hobbs MM. Persistent and recurrent Trichomonas vaginalis infections: epidemiology, treatment and management considerations. Expert Rev Anti Infect Ther. 2014;12(6):673-85.
149. Sangkomkamhang US, Lumbiganon P, Prasertcharoensuk W, Laopaiboon M. Antenatal lower genital tract infection screening and treatment programs for preventing preterm delivery. Cochrane Database Syst Rev. 2015;2015(2):Cd006178.
150. Nyemba DC, Peters RPH, Medina-Marino A, Klausner JD, Ngwepe P, Myer L, et al. Impact of aetiological screening of sexually transmitted infections during pregnancy on pregnancy outcomes in South Africa. BMC Pregnancy Childbirth. 2022;22(1):194.
151. Ross SM, van Middelkoop A. Trichomonas infection in pregnancy--does it affect perinatal outcome? South African medical journal = Suid-Afrikaanse tydskrif vir geneeskunde. 1983;63(15):566-7.

152. Okun N, Gronau KA, Hannah ME. Antibiotics for bacterial vaginosis or Trichomonas vaginalis in pregnancy: a systematic review. Obstetrics and gynecology. 2005;105(4):857-68.
153. Klebanoff MA, Carey JC, Hauth JC, Hillier SL, Nugent RP, Thom EA, et al. Failure of metronidazole to prevent preterm delivery among pregnant women with asymptomatic Trichomonas vaginalis infection. N Engl J Med. 2001;345(7):487-93.
154. Kigozi GG, Brahmbhatt H, Wabwire-Mangen F, Wawer MJ, Serwadda D, Sewankambo N, et al. Treatment of Trichomonas in pregnancy and adverse outcomes of pregnancy: a subanalysis of a randomized trial in Rakai, Uganda. Am J Obstet Gynecol. 2003;189(5):1398-400.
155. Carey JC, Klebanoff MA, Hauth JC, Hillier SL, Thom EA, Ernest JM, et al. Metronidazole to prevent preterm delivery in pregnant women with asymptomatic bacterial vaginosis. National Institute of Child Health and Human Development Network of Maternal-Fetal Medicine Units. N Engl J Med. 2000;342(8):534-40.
156. Hauth JC, Goldenberg RL, Andrews WW, DuBard MB, Copper RL. Reduced incidence of preterm delivery with metronidazole and erythromycin in women with bacterial vaginosis. N Engl J Med. 1995;333(26):1732-6.
157. McDonald HM, O'Loughlin JA, Vigneswaran R, Jolley PT, Harvey JA, Bof A, et al. Impact of metronidazole therapy on preterm birth in women with bacterial vaginosis flora (Gardnerella vaginalis): a randomised, placebo controlled trial. British journal of obstetrics and gynaecology. 1997;104(12):1391-7.
158. Morales WJ, Schorr S, Albritton J. Effect of metronidazole in patients with preterm birth in preceding pregnancy and bacterial vaginosis: a placebo-controlled, double-blind study. Am J Obstet Gynecol. 1994;171(2):345-7; discussion 8-9.
159. Stringer E, Read JS, Hoffman I, Valentine M, Aboud S, Goldenberg RL. Treatment of trichomoniasis in pregnancy in sub-Saharan Africa does not appear to be associated with low birth weight or preterm birth. SAMJ South African Medical Journal. 2010;100(1):58-64.
160. Adjei C, Boateng R, Dompreh A, Okyere B, Owiredu EW. Prevalence and the evaluation of culture, wet mount, and ELISA methods for the diagnosis of Trichomonas vaginalis infection among Ghanaian women using urine and vaginal specimens. Trop Med Health. 2019;47:33.
161. Patil MJ, Nagamoti JM, Metgud SC. Diagnosis of Trichomonas Vaginalis from Vaginal Specimens by Wet Mount Microscopy, In Pouch TV Culture System, and PCR. J Glob Infect Dis. 2012;4(1):22-5.
162. Hobbs MM, Seña AC. Modern diagnosis of Trichomonas vaginalis infection. Sex Transm Infect. 2013;89(6):434-8.
163. Sood S, Mohanty S, Kapil A, Tolosa J, Mittal S. InPouch TV culture for detection of Trichomonas vaginalis. Indian J Med Res. 2007;125(4):567-71.
164. Gaydos CA, Klausner JD, Pai NP, Kelly H, Coltart C, Peeling RW. Rapid and point-of-care tests for the diagnosis of Trichomonas vaginalis in women and men. Sex Transm Infect. 2017;93(S4):S31-s5.
165. Campbell L, Woods V, Lloyd T, Elsayed S, Church DL. Evaluation of the OSOM Trichomonas rapid test versus wet preparation examination for detection of Trichomonas vaginalis vaginitis in specimens from women with a low prevalence of infection. J Clin Microbiol. 2008;46(10):3467-9.
166. Darani HY, Ahmadi F, Zabardast N, Yousefi HA, Shirzad H. Development of a Latex Agglutination Test as a Simple and Rapid Method for Diagnosis of Trichomonas vaginalis Infection. Avicenna J Med Biotechnol. 2010;2(1):63-6.
167. Lillis RA, Parker RL, Ackerman R, Ackerman J, Young S, Weissfeld A, et al. Clinical Evaluation of a New Molecular Test for the Detection of Organisms Causing Vaginitis and Vaginosis. J Clin Microbiol. 2023;61(3):e0174822.
168. Van Gerwen OT, Muzny CA. Recent advances in the epidemiology, diagnosis, and management of Trichomonas vaginalis infection. F1000Res. 2019;8.
169. Kairys N, Garg M. Bacterial Vaginosis. StatPearls. Treasure Island (FL): StatPearls Publishing

170. Unemo M, Bradshaw CS, Hocking JS, de Vries HJC, Francis SC, Mabey D, et al. Sexually transmitted infections: challenges ahead. Lancet Infect Dis. 2017;17(8):e235-e79.
171. Dingens AS, Fairfortune TS, Reed S, Mitchell C. Bacterial vaginosis and adverse outcomes among full-term infants: a cohort study. BMC Pregnancy Childbirth. 2016;16(1):278.
172. Coudray MS, Madhivanan P. Bacterial vaginosis-A brief synopsis of the literature. Eur J Obstet Gynecol Reprod Biol. 2020;245:143-8.

173. Bautista CT, Wurapa E, Sateren WB, Morris S, Hollingsworth B, Sanchez JL. Bacterial vaginosis: a synthesis of the literature on etiology, prevalence, risk factors, and relationship with chlamydia and gonorrhea infections. Mil Med Res. 2016;3:4.
174. Svare JA, Schmidt H, Hansen BB, Lose G. Bacterial vaginosis in a cohort of Danish pregnant women: prevalence and relationship with preterm delivery, low birthweight and perinatal infections. BJOG. 2006;113(12):1419-25.
175. Donders G. Diagnosis and management of bacterial vaginosis and other types of abnormal vaginal bacterial flora: a review. Obstet Gynecol Surv. 2010;65(7):462-73.
176. Afolabi BB, Moses OE, Oduyebo OO. Bacterial Vaginosis and Pregnancy Outcome in Lagos, Nigeria. Open Forum Infect Dis. 2016;3(1):ofw030.
177. Leitich H, Bodner-Adler B, Brunbauer M, Kaider A, Egarter C, Husslein P. Bacterial vaginosis as a risk factor for preterm delivery: a meta-analysis. Am J Obstet Gynecol. 2003;189(1):139-47.
178. Leitich H, Kiss H. Asymptomatic bacterial vaginosis and intermediate flora as risk factors for adverse pregnancy outcome. Best practice & research Clinical obstetrics & gynaecology. 2007;21(3):375-90.
179. Klebanoff MA, Schuit E, Lamont RF, Larsson PG, Odendaal HJ, Ugwumadu A, et al. Antibiotic treatment of bacterial vaginosis to prevent preterm delivery: Systematic review and individual participant data meta-analysis. Paediatr Perinat Epidemiol. 2023;37(3):239-51.
180. McGregor JA, French JI, Parker R, Draper D, Patterson E, Jones W, et al. Prevention of premature birth by screening and treatment for common genital tract infections: results of a prospective controlled evaluation. Am J Obstet Gynecol. 1995;173(1):157-67.
181. Brocklehurst P, Gordon A, Heatley E, Milan SJ. Antibiotics for treating bacterial vaginosis in pregnancy. Cochrane Database Syst Rev. 2013(1):Cd000262.
182. Koumans EH, Lane SD, Aubry R, Demott K, Webster N, Levandowski BA, et al. Evaluation of Syracuse Healthy Start's program for abnormal flora management to reduce preterm birth among pregnant women. Maternal and child health journal. 2011;15(7):1020-8.
183. Lamont RF, Duncan SL, Mandal D, Bassett P. Intravaginal clindamycin to reduce preterm birth in women with abnormal genital tract flora. Obstetrics and gynecology. 2003;101(3):516-22.
184. Nygren P, Fu R, Freeman M, Bougatsos C, Klebanoff M, Guise JM. Evidence on the benefits and harms of screening and treating pregnant women who are asymptomatic for bacterial vaginosis: an update review for the U.S. Preventive Services Task Force. Annals of internal medicine. 2008;148(3):220-33.
185. Bhujel R, Mishra SK, Yadav SK, Bista KD, Parajuli K. Comparative study of Amsel's criteria and Nugent scoring for diagnosis of bacterial vaginosis in a tertiary care hospital, Nepal. BMC Infect Dis. 2021;21(1):825.
186. Chawla R, Bhalla P, Chadha S, Grover S, Garg S. Comparison of Hay's criteria with Nugent's scoring system for diagnosis of bacterial vaginosis. Biomed Res Int. 2013;2013:365194.
187. Coleman JS, Gaydos CA. Molecular Diagnosis of Bacterial Vaginosis: an Update. J Clin Microbiol. 2018;56(9).
188. Rodrigues FS, Peixoto S, Adami F, Alves Bda C, Gehrke Fde S, Azzalis LA, et al. Proposal of a new cutoff for Nugent criteria in the diagnosis of bacterial vaginosis. J Microbiol Methods. 2015;115:144-6.
189. Batura N, Saweri OP, Vallely A, Pomat W, Homer C, Guy R, et al. Point-of-care testing and treatment of sexually transmitted and genital infections during pregnancy in Papua New Guinea (WANTAIM trial): protocol for an economic evaluation alongside a cluster-randomised trial. BMJ Open. 2021;11(8):e046308.
190. Centers for Disease Control and Prevention. Sexually transmitted diseases treatment guidelines: MMWR Recommendations. Centers for Disease Control and Prevention. ; 2002.
191. World Health Organization. Guidelines for the Management of Sexually Transmitted Infections. Geneva: World Health Organisation; 2001.
192. Agency TZS. The 2018 Zambia Demographic and Health Survey. 2018.
193. Shahesmaeili A, Karamouzian M, Shokoohi M, Kamali K, Fahimfar N, Nadji SA, et al. Symptom-Based Versus Laboratory-Based Diagnosis of Five Sexually Transmitted Infections in Female Sex Workers in Iran. AIDS Behav. 2018;22(Suppl 1):19-25.
194. WHO. Trichomoniasis and bacterial vaginosis in pregnancy: inadequately managed with the syndromic approach. 2007;85(4):245-324.

195. Joyisa N, Moodley D, Nkosi T, Talakgale R, Sebitloane M, Naidoo M, et al. Asymptomatic Bacterial Vaginosis in Pregnancy and Missed Opportunities for Treatment: A Cross-Sectional Observational Study. Infect Dis Obstet Gynecol. 2019;2019:7808179.
196. Romoren M, Velauthapillai M, Rahman M, Sundby J, Klouman E, Hjortdahl P. Trichomoniasis and bacterial vaginosis in pregnancy: inadequately managed with the syndromic approach. Bull World Health Organ. 2007;85(4):297-304.
197. Romoren M, Sundby J, Velauthapillai M, Rahman M, Klouman E, Hjortdahl P. Chlamydia and gonorrhoea in pregnant Batswana women: time to discard the syndromic approach? BMC Infect Dis. 2007;7:27.
198. World Health Organization. Global Prevalence and Incidence of selected curable Sexually Transmitted Infection: Overview and Estimates. Geneva: World Health Organisation; 1991.
199. Msuya SE, Uriyo J, Stray-Pedersen B, Sam NE, Mbizvo EM. The effectiveness of a syndromic approach in managing vaginal infections among pregnant women in northern Tanzania. East African journal of public health. 2009;6(3):263-7.
200. Baisley K, Changalucha J, Weiss HA, Mugeye K, Everett D, Hambleton I, et al. Bacterial vaginosis in female facility workers in north-western Tanzania: prevalence and risk factors. Sex Transm Infect. 2009;85(5):370-5.
201. World Health Organisation. A Strategic Framework for Malaria Prevention and Control during Pregnancy in the African Region Brazzaville: WHO Regional Office for Africa; 2004.
202. Chico RM, Chaponda EB, Ariti C, Chandramohan D. Sulfadoxine-Pyrimethamine Exhibits Dose-Response Protection Against Adverse Birth Outcomes Related to Malaria and Sexually Transmitted and Reproductive Tract Infections. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2017;64(8):1043-51.
203. Njagi JK, Magnussen P, Estambale B, Ouma J, Mugo B. Prevention of anaemia in pregnancy using insecticide-treated bednets and sulfadoxine-pyrimethamine in a highly malarious area of Kenya: a randomized controlled trial. Trans R Soc Trop Med Hyg. 2003;97(3):277-82.
204. Kayentao K, Kodio M, Newman RD, Maiga H, Doumtabe D, Ongoiba A, et al. Comparison of intermittent preventive treatment with chemoprophylaxis for the prevention of malaria during pregnancy in Mali. J Infect Dis. 2005;191(1):109-16.
205. Schultz LJ, Steketee RW, Macheso A, Kazembe P, Chitsulo L, Wirima JJ. The efficacy of antimalarial regimens containing sulfadoxine-pyrimethamine and/or chloroquine in preventing peripheral and placental Plasmodium falciparum infection among pregnant women in Malawi. Am J Trop Med Hyg. 1994;51(5):515-22.
206. Shulman CE, Dorman EK, Cutts F, Kawuondo K, Bulmer JN, Peshu N, et al. Intermittent sulphadoxine-pyrimethamine to prevent severe anaemia secondary to malaria in pregnancy: a randomised placebo-controlled trial. Lancet. 1999;353(9153):632-6.
207. ter Kuile FO, van Eijk AM, Filler SJ. Effect of sulfadoxine-pyrimethamine resistance on the efficacy of intermittent preventive therapy for malaria control during pregnancy: a systematic review. Jama. 2007;297(23):2603-16.
208. Fried M, Nosten F, Brockman A, Brabin BJ, Duffy PE. Maternal antibodies block malaria. Nature. 1998;395(6705):851-2.
209. Mehaffey PC, Barrett MS, Putnam SD, Jones RN. Antigonococcal activity of 11 drugs used for therapy or prophylaxis of malaria. Diagnostic microbiology and infectious disease. 1995;23(1-2):11-3.
210. Sombie I, Meda N, Cartoux M, Tiendrebeogo S, Ouangre A, Yaro S, et al. Seroprevalence of syphilis among women attending urban antenatal clinics in Burkina Faso, 1995-8. The DITRAME Study Group. DIminunation de la TRAnsmission Mere-Enfant. Sex Transm Infect. 2000;76(4):314-6.
211. WHO. Policy brief for the implementation of intermittent preventive treatment of malaria in pregnancy using sulfadoxine-pyrimethamine (IPTp-SP). 2013.
212. Moore JM, Nahlen BL, Lal AA, Udhayakumar V. Immunologic memory in the placenta: a lymphocyte recirculation hypothesis. Medical hypotheses. 2000;54(3):505-10.
213. Chico RM, Cano J, Ariti C, Collier TJ, Chandramohan D, Roper C, et al. Influence of Malaria Transmission Intensity and the 581G Mutation on the Efficacy of Intermittent Preventive Treatment in Pregnancy: Systematic Review and Meta-analysis. Trop Med Int Health. 2015.
214. Chico RM, Cano J, Ariti C, Collier TJ, Chandramohan D, Roper C, et al. Influence of malaria transmission intensity and the 581G mutation on the efficacy of intermittent preventive treatment in pregnancy: systematic review and meta-analysis. Trop Med Int Health. 2015;20(12):1621-33.

215. Chico RM, Chandramohan D. Intermittent preventive treatment of malaria in pregnancy: at the crossroads of public health policy. Tropical medicine & international health : TM & IH. 2011;16(7):774-85.
216. Vallely A, Vallely L, Changalucha J, Greenwood B, Chandramohan D. Intermittent preventive treatment for malaria in pregnancy in Africa: what's new, what's needed? Malar J. 2007;6:16.
217. Anchang-Kimbi JK, Kalaji LN, Mbacham HF, Wepnje GB, Apinjoh TO, Ngole Sumbele IU, et al. Coverage and effectiveness of intermittent preventive treatment in pregnancy with sulfadoxine-pyrimethamine (IPTp-SP) on adverse pregnancy outcomes in the Mount Cameroon area, South West Cameroon. Malar J. 2020;19(1):100.
218. Braun V, Rempis E, Schnack A, Decker S, Rubaihayo J, Tumwesigye NM, et al. Lack of effect of intermittent preventive treatment for malaria in pregnancy and intense drug resistance in western Uganda. Malar J. 2015;14:372.
219. Kissinger PJ, Gaydos CA, Seña AC, Scott McClelland R, Soper D, Secor WE, et al. Diagnosis and Management of Trichomonas vaginalis: Summary of Evidence Reviewed for the 2021 Centers for Disease Control and Prevention Sexually Transmitted Infections Treatment Guidelines. Clin Infect Dis. 2022;74(Suppl_2):S152-s61.
220. Nygren P, Fu R, Freeman M, Bougatsos C, Guise JM. U.S. Preventive Services Task Force Evidence Syntheses, formerly Systematic Evidence Reviews. Screening and Treatment for Bacterial Vaginosis in Pregnancy: Systematic Review to Update the 2001 US Preventive Services Task Force Recommendation. Rockville (MD): Agency for Healthcare Research and Quality (US); 2008.
221. Wi TE, Ndowa FJ, Ferreyra C, Kelly-Cirino C, Taylor MM, Toskin I, et al. Diagnosing sexually transmitted infections in resource-constrained settings: challenges and ways forward. J Int AIDS Soc. 2019;22 Suppl 6(Suppl Suppl 6):e25343.
222. organization WH. Trichomoniasis and bacterial vaginosis in pregnancy: inadequately managed with the syndromic approach. 2007;85(4):245-324.
223. Valsangkar S, Selvaraju D, Rameswarapu R, Kamutapu S. Impairment of quality of life in symptomatic reproductive tract infection and sexually transmitted infection. J Reprod Infertil. 2014;15(2):87-93.
224. Chaponda EB, Bruce J, Michelo C, Chandramohan D, Chico RM. Assessment of syndromic management of curable sexually transmitted and reproductive tract infections among pregnant women: an observational cross-sectional study. BMC Pregnancy Childbirth. 2021;21(1):98.
225. Freeman CD, Klutman NE, Lamp KC. Metronidazole. A therapeutic review and update. Drugs. 1997;54(5):679-708.
226. Cenkowski M, Wudel B, Poliquin V. Vaginal trichomoniasis. Cmaj. 2022;194(6):E217.
227. Kissinger P, Muzny CA, Mena LA, Lillis RA, Schwebke JR, Beauchamps L, et al. Single-dose versus 7-day-dose metronidazole for the treatment of trichomoniasis in women: an open-label, randomised controlled trial. Lancet Infect Dis. 2018;18(11):1251-9.
228. Kissinger P, Muzny CA. Treatment recommendations for trichomoniasis in women. Lancet Infect Dis. 2019;19(1):20-1.
229. Muzny CA, Schwebke JR. Asymptomatic Bacterial Vaginosis: To Treat or Not to Treat? Curr Infect Dis Rep. 2020;22(12).
230. Esposito R. Emetine and metronidazole as antimalarial drugs. Lancet. 1985;2(8458):784.
231. Pallangyo KJ, Minjas JN, Sarda RK. Metronidazole for falciparum malaria. Lancet. 1986;1(8486):922.
232. Chaponda EB, Mharakurwa S, Michelo C, Bruce J, Chandramoha D, Matthew Chico R. Sulfadoxine-pyrimethamine parasitological efficacy against Plasmodium falciparum among pregnant women and molecular markers of resistance in Zambia: an observational cohort study. Malar J. 2021;20(1):61.
233. Zambia Demographic and Health Survey 2013–14. Rockville, MD, USA: CSO / MOH Zambia & ICF Int'l; 2014.
234. ClinicalTrials.gov: NCT01103063. Phase III: Evaluate azithromycin plus chloroquine and sulfadoxine plus pyrimethamine for IPT of falciparum malaria infection in pregnant women in Africa. (Access date: 14 April 2015).
235. Mukonka VM, Chanda E, Haque U, Kamuliwo M, Mushinge G, Chileshe J, et al. High burden of malaria following scale-up of control interventions in Nchelenge District, Luapula Province, Zambia. Malaria Journal. 2014;13(1):153.

236. Pinchoff J, Chaponda M, Shields TM, Sichivula J, Muleba M, Mulenga M, et al. Individual and Household Level Risk Factors Associated with Malaria in Nchelenge District, a Region with Perennial Transmission: A Serial Cross-Sectional Study from 2012 to 2015. PLoS One. 2016;11(6):e0156717.
237. Das S, Muleba M, Stevenson JC, Norris DE. Habitat Partitioning of Malaria Vectors in Nchelenge District, Zambia. Am J Trop Med Hyg. 2016;94(6):1234-44.
238. Stevenson JC, Pinchoff J, Muleba M, Lupiya J, Chilusu H, Mwelwa I, et al. Spatio-temporal heterogeneity of malaria vectors in northern Zambia: implications for vector control. Parasit Vectors. 2016;9(1):510.
239. Choi KS, Christian R, Nardini L, Wood OR, Agubuzo E, Muleba M, et al. Insecticide resistance and role in malaria transmission of Anopheles funestus populations from Zambia and Zimbabwe. Parasit Vectors. 2014;7:464.
240. Ippolito MM, Gebhardt ME, Ferriss E, Schue JL, Kobayashi T, Chaponda M, et al. Scientific Findings of the Southern and Central Africa International Center of Excellence for Malaria Research: Ten Years of Malaria Control Impact Assessments in Hypo-, Meso-, and Holoendemic Transmission Zones in Zambia and Zimbabwe. Am J Trop Med Hyg. 2022;107(4_Suppl):55-67.
241. Nchelenge District Health Management Team. Nchelenge District Population-2014. 2014.
242. Health Mo. Zambia National Malria Indicator Survey 2021. 2021.
243. Nchelenge District Health Management Team. Distric Maternal & Child HEalth Report. 2022.
244. Hsiang MS, Lin M, Dokomajilar C, Kemere J, Pilcher CD, Dorsey G, et al. PCR-based pooling of dried blood spots for detection of malaria parasites: optimization and application to a cohort of Ugandan children. J Clin Microbiol. 2010;48(10):3539-43.
245. Duraisingh MT, Curtis J, Warhurst DC. Plasmodium falciparum: detection of polymorphisms in the dihydrofolate reductase and dihydropteroate synthetase genes by PCR and restriction digestion. Experimental parasitology. 1998;89(1):1-8.
246. Mkulama MA, Chishimba S, Sikalima J, Rouse P, Thuma PE, Mharakurwa S. Escalating Plasmodium falciparum antifolate drug resistance mutations in Macha, rural Zambia. Malar J. 2008;7:87.
247. Mharakurwa S, Simoloka C, Thuma PE, Shiff CJ, Sullivan DJ. PCR detection of Plasmodium falciparum in human urine and saliva samples. Malar J. 2006;5:103.
248. Nag S, Dalgaard MD, Kofoed PE, Ursing J, Crespo M, Andersen LO, et al. High throughput resistance profiling of Plasmodium falciparum infections based on custom dual indexing and Illumina next generation sequencing-technology. Sci Rep. 2017;7(1):2398.
249. Ewels P, Magnusson M, Lundin S, Käller M. MultiQC: summarize analysis results for multiple tools and samples in a single report. Bioinformatics. 2016;32(19):3047-8.
250. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 2014;30(15):2114-20.
251. T. R. Haemagglutination tests utilizing antigens from pathogenic and apathogenic Treponema pallidum WHO/VDT/RES 1965;77:65.
252. Tomizawa T. Hemagglutination tests for diagnosis of syphilis. A preliminary report. Jpn J Med Sci Biol. 1966;19(6):305-8.
253. Rathlev T. Haemagglutination test utilizing pathogenic Treponema pallidum for the sero-diagnosis of syphilis. The British journal of venereal diseases. 1967;43(3):181-5.
254. Tomizawa T, Kasamatsu S, Yamaya SI. Usefulness of the hemagglutination test using Treponema pallidum antigen (TPHA) for the serodiagnosis of syphilis. Jpn J Med Sci Biol. 1969;22(6):341-50.
255. Abou Tayoun AN, Burchard PR, Caliendo AM, Scherer A, Tsongalis GJ. A multiplex PCR assay for the simultaneous detection of Chlamydia trachomatis, Neisseria gonorrhoeae, and Trichomonas vaginalis. Exp Mol Pathol. 2015;98(2):214-8.
256. Nugent RP, Krohn MA, Hillier SL. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. J Clin Microbiol. 1991;29(2):297-301.
257. WHO. Malaria Microscopy Quality Assurance Manual. 2016.
258. S. Kin Suvarna CL, John D. Bancroft. Bancroft's Theory and Practice of Histological Techniques. 8th Edition ed: Churchill, Livingstone; 2019.
259. Bancroft JD GM. Theory and Practice of Histological Techniques. 6th Edition ed: Churchill Livingstone; 2008.
260. WHO. Malaria chemoprevention efficacy study protocol. 2022.

261. Clerk CA, Bruce J, Affipunguh PK, Mensah N, Hodgson A, Greenwood B, et al. A randomized, controlled trial of intermittent preventive treatment with sulfadoxine-pyrimethamine, amodiaquine, or the combination in pregnant women in Ghana. J Infect Dis. 2008;198(8):1202-11.
262. Craig-Kuhn MC, Granade C, Muzny CA, Van Der Pol B, Lillis R, Taylor SN, et al. Optimal Timing for Trichomonas vaginalis Test of Cure Using Nucleic Acid Amplification Testing. Sex Transm Dis. 2019;46(5):312-6.
263. Howe K, Kissinger PJ. Single-Dose Compared With Multidose Metronidazole for the Treatment of Trichomoniasis in Women: A Meta-Analysis. Sexually transmitted diseases. 2017;44(1):29-34.
264. Mtove G, Chico RM, Madanitsa M, Barsosio HC, Msemo OA, Saidi Q, et al. Fetal growth and birth weight are independently reduced by malaria infection and curable sexually transmitted and reproductive tract infections in Kenya, Tanzania, and Malawi: A pregnancy cohort study. Int J Infect Dis. 2023.
265. Stoner MC, Vwalika B, Smid M, Kumwenda A, Stringer E, Chi BH, et al. Dosage of Sulfadoxine-Pyrimethamine and Risk of Low Birth Weight in a Cohort of Zambian Pregnant Women in a Low Malaria Prevalence Region. Am J Trop Med Hyg. 2017;96(1):170-7.
266. Grunberg E. The effect of trimethoprim on the activity of sulfonamides and antibiotics in experimental infections. J Infect Dis. 1973;128:Suppl:478-85 p.
267. Burman LG. The antimicrobial activities of trimethoprim and sulfonamides. Scand J Infect Dis. 1986;18(1):3-13.
268. Sundararaman SA, Odom John AR. Prevention of malaria in pregnancy: The threat of sulfadoxine-pyrimethamine resistance. Front Pediatr. 2022;10:966402.
269. Madanitsa M, Kalilani L, Mwapasa V, van Eijk AM, Khairallah C, Ali D, et al. Scheduled Intermittent Screening with Rapid Diagnostic Tests and Treatment with Dihydroartemisinin-Piperaquine versus Intermittent Preventive Therapy with Sulfadoxine-Pyrimethamine for Malaria in Pregnancy in Malawi: An Open-Label Randomized Controlled Trial. PLoS Med. 2016;13(9):e1002124.
270. Olaleye A, Okusanya BO, Oduwole O, Esu E, Meremikwu M. A systematic review and meta-analysis of dihydroartemisinin-piperaquine versus sulphadoxine-pyrimethamine for malaria prevention in pregnancy. International journal of gynaecology and obstetrics: the official organ of the International Federation of Gynaecology and Obstetrics. 2019;146(1):43-55.
271. Cutts JC, Agius PA, Zaw L, Powell R, Moore K, Draper B, et al. Pregnancy-specific malarial immunity and risk of malaria in pregnancy and adverse birth outcomes: a systematic review. BMC Med. 2020;18(1):14.
272. Ruizendaal E, van Leeuwen E, Mens PF. Peripheral and placental biomarkers in women with placental malaria: a systematic review. Biomark Med. 2015;9(3):217-39.
273. Min AM, Saito M, Simpson JA, Kennedy SH, Nosten FH, McGready R. Placental histopathology in preterm birth with confirmed maternal infection: A systematic literature review. PLoS One. 2021;16(8):e0255902.
274. Mlugu EM, Minzi O, Kamuhabwa AAR, Aklillu E. Effectiveness of Intermittent Preventive Treatment With Dihydroartemisinin-Piperaqunine Against Malaria in Pregnancy in Tanzania: A Randomized Controlled Trial. Clin Pharmacol Ther. 2021;110(6):1478-89.
275. WHO. Anaemia2023. Available from: https://www.who.int/news-room/fact-sheets/detail/anaemia#:~:text=During%20pregnancy%2C%20anaemia%20has%20been,%2C%20families%2C%20communities%20and%20countries.
276. Poespoprodjo JR, Fobia W, Kenangalem E, Lampah DA, Sugiarto P, Tjitra E, et al. Treatment policy change to dihydroartemisinin-piperaquine contributes to the reduction of adverse maternal and pregnancy outcomes. Malar J. 2015;14:272.
277. Chaponda EB, et al. Malarial infection and curable STIs/RTIs among pregnant women in a rural district of Zambia. AJTMH. 2016;95(5):1069-76.
278. Gatton ML, Martin LB, Cheng Q. Evolution of resistance to sulfadoxine-pyrimethamine in Plasmodium falciparum. Antimicrob Agents Chemother. 2004;48(6):2116-23.
279. Marques-Silva M, Lisboa C, Gomes N, Rodrigues AG. Trichomonas vaginalis and growing concern over drug resistance: a systematic review. J Eur Acad Dermatol Venereol. 2021;35(10):2007-21.
280. Workowski KA, Bachmann LH, Chan PA, Johnston CM, Muzny CA, Park I, et al. Sexually Transmitted Infections Treatment Guidelines, 2021. MMWR Recomm Rep. 2021;70(4):1-187.

281. Ralph ED, Amatnieks YE. Relative susceptibilities of Gardnerella vaginalis (Haemophilus vaginalis), Neisseria gonorrhoeae, and Bacteroides fragilis to Metronidazole and its two major metabolites. Sexually transmitted diseases. 1980;7(4):157-60.
282. Briand V, Bottero J, Noël H, Masse V, Cordel H, Guerra J, et al. Intermittent treatment for the prevention of malaria during pregnancy in Benin: a randomized, open-label equivalence trial comparing sulfadoxine-pyrimethamine with mefloquine. J Infect Dis. 2009;200(6):991-1001.
283. González R, Mombo-Ngoma G, Ouédraogo S, Kakolwa MA, Abdulla S, Accrombessi M, et al. Intermittent preventive treatment of malaria in pregnancy with mefloquine in HIV-negative women: a multicentre randomized controlled trial. PLoS Med. 2014;11(9):e1001733.
284. González R, Desai M, Macete E, Ouma P, Kakolwa MA, Abdulla S, et al. Intermittent preventive treatment of malaria in pregnancy with mefloquine in HIV-infected women receiving cotrimoxazole prophylaxis: a multicenter randomized placebo-controlled trial. PLoS Med. 2014;11(9):e1001735.
285. Denoeud-Ndam L, Zannou DM, Fourcade C, Taron-Brocard C, Porcher R, Atadokpede F, et al. Cotrimoxazole prophylaxis versus mefloquine intermittent preventive treatment to prevent malaria in HIV-infected pregnant women: two randomized controlled trials. J Acquir Immune Defic Syndr. 2014;65(2):198-206.
286. Akinyotu O, Bello F, Abdus-Salam R, Arowojolu A. Comparative study of mefloquine and sulphadoxine-pyrimethamine for malaria prevention among pregnant women with HIV in southwest Nigeria. International journal of gynaecology and obstetrics: the official organ of the International Federation of Gynaecology and Obstetrics. 2018;142(2):194-200.
287. Natureeba P, Kakuru A, Muhindo M, Ochieng T, Ategeka J, Koss CA, et al. Intermittent Preventive Treatment With Dihydroartemisinin-Piperaquine for the Prevention of Malaria Among HIV-Infected Pregnant Women. J Infect Dis. 2017;216(1):29-35.
288. Divala TH, Mungwira RG, Mawindo PM, Nyirenda OM, Kanjala M, Ndaferankhande M, et al. Chloroquine as weekly chemoprophylaxis or intermittent treatment to prevent malaria in pregnancy in Malawi: a randomised controlled trial. Lancet Infect Dis. 2018;18(10):1097-107.
289. Akinyotu O, Bello F, Abdus-Salam R, Arowojolu A. A randomized controlled trial of azithromycin and sulphadoxine-pyrimethamine as prophylaxis against malaria in pregnancy among human immunodeficiency virus-positive women. Trans R Soc Trop Med Hyg. 2019;113(8):463-70.
290. Luntamo M, Kulmala T, Mbewe B, Cheung YB, Maleta K, Ashorn P. Effect of repeated treatment of pregnant women with sulfadoxine-pyrimethamine and azithromycin on preterm delivery in Malawi: a randomized controlled trial. Am J Trop Med Hyg. 2010;83(6):1212-20.
291. Kimani J, Phiri K, Kamiza S, Duparc S, Ayoub A, Rojo R, et al. Efficacy and Safety of Azithromycin-Chloroquine versus Sulfadoxine-Pyrimethamine for Intermittent Preventive Treatment of Plasmodium falciparum Malaria Infection in Pregnant Women in Africa: An Open-Label, Randomized Trial. PLoS One. 2016;11(6):e0157045.

9 Appendices

Appendix 9.1. Table of drugs for Intermittent Preventive Treatment of Malaria.

Adopted and edited from Figueroa-Romero *et al* 2022

Drug	Study	Study Design	Study Year and Location	Malaria Indicators	Safety on Pregnancy Outcomes	Tolerability	Conclusion
1.AQ	Clerk *et al.*, 2008 (261)	Double-blind, three-arm RCT 1.IPTp-AQ 2.IPTp-AQSP 3.IPTp-SP (N = 3643)	2004–2007 Ghana	The prevalence of peripheral and placental malaria and anemia at delivery was similar groups.	There was no difference between groups regarding the incidences of LBW.	Women who received AQ or SPAQ were more likely to report adverse events than were those who received SP. symptoms were usually mild, including bodily pains and weakness, dizziness, vomiting, and nausea.	The effects of IPTp-AQ or SPAQ were comparable to the effects of IPTp-SP.
2.MQ	Briand *et al.*, 2009 (282)	Open-label equivalence RCT 1.IPTp-MQ 2.IPTp-SP (N = 1601)	2005 –2008 Benin	Placental and peripheral parasitemia at delivery were significantly less prevalent in the MQ group than in the SP group. Women in the MQ group were less likely to have anemia than were women in the SP group, (difference only marginally significant	The incidences of spontaneous abortions, stillbirths, and congenital anomalies did not differ significantly between groups. Prevalence of LBW among infants born to women receiving MQ and to women receiving SP was not statistically different.	The proportion of women who reported an AE was significantly higher in the MQ group than in the SP group. The most common complaints were vomiting, dizziness, tiredness, and nausea	MQ proved to be highly efficacious for use as IPTp. Its low tolerability might impair its effectiveness.
3.MQ	González *et al.*, 2014 (283)	Open label, Three-arm, RCT 1 IPTp-SP 2. IPTp-MQ full dose 3 IPTp-MQ split-dose (N = 4749)	2009–2013 Benin, Gabon, Tanzania, Mozambique	IPTp-MQ was associated with lower rates of Peripheral malaria, parasitemia at delivery, Maternal anemia at delivery, Clinical malaria episodes', All-cause outpatient attendances during pregnancy. There were no differences between groups in the prevalence of	There were no significant differences between the MQ and SP groups in either the prevalence of LBW infants or in mean birth weight. There was no difference in the prevalence of	The immediate tolerability of IPTp was poorer in the two MQ groups as compared to the SP group, with no difference between the MQ full and split-dose groups. The most	The results of this study do not support a change in the current recommended IPTp policy

Drug	Study	Study Design	Study Year and Location	Malaria Indicators	Safety on Pregnancy Outcomes	Tolerability	Conclusion
				placental infection, neonatal parasitemia and neonatal anemia	adverse pregnancy outcomes between groups, including miscarriages, stillbirths, and congenital malformations	frequently reported related AEs were dizziness and vomiting	
4.MQ	González *et al.*, 2014 (284)	Double-blind two arm RCT: 1.IPTp-Placebo + CTX 2.IPTp-MQ + CTX (N = 1071 HIV-infected women)	2009–2013 Kenya, Tanzania and Mozambique	IPTp-MQ was associated with reduced rates of - maternal parasitemia - placental malaria - hospital admissions HIV viral load at delivery was higher in the MQ group compared to the control group.	There were no differences in the prevalence of adverse pregnancy outcomes between groups. The mother-to-child transmission of HIV was twofold higher in the IPTp-MQ group	Drug tolerability was poorer in the MQ group compared to the control group (dizziness and vomiting after the first IPTp-MQ administration)	Its potential for IPTp is limited given poor drug tolerability and given that MQ was associated with an increased risk of mother-to-child transmission of HIV.
5.MQ	Denoueud-Ndam 2014 *et al*. (I) (285)	Open label, non-inferiority RCT 1.daily CTX 2.daily CTX + IPTp-MQ (N = 140 HIV-infected women)	2009–2011 Benin	CTX efficacy for the prevention of placental parasitemia was not more than 5% inferior to the association of CTX + MQ-IPTp. No differences were either observed regarding peripheral parasitemia at delivery and maternal hemoglobin between groups. Because of the small sample size obtained, noninferiority could not be conclusively assessed. No statistically significant differences were observed regarding peripheral parasitemia at delivery and maternal hemoglobin	No statistically significant differences were either observed regarding birth weight, or prematurity.	Vomiting, nausea, dizziness, and fatigue were more frequently reported with MQ.	Small sample size MQ-IPTp may be an effective alternative given concern about parasite resistance to CTX

Drug	Study	Study Design	Study Year and Location	Malaria Indicators	Safety on Pregnancy Outcomes	Tolerability	Conclusion
6.MQ	Akinyotu *et al*., 2018 (286)	Open label RCT 1.IPTp-MQ 2.IPTp-SP (N = 142 HIV-infected women)	2016 Nigeria	Presence of malaria parasites in peripheral blood at delivery or enrolment.	No statistically significant differences were found in the incidence of preterm birth and LBW.	There was no significant difference in the occurrence of vomiting, gastric pain, headache, and dizziness. Nausea was eight times more likely to occur in the MQ group.	Outcomes following use of IPTp-PQ were comparable to IPTp-SP treatment. The authors concluded that MQ is a feasible alternative therapy.
7.DP	Kakuru *et al*., 2016 (102)	Three-arm, double-blind, RCT 1. IPTp-SP 2. 3-dose IPTp-DP or monthly IPTp-DP (N = 300)	2014 Uganda	The prevalence of placental malaria was significantly higher in the SP group than in the three-dose DP group or the monthly DP group. - During pregnancy, the incidence of symptomatic malaria was significantly higher in the SP group than in the three-dose DP or the monthly DP	The prevalence of a composite adverse birth outcome was lower in the monthly DP group than in the SP group or the three-dose DP group	In each treatment group, the risk of vomiting after administration of any dose of the study agents was very low. - There were no significant differences among the groups in the risk of adverse events	IPTp-DP during pregnancy resulted in a lower burden of malaria than did treatment with SP.
8.DP	Desai *et al*., 2016 (103)	Three-arm, open-label RCT 1.IPTp-DP 2.IPTp-SP 3.IST-DP (N = 1546)	2012–2014 Kenya	Compared with women who received IPTp-SP, prevalence of malaria infection at the time of delivery was lower in the IPTp-DP group Women in IPTp-DP group had fewer malaria infections lower incidence of clinical malaria Fewer all-cause sick-clinic visits during pregnancy than those in the IPTp-SP group	Women in the IPTp-DP group had fewer stillbirths, and infant mortality than those in the IPTp-SP group. Prevalence of LBW, small for gestational age, and preterm delivery did not differ significantly between groups.	DP was well tolerated by most women. Adverse events were more frequent in the IPTp-DP group	DP is a promising alternative drug to replace SP for IPTp.
9.DP	Natureeba *et al*., 2017 (287)	Double-blinded, RCT 1. daily CTX + monthly DP 2. daily CTX (N = 200 HIV-positive women)	2014–2015 Uganda	No statistically significant difference in Risk of placental malarial infection incidence of malaria and parasite prevalence among both arms	No statistically significant difference in the incidence of adverse birth outcomes among both arms	There were no significant differences in the incidence of adverse events of any severity.	Adding monthly DP to daily CTX did not reduce the risk of placental or maternal malaria or improve birth outcomes.

Drug	Study	Study Design	Study Year and Location	Malaria Indicators	Safety on Pregnancy Outcomes	Tolerability	Conclusion
10.DP	Kajubi *et al*., 2019 HIV-positive women) (101)	Double-blind, RCT 1.IPTp-SP 2.IPTp-DP (N = 782)	2016–2017 Uganda	IPTp-DP was associated with lower: - incidence of symptomatic malaria during pregnancy. - prevalence of parasitaemia at the time of each routine visit. - risk of maternal anemia during pregnancy	There was no significant difference in the risk of LBW, preterm birth, small for gestational age, or composite adverse birth outcome between the treatment groups	Both drug regimens were well tolerated, with no significant differences in adverse events between the groups, with the exception of asymptomatic corrected QT interval prolongation (significantly higher in the DP group).	Monthly IPTp-DP was safe but did not lead to significant improvements in birth outcomes compared with SP
11.DP	Mlugu *et al*., 2021 (274)	Open-label RCT 1.IPTp-DP 2.IPTp-SP (N = 956)	2017–2019 Tanzania	IPTp-DP was associated with lower prevalence of maternal malaria at delivery. Incidence of symptomatic-malaria and parasitemia during pregnancy	The prevalence of any adverse birth outcomes was not significantly different between groups. The prevalence of LBW was significantly lower in IPTp-DP.	There was no significant difference in the prevalence of adverse drug events between the treatment groups.	There was a significantly higher protective efficacy of IPTp-DP compared to monthly IPTp-SP.
12.DP	Madanitsa *et al*., 2023 (104)	Three-arm, randomized, double-blind, partly placebo-controlled trial 1.SP 2.IPTp-DP 3.IPTp-DP +AZT	2018-2019 Kenya, Malawi, Tanzania	The use of DP was associated with reductions in the incidence of malaria. However, the risk of adverse pregnancy outcomes was significantly lower in the SP group than in both DP groups.	Compared with women in the SP group, the primary composite endpoint of adverse pregnancy outcomes was reported more frequently in the DP group and in DP plus AZ group. The incidence of SAEs was similar in all the 3 groups	Drug regimens were well tolerated in all study arms. Adding AZ at the enrolment visit resulted in significantly longer QT prolongation compared with DP alone. All QTc prolongation was asymptomatic.	Monthly IPTp-DP did not improve pregnancy outcomes, and the addition of a single course of AZ did not enhance the effect of monthly IPTp-DP
13.CQ	Divala *et al*., 2018 (288)	Three arm, open-label, RCT 1. CQ-IPTp 2.CQ chemoprophylaxis 3.SP-IPTp (N = 900)	2012–2014 Malawi	There was no difference in the risk of placental malaria detected by histopathology. Malaria infection or clinical malaria illness.	There were no differences in adverse pregnancy outcomes between arms.	Both CQ treatment regimens were associated with higher rates of treatment-related adverse events than the SP-IPTp regimen.	This study did not have enough superiority evidence of chloroquine either as IPTp or as chemoprophylaxis versus SP-IPTp for prevention of malaria during pregnancy and

Drug	Study	Study Design	Study Year and Location	Malaria Indicators	Safety on Pregnancy Outcomes	Tolerability	Conclusion
							associated maternal and infant adverse outcomes.
14.AZ	Akinyotu *et al*., 2019 (289)	Open-label RCT 1.IPTp-SP 2.AZ (N = 180 HIV-infected women)	2015–2016 Nigeria	No statistically significant difference in the incidence of malaria parasitaemia at delivery and placental parasitization among arms.	No significant difference in preterm birth and LBW between both arms.	Nausea was significantly higher in the AZ group compared with the SP group. There were no statistically significant differences among groups in the presence of dizziness and headache.	The use of AZ for malaria prevention in HIV-positive pregnant women has a comparable outcome to SP. It is tolerable and has few maternal and foetal adverse effects
15.AZSP	Luntamo *et al.*, 2010 (290)	RCT 1.Two-dose IPTp-SP 2.Monthly IPTp-SP 3.Monthly IPTp-AZSP (N = 1320)	2003–2006 Malawi	Compared with the controls, participants in the monthly SP and AZSP groups had a statistically significant lower prevalence of peripheral malaria parasitemia at 32 gestation weeks.	IPTp-SPAZ was associated with lower incidence of preterm delivery and LBW IPTp-SPAZ and monthly IPTp-SP were associated with higher mean duration of pregnancy.	Incidence of serious adverse events was low in all groups.	This intervention could be efficacious, but the impact would heavily depend on the local epidemiology and resistance of malaria.
16.AZCQ	Kimani *et al*., 2016 (291)	Open-label RCT 1.IPTp-SP 2.IPTp-AZCQ (N = 2891)	2010–2013 Benin, Kenya, Tanzania, Uganda	Statistically significant reduction in symptomatic malaria episodes Incidence of peripheral parasitemia at w. 36–38.	There was no significant difference in the incidence of LBW between treatment groups in the IPTp-AZCQ group.	AEs such as vomiting, dizziness, headache, and asthenia were reported more frequently by women receiving IPTp-AZCQ than those receiving IPTp-SP.	IPTp-AZCQ was not superior to IPTp-SP. The study was terminated earlier due to futility.

AQ: amodiaquine; AQSP: amodiaquine-sulfadoxine/pyrimethamine; IPTp: intermittent preventive treatment of malaria in pregnancy; LBW: low birth weight; MQ: mefloquine; RCT: randomized clinical trial; SP: sulfadoxine-pyrimethamine. CTX: cotrimoxazole; IPTp: intermittent preventive treatment of malaria in pregnancy; LBW: low birth weight; MQ: mefloquine; RCT: randomized clinical trial; SP: sulfadoxine-pyrimethamine. CTX: cotrimoxazole; DP: dihydroartemisinin-piperaquine; IPTp: intermittent preventive treatment of malaria in pregnancy; IST: intermittent screening and treatment; RCT: randomized clinical trial; SP: sulfadoxine-pyrimethamine. CQ: chloroquine; DP: dihydroartemisinin-piperaquine; IPTp: intermittent preventive treatment of malaria in pregnancy; LBW: low birthweight; RCT: randomized clinical trial; SP: sulfadoxine-pyrimethamine. AZ: azithromycin; AZCQ: azithromycin/chloroquine; AZSP: azithromycin/sulfadoxine-pyrimethamine; IPTp: intermittent preventive treatment of malaria in pregnancy; LBW: low birthweight; RCT: randomized clinical trial; SP: sulfadoxine-pyrimethamine; w.: week.

15.5 PARTICIPANT INFORMATION SHEETS / INFORMED CONSENT

The ASPIRE Trial

"Aiming for Safe Pregnancies by Reducing Malaria and Infections of the Reproductive Tract"

Participant Information Sheet for main trial (all women)

Full Title: Effects of metronidazole plus intermittent preventive treatment of malaria in pregnancy on birth outcomes: a randomised controlled trial in Zambia

Dr Mike Chaponda, MBChB, MSc, DTMH[1]
Dr Modest Mulenga, MBChB, MSc, DTH&M, DLHTM, PhD[1]
Dr Sebastian Hachizovu, MBChB, MPH[1]
Dr Enesia Banda Chaponda Ngulube, BSc, MSc, PhD[2]

Dr Matthew Chico, MPH, PhD[3]
Prof Daniel Chandramohan, MBBS, MSc, PhD[3]
Ms Jane Bruce, MSc[3]
Prof Philippe Mayaud, MD, MSc, DMTH[3]
Dr Suzanna Francis, FNP, MPH, PhD[3]
Prof Nigel Klein, MBBS, PhD[4]
Dr David MacIntyre, BSc, BSc, PhD[5]
Dr Antonieta Medina-Lara, BSc(Hon), MSc, PhD[6]

Institutions

[1] Tropical Diseases Research Centre, Zambia
[2] University of Zambia
[3] London School of Hygiene and Tropical Medicine, UK
[4] University College London, UK
[5] Imperial College London, UK
[6] University of Exeter, UK

Introduction

The Tropical Diseases Research Centre (TDRC) and the University of Zambia (UNZA) are conducting research to find the best way to prevent malaria and reproductive tract infections in pregnancy. Together they are working with partners overseas led by the London School of Hygiene and Tropical Medicine in the United Kingdom. We invite you to take part in this study. Before you decide whether to take part, it is important for you to understand why the study is being done.

What is the purpose of the study?

Malaria and reproductive tract infections put the health and lives of pregnant women and their babies at risk and can result in the loss of the pregnancy or cause the baby to be born small and weak. Many pregnant women who have these infections do not feel ill but can still have bad birth outcomes such as premature delivery and low birthweight. To help prevent malaria, the Government of Zambia recommends three tablets of sulphadoxine-pyrimethamine (SP or Fansidar) taken several times during pregnancy. This is called IPTp-SP

However, the malaria parasites are now becoming resistant to SP. This means the SP may not work well against malaria parasites. A new treatment called dihydroartemisinin-piperaquine or 'DP' for short has been tested in studies in Uganda and Kenya. DP was better than SP alone against malaria parasites. However, SP was better than DP at reducing bad birth outcomes such as premature delivery and low birth weight. We think this was due to the fact that SP may reduce the effect of other reproductive tract infections. Therefore, DP will need a partner medicine that will reduce the bad effects of other reproductive tract infections. The most common reproductive tract infections in Zambia are bacterial vaginosis followed by trichomoniasis, and they both can be treated with metronidazole (MTZ). For this reason, we want to see if the addition of MTZ to SP or to DP might be better than SP alone. MTZ is used to treat other common infections and is known to be safe in pregnancy.

Why have I been chosen?

The study will recruit women who are between 16 and 28 weeks pregnant and have not yet received IPTp-SP. If you are less than 16 weeks pregnant, we will ask you to join the study at your next visit when you are at least 16 weeks.

How many people will participate in this study?

The study will involve 5,436 pregnant women. The last visit of the last study participant will take place in late 2022.

What will happen if I want to take part?

If you agree to join the study and you are eligible to join, you will be given one of the three different treatments. One group of women will receive the usual IPTp with SP during antenatal care visits. On two following visits, these same women in the first group will also be given tablets that look like MTZ. In a second group, women will receive the usual IPTp with SP plus; and on two occasions they will also receive tablets of MTZ. In a third group, women will receive IPTp with DP; and on two occasions will also receive tablets of MTZ. Tablets of SP and MTZ will be given during your visit under direct observation by the study staff. The MTZ

or tablets that look like MTZ, will be given along with a flavoured candy because these tablets can cause a metallic taste. The DP tablets have to be taken over three days. The day one dose will be given under observation by the study staff and days 2 and 3 tablets you have to take at home.

For all the treatments to which you may be selected to receive, the first dose will start today. If you are chosen to receive the third treatment with DP, you will be provided with 2 extra sets of tablets of DP which you will take at home, one set of tablets each day for the next 2 days. We will ask you to come to for antenatal care every month during your pregnancy where at each visit you will be given the same treatment as you will receive today. It is important for the study that you are able to come to each visit. We will also document details of the delivery and take a blood sample. Your medical records may be reviewed by authorized personnel/third parties, confidentiality will be maintained at all times. We will examine the baby at birth, and again when the baby is about 1 week old, and about 1 to 2 months old.

What we will do today?

If you agree to join the study, we will first take a small amount of blood from your arm or finger (no more than two teaspoons). This blood will first be used to do all the other routine tests that pregnant women usually have. This includes tests for HIV, anaemia and for syphilis.

If the blood tests show that you can join the study, and if you agree to take part, then the rest of the blood will be stored and later transported to the laboratory. It will be used for later research tests. This includes tests for malaria and other reproductive tract infections, and tests for factors that protect against these infections.

We will also ask you some questions about your health and your background so we understand the results of the study better. We will examine your belly, take your blood pressure, measure your weight, and copy some information from your health card (we may photograph your ANC card, no identifying information (your name, DOB, address etc) will be included in this photograph) and from the lab books.

We will also do an ultrasound scan of your baby. An ultra-sound scan is a test which shows a moving picture of the baby inside you. You will be asked to lie down, a cool gel will be spread over

your belly and a small machine will be moved gently over it. We will use the ultrasound picture to know when your baby is due to be born. Ultra-sound scans are completely painless and harmless to you and the baby. They are very common in Europe and America.

In some clinics, women will be asked to provide a sample from their reproductive area using a swab which they will be able to do themselves. This will help us find out if women have some infections in these areas that may affect pregnancy. The samples will be stored in the freezer and then sent to the lab for specific tests to determine the presence of organisms that would cause infections in this area.

We will also ask some women to provide us with a sample of your stool so we may know the organisms that are in your gut and how they may affect your nutrition in pregnancy or cause any infections. If you agree, this stool sample will be frozen and sent to a lab abroad for testing. If you are not able to provide a stool sample today, we will ask you to take the bottle home and bring your sample the following day.

We will then open an envelope containing the study treatment that you will get. Each woman will have one of the three different treatments. The treatment you get is decided by chance, like flipping a coin. These are SP tablets (standard treatment) with tablets that look like MTZ, or SP plus MTZ (a new combination treatment), or DP plus MTZ (another new combination treatment). Thus, one third of women will get the standard treatment with SP, and two thirds will get one of the new combination treatments. Women who get the standard treatment in the first group will also get tablets that will look identical to the metronidazole tablet. These tablets are called placebos. This is done to disguise who gets the new combination treatment and who gets the standard treatment. Even though we do not know which tablets you are getting, each woman gets medicine that we think are good for you and the baby – either the treatment you would normally receive if you did not participate in the study or something that we believe might be better.

You will be given tablets at the clinic with clean water. The nurse will observe you taking the tablets. You will be asked to stay for a further 30 minutes to ensure you do not vomit the tablets. Women who will be allocated to the DP plus metronidazole group will be given two more packets of DP to take at home. Each packet will contain 3 tablets each. You have to take one packet of tablets tomorrow and the other packet the day after tomorrow. A community health worker will come to visit some of the women to ensure that they have taken the tablets.

At the end of your visit today, you will then be given a date for your next visit to the clinic for your antenatal care. All the processes will take no longer than 1 to 1.5 hours altogether.

What will happen in the next scheduled visits?

On your next scheduled visit, we will give you again the same drugs you received when you entered the study. You will be asked to take the first dose under supervision in the clinic. We will follow the same monitoring system as on your previous visit.

We will also ask for permission to take blood sample from your arm or finger which will be no more than 2 teaspoons. This will be used for testing of malaria and other tests later in the laboratory. We will do this every month during pregnancy.

During your clinic visits you will also receive all other health care that pregnant women attending the clinic would usually receive including vaccinations. If you feel sick in between the monthly visits, we ask you to come and visit our clinic or inform us by phone. We will then examine you, take a blood sample to test for malaria on the same day and if found to have malaria you will receive treatment. The list on the next page shows what happens at each visit. The last time we will collect data on you and your baby is at 28 days (4 weeks) after delivery, after this time-point we will not collect any further data on your or your baby.

What happens at each visit	Visit 1 Wk 16-19	Visit 2 Wk 20-24	Visit 3 Wk 25-29	Visit 4 Wk 30-34	Visit 5 Delivery	Visit 6 Wk 4
Medical history and ultrasound	■					
General medical exam	■					
Test and treat HIV and syphilis	■					
Measure strength of blood (haemoglobin)	■				■	
Measure sugar and protein in urine	■				■	
Collect pin-prick blood spots for malaria	■	■	■	■	■	
Collect swabs (all women)	■	■	■	■		
Collect stool (some women)	■	■				
Provide treatment for the study	■	■	■	■		
Provide general care	■	■	■	■	■	■
Record pregnancy outcome					■	
Measure malaria in the placenta					■	
Collect placental tissue					■	
Check baby health					■	■

What will happen at delivery and for the baby?

We would like you to give birth to your baby in the study hospital so that we can check how you and the baby are doing. After your baby is born we will take samples from the placenta to check for malaria. We will not take any samples from your baby's body at the time of birth. We will measure your baby's weight and size, and give the baby a health check. If you give birth to your baby at home, we would like you to come to the clinic as soon as possible so that a midwife can check the health of you and the baby, and collect information for the study. If your baby is born with any abnormality we would like to ask your permission to take a photograph of the abnormality on the baby. This will help us to get experts to identify the abnormality that will help in providing care to your baby by the hospital. The photograph will be used only for research purposes. No names will appear in the photograph; only your study identification number will be used and baby's face will be blurred in the photo so identification is not possible. In extreme cases, photography may need to be abandoned and rescheduled if the child is too distressed or unwell.

Additional measurement to assess the size of new-born babies will be conducted in some of the clinics where this study is being done. These additional measurements will be done when the baby is born and again on the visit where you and the baby will finish the study at 4 weeks after delivery.

What will happen after the delivery visit to me and the baby?

We will ask you to bring your baby to the clinic when your baby is seven days old and again at about 4 weeks old. We will check that you and your baby are still doing well and check the baby's weight and size. We will also take these measures of body size from some of the mothers.

What else will be expected of me if I agree to be in the study?

If you enrol in the study, we would prefer that you do not take any medicine that is not provided by the clinic. If you or your baby feels unwell anytime during the study period, you can come to the clinic. You will be seen by a qualified member of staff and will be given any treatment that you need free of charge. The information collected will be used by staff and

scientists involved in the study. Your information will be kept confidential; no names nor addresses will ever appear on any of the study reports.

What are the possible disadvantages and risks of taking part?

DP is a very effective treatment for malaria. It has already been tested in pregnant women and it does not seem to cause any harm to women or their babies. However, it is still quite a new drug and we cannot be completely sure that it will not have any side-effects. If you appear to suffer bad side-effects during the study, we will stop your treatment. Once you go back home, if you feel bad or you should return to the clinic right away, even if you have not been asked to return on that day. There will be a doctor at the clinic every day and every night who will give you treatment for these problems. You will not have to pay for any of the treatments you receive during the course of this study, even on days when we did not tell you to come.

All babies will be given a thorough health check and any problems will be recorded. The study will be stopped if we suspect that DP is causing health problems in the babies.

SP and metronidazole have been used previously in pregnancy and were found to be safe. You will also be given SP if you do not take part in the study. If you become ill after taking any of the study drugs, and we think it might be because of the drug, we may stop your treatment and we will not give you the same drug again. However, if this happens, we would still like you to continue in the study.

All three drugs (SP, MTZ, DP) in the trial are considered safe for mother and fetus during the second and third trimesters of pregnancy, and all three have been used extensively in research settings with favourable safety profiles within the proposed doses. However, no previous trials have used the combination of SP plus MTZ, nor DP plus MTZ. To ensure the safety of providing the proposed study drugs in combination, we conducted a review of the WHO database of suspected adverse drug reactions. There are presently 15 million reports in the database. Over 94,000 mention MTZ or one of the anti-malarial drugs of interest; 195 of the reports have both MTZ and one of the anti-malarial drugs of interest mentioned on the same report; only 12 of these are from sub-Saharan Africa. We compared the proportion of case reports across different classes of adverse events among the drugs of

interest, as well as their combination. We did not find any excess incidence of adverse events for use of any combination. Thus, there is no evidence to suggest the proposed drug combinations cannot be co-administered safely.

When we take blood, you may get a small bruise or mild pain on the arm or finger where the blood is taken. There is also a very small chance of infection; the chance is very small because we always use clean materials.

What are the possible benefits of taking part?

Treatment will be offered to anyone found with symptoms of malaria and STIs/RTIs to protect both you and your unborn baby. This study will benefit pregnant women in Zambia, their children, and the individual participants in the study. We intend to share the results of this study with the Ministry of Health so that they can have evidence for the need to improve their recommendations on management and control of malaria and other infections that put the

lives of pregnant women and their unborn babies at risk. This study has the potential to contribute to the improvement of health care which pregnant women and babies born to them receive.

Costs and compensation for being in the study

You will not be asked to pay anything to participate in this study. You will receive reimbursement of K20 for your transport to come to the clinic and/or to deliver in the hospital. You also receive transport reimbursement to go back home. When you visit the clinic during your pregnancy, we may sometimes ask you to wait. If this takes more than half a day, you will be provided some snacks and refreshments or the cash equivalent.

What if I don't want to be in the study?

You are free to choose to be part of this study and also have the right to refuse. If you decide not to join the study, you will receive all the usual health care that pregnant women attending the clinic usually receive. If you do choose to participate in the study, you are fee to change your mind and withdraw from the study at any time, and you do not have to give a reason if you do not want to. Even if you withdraw from the study you will still get the usual care pregnant women receive in the antenatal clinic.

Consent for shipping of samples

Some of the samples collected from you and your baby will be stored in the freezer before they can be tested. We request for your permission to ship some of your and your baby's samples to laboratories outside the country for further tests. Your/your baby's samples will not have your/your baby's name but only your study number and therefore no one will be able to link the sample with you or your baby.

Consent for long-term sample storage for future studies

We are also asking people who join this study if they will let the researchers' use their blood sample for future studies. These future studies may help to find new ways to prevent malaria or other diseases. If you agree, we will store your blood in the laboratory with a unique

number and not with your name. Your sample will be stored for up to 10 years. If we need to keep the samples for longer than 10 years, we will apply for authorization from the National Health Research Authority (NHRA) in Zambia. We may share test results with researchers at other organizations but we will not give them your name, address, or any information that could identify you. After the study has ended, we will remove any means to link the sample to you. If you do not wish to have your samples stored for future tests, you may still participate in our study.

Study ethical approvals

The protocol and this information sheet have been reviewed and approved by all members of the Research Committees of: Tropical Diseases Research Centre (TDRC), National Health Research Authority-Zambia (NHRA, Zambia Medical Research Authority (ZAMRA), and London School of Hygiene and Tropical Medicine (please see separate page with names of all members of these committees).

Contact information for questions or concerns

If you have any questions about this study, or if you want you/your child to stop being part of the study, please contact the local principal investigator (study doctor).

Dr Mike Chaponda
Tel: +260 966849622
Tropical Diseases Research Centre
P.O. Box 71769
Ndola, Zambia

You can also contact any of our study staff at the hospital. If you have any questions about your rights as a study patient, or if you think you or your child has been injured because of this study, please contact:

The Secretary

TDRC Ethical Review Committee

P.O Box 71769

Ndola, Zambia
Tel: +260 212 620737

This completes Part 1 of the information sheet.

If the information in Part 1 has interested you, then please continue to read Part 2 of the information sheet.

Part 2 of the Information Sheet

What happens if relevant new information becomes available?

You or your legally acceptable representative will be kept informed, in a timely manner, of any information that may relate to your willingness to continue participation in the study. At the discretion of the clinical staff, you or your legally acceptable representative may be asked to sign a revised informed consent or consent addendum that provides this information.

Complaints

If you have concerns about any aspect of this study you should ask to speak with the study doctor of the trial who will do his best to answer your questions or give you the procedure for making a complaint.

Harm

If you think you have an injury/illness that is related to the study, you should immediately notify the study doctor of the trial. The study doctor may be reached at the telephone number indicated in Part 1 of this document. If you have a study-related injury/illness, the study doctor and the study staff will make sure that you receive necessary treatment. The sponsor has contracted liability insurance for this study that will compensate you for reasonable medical expenses for the treatment of any injury/illness due to your participation on the study.

Please know that you are not waiving any legal rights by participating in this study.

Effects of metronidazole plus intermittent preventive treatment of malaria in pregnancy on birth outcomes: a randomised controlled trial in Zambia

ASPIRE STUDY

Consent Statement for Screening and Participation in the Main Trial

Protocol Version. 1.2 dated 29 April 2022

Local Principal Investigator: Dr Mike Chaponda, TDRC

PARTICIPANT SCREENING NUMBER: _____

I (name of the participant): _____

	Initial **boxes**
I have read the participant information sheet, version 1.2, dated 29 April 2022, for this study.	
I have received an explanation of the nature, purpose, duration of the study and the foreseeable effects and risks of the study and what I will be expected to do. My questions have been answered satisfactorily.	
I have been given ample time to read and opportunity to enquire about the details of the study.	
I agree to take part in this study and to cooperate fully with the staff conducting the study and contact the study doctor immediately if I suffer any unexpected or unusual symptoms during the study.	
For the duration of the study, I will notify the study doctor of any other medical treatments that may be necessary for me to undergo.	
I understand that, before final inclusion in the study, laboratory and scan tests will be performed and if any of these results show that I am not eligible for the study I will not be included in the study.	
I have informed the clinical staff conducting the study of all my previous or present illnesses and medication and of any consultations that I have had with medical staff prior to today.	

I have further informed the clinical staff conducting the study about my participation in any other clinical studies recently. ☐

I am aware that if I do not cooperate fully with the study doctor's requests and directions, I may harm myself by participating in the study. ☐

I understand that I do not have to sign this consent form. I also understand that if I do not sign this form, I will not be able to participate in this study. ☐

I understand that my participation in the study is voluntary and that I am free to withdraw at any time without giving any reason and without my medical care or legal rights being affected. ☐

I understand that any information that becomes available during the course of the study that may affect my willingness to take part will be disclosed to me as soon as possible. ☐

I understand that relevant sections of my medical notes and data collected from the study may be looked at by regulatory authorities or by persons from London School of Hygiene & Tropical Medicine where it is relevant to my taking part in this study. I give permission for these individuals to have access to this information. ☐

I agree that my anonymous data may be sent to countries where data protection laws do not protect privacy to the same extent as in Zambia but that the London School of Hygiene & Tropical Medicine will take all reasonable steps to protect my privacy. ☐

Participant statement: I hereby freely consent to take part in the study.

Signature of the participant: _____ Date: _____
(DD-MM-YYYY)

Printed name or thumb print of participant: _____

Impartial witness statement: (needed in case of thumb print) This form has been read / explained by / signed in the presence of:

Printed name of the impartial witness: _____

Printed name of the consenter: _____

in the language of _____ which is understood by the participant. I believe that she has understood what I explained and that she has freely given consent to take part in the study.

Signature of Consenter: _____ Date: _____
(DD-MM-YYYY)

Signature of the witness: _____ Date: _____

(DD-MM-YYYY)

Investigator's statement: I confirm that I have explained the nature, purpose and possible effects of the study to the participant whose name is printed above. They have agreed to take part by signing and dating above.

Signature of the Investigator (or delegated study staff): _____ Date:_____

Printed name of the Investigator (or delegate study staff): _____

Consent and Assent statement for Screening and Participation in the Main Trial

<u>This page is to be completed if the participant is a minor</u>
The legal guardian will consent and the minor will assent.

Signature of the participant (a minor): _____ Date _____

(DD-MM-YYYY)

Printed name or thumb print of participant (a minor): _____

Signature of legal guardian of the minor: _____ Date: _____
(DD-MM-YYYY)

Printed name or thumb print of legal guardian: _____

Signature of legal guardian: _____ Date: _____
(DD-MM-YYYY)

Impartial witness statement (needed in case of thumb print)

This form has been read/explained by/signed in the presence of

Printed name of the impartial witness: _____

Signature of impartial witness: _____ Date: _____
(DD-MM-YYYY)

Next steps:

- File original in the Trial Master File
- Give one copy for the participant
- File one copy along with the medical notes of the participant

Consent Statement for Long Term Anonymised Storage of Samples and Use in Future Studies

Your signature below means that you voluntarily agree to allow for long term storage and future research of your samples.

Participant statement: I hereby freely consent to allow for long term storage and future research of my samples.

Signature of the participant: _____ Date: _____
(DD-MM-YYYY)

Printed name or thumb print of participant: _____

Impartial witness statement: (needed in case of thumb print) This form has been read / explained by / signed in the presence of:

Printed name of the impartial witness: _____

Printed name of the consenter: _____

in the language of _____ which is understood

by the participant. I believe that she has understood what I explained and that she has freely given consent to take part in the study.

Signature of the Consenter: _____ Date: _____
(DD-MM-YYYY)
Signature of the witness: _____ Date: _____
(DD-MM-YYYY)

Investigator's statement: I confirm that I have explained the nature, purpose and possible effects of the study to the participant whose name is printed above. They have agreed to take part by signing and dating above.

Signature of the Investigator (or delegated staff): _____ Date: _____
(DD-MM-YYY
Printed name of the Investigator (or delegated staff): _____

Next steps:

- File original in the Trial Master File
- Give one copy for the participant
- File one copy along with the medical notes of the participant

Consent Statement for Provision of Stool Samples

Your signature below means that you voluntarily agree to provide stool samples for this study and possible future research.

Participant statement: I hereby freely consent to provide stool samples.

Signature of the participant: _____ Date: _____
(DD-MM-YYYY)

Printed name or thumb print of participant: _____

Impartial witness statement: (needed in case of thumb print) This form has been read / explained by / signed in the presence of:

Printed name of the impartial witness: _____

Printed name of the consenter: _____

in the language of _____ which is understood

by the participant. I believe that she has understood what I explained and that she has freely given consent to take part in the study.

Signature of the Consenter: _____ Date: _____
(DD-MM-YYYY)
Signature of the witness: _____ Date: _____
(DD-MM-YYYY)

Investigator's statement: I confirm that I have explained the nature, purpose and possible effects of the study to the participant whose name is printed above. They have agreed to take part by signing and dating above.

Signature of the Investigator (or delegated staff): _____
Date: _____
(DD-MM-YYYY)

Printed name of the Investigator (or delegated staff): _____

Next steps:

- File original in the Trial Master File
- Give one copy for the participant
- File one copy along with the medical notes of the participant

Consent Statement for Vaginal Swab from Symptomatic Women and Suspected of a Reproductive Tract Infection

Your signature below means that you voluntarily agree to provide a vaginal swab as you are symptomatic and have a suspected reproductive tract infection.

Participant statement: I hereby freely consent to provide a vaginal swab as described above and care for a suspected reproductive tract infection.

Signature of the participant: _____ Date: _____
(DD-MM-YYYY)

Printed name of the participant: _____

(If participant is illiterate)

Impartial witness statement: This form has been read/explained by/signed in the presence of:

Printed name of the impartial witness: _____

Printed name of the consenter: _____

in the language of _____ which is understood

by the participant. I believe that she has understood what I explained and that she has freely given consent to take part in the study.

Signature of the Consenter: _____ Date: _____
(DD-MM-YYYY)

Signature of the witness: _____ Date: _____
(DD-MM-YYYY)

Investigator's statement: I confirm that I have explained the nature, purpose and possible effects of the study to the participant whose name is printed above. They have agreed to take part by signing and dating above.

Signature of the Investigator (or delegated staff): _____Date: _____

Printed name of the Investigator (or delegated staff): _____

Next steps:

- File original in the Trial Master File
- Give one copy for the participant
- File one copy along with the medical notes of the participant

Appendix 9.3 Drug Product characteristics

Sulfadoxine-pyrimethamine

SULPHADOXINE + PYRIMETHAMINE TABLETS

Registration number
Gamsidar Tablets: 009/092

Pharmacological classification
Anti-malarial

Category of distribution
Prescription Only Medicine (POM)

Proprietary name & strength
Gamsidar Tablets (sulphadoxine 500mg +Pyrimethamine 25mg)

Identification, composition & presentation
A circular, flat, bevel edged tablet with "g" embossed on one side and "GS/DR" separated by a break line embossed on the other side.
Each tablet contains Sulphadoxine BP 500mg and Pyrimethamine BP 25mg.
The tablets are packed in plastic containers in packs of 100's & 1000's

Pharmacological action
Gamsidar acts against the asexual intra-erythrocytic form of the malaria parasite Plasmodium falciparum in humans. The synergistic action of the two components sulfadoxine and pyrimethamine results in inhibition of two enzymes that are involved in the biosynthesis of folinic acid in the parasites. Gamsidar is also active against strains of P. falciparum that are resistant to chloroquine. Nevertheless, strains of P. falciparum that are clinically resistant to Gamsidar are commonly found in parts of South East Asia and South America and to some extent also in East and Central Africa, hence caution is required with the use of Gamsidar in these areas. Gamsidar is also effective in infections due to Toxoplasma gondii and Pneumocystis carinii.

Pharmacokinetics
Absorption: After ingestion of one tablet, maximum plasma levels of pyrimethamine (approximately 0.2 mg/l) and sulfadoxine (approximately 60 mg/l) are reached after about four hours.
Distribution: The volume of distribution is 0.14 l/kg for sulfadoxine and 2.3 l/kg for pyrimethamine.
Ingestion of one tablet per week (the recommended adult dose for malaria prophylaxis) can be expected to lead to mean steady state plasma concentrations of 0.15mg/l for pyrimethamine (reached after about four weeks) ,and approximately 98 mg/l for sulfadoxine (reached after about seven weeks).The concentration of sulfadoxine in the erythrocyte fraction of blood is about 3% of that in plasma. The concentration of pyrimethamine in erythrocytes is about 50% of that in plasma. Pyrimethamine and sulfadoxine are about 90% bound to plasma proteins. Pyrimethamine and sulfadoxine cross the placenta and are excreted in breast milk.
Metabolism: Approximately 5% of sulfadoxine appears in the blood as acetylated metabolite and approximately 2 to 3% as glucuronide. Pyrimethamine is converted into various metabolites.
Elimination: Both components have relatively long elimination half-lives, the mean values being about 100 hours for pyrimethamine and about 200 hours for sulfadoxine. Both pyrimethamine and sulfadoxine are excreted mostly via the kidneys.
Pharmacokinetics in special clinical situations
Depending on the population being studied, the individual pharmacokinetic parameters of malaria patients can differ from those of healthy subjects.
Delayed elimination of the active ingredients of Gamsidar must be expected in patients with renal failure.

Indications
Established indications: Malaria
Treatment of malaria: Gamsidar is indicated for the treatment of malaria, particularly in cases due to

strains of P. falciparum that are resistant to chloroquine.
Malaria prophylaxis: Gamsidar is not routinely recommended for malaria prophylaxis. Prophylaxis with Gamsidar can only be considered for areas where Plasmodium falciparum malaria is endemic and sensitive to Gamsidar, and when alternative drugs are not available or are contra-indicated (see also restrictions on use).
Self-treatment of malaria in emergencies: Gamsidar may be prescribed as a treatment option for emergencies in which malaria is suspected and immediate medical assistance is unavailable.
Possibilities for use: Opportunistic infections, infections with Toxoplasma gondii, prophylaxis of Pneumocystis carinii pneumonia.

Dosage and administration
The tablets are to be swallowed whole with plenty of liquid after a meal.

a. Curative treatment of uncomplicated malaria with a single dose

	Tablets
Children	
<10 kg (up to approx. 2 years)	1/2 tablet
>10-20 kg (approx. 2-5 years)	1 tablet
>20-30 kg (approx. 5-10 years)	*11/2* tablets
>30-45 kg (approx. 10-14 years)	2 tablets
Adults	
<45 kg	2 tablets
> 45 kg	3 tablets

b. Curative treatment of complicated malaria
The standard treatment for severe and cerebral malaria is administration of quinine for 7 to 10 days. The duration of treatment with quinine can be reduced to 2 to 3 days if a single dose of Gamsidar is given after the quinine therapy. Sequential therapy is also effective at reducing the relapses that commonly occur after monotherapy with quinine. The risk of developing malaria must be carefully weighed against that of developing severe side effects. Where Gamsidar is prescribed for malaria prophylaxis the physician must ascertain whether the patient has a history of sulfonamide intolerance and must point out the associated risk. Should skin reactions occur, the drug must be withdrawn immediately.

The following doses are recommended for prophylaxis

Children	
<10 kg (up to approx. 2 years)	½ tablet every two weeks
>10-30 kg (approx. *2-10* years)	1 tablet every two weeks
>30-45 kg (approx. 10-14 years)	1 ½ tablets every two weeks
Adults	
<45 kg	1 ½ tablets every 2 weeks
>45 kg	1 tablet once weekly

c. For malaria prophylaxis the first dose of Gamsidar should be taken one week before arrival in the endemic area. Treatment should then be continued in accordance with the above dosage schedule throughout the stay in the malarious region and for four weeks thereafter. As no experience is available on more prolonged use, prophylaxis should not be given for longer than two years.
d. Self-treatment in emergencies
Gamsidar may be prescribed as a treatment option for emergencies in which immediate medical assistance

is unavailable. Self-treatment consists of a single dose of Gamsidar in accordance with the dosage schedule indicated under (a) above.
Curative treatment of uncomplicated malaria with a single dose.
Patients should be advised to refer to a physician at the earliest opportunity even if they believe themselves to be fully recovered. This ensures that the diagnosis is confirmed and that the patient receives any further medical care that may be required.
e. Infections due to Toxoplasma
The following dosage schedule has proved effective in the treatment of toxoplasmosis in adults: two tablets once weekly for six weeks (in patients with CNS involvement in conjunction with spiramycin 3 g daily for three to four weeks).Only a very small number of neonates with congenital toxoplasmosis who were classified as hardship cases have received Gamsidar for this indication.
f. Prophylaxis of Pneumocystis carinii pneumonia
Infants over two months of age and children: 40 mg/kg (in terms of sulfadoxine) every two weeks.
Dosage recommendation for adults: 1-2 tablets per week.

Restrictions on use
Contra-indications
Gamsidar is contra-indicated in patients with sulfonamide intolerance or intolerance to one or more constituents of the product. If skin reactions are observed, Gamsidar must be withdrawn immediately, as these may be indicative of a life-threatening reaction to the drug. Prophylactic (repeated) use of Gamsidar is contra-indicated in patients with severe renal failure, pronounced liver parenchymal damage, or blood dyscrasias.

Precautions
Excessive exposure to the sun must be strictly avoided. Patients should be advised that sore throat, fever, cough, dyspnea, or purpura may be early indications of severe side effects. In particular, Gamsidar must be withdrawn at the first sign of any skin rash, significant reduction in blood count, or super-infection with bacteria or fungi. Where Gamsidar is given for more than three months, regular hematologic tests are required. Where higher doses are given for prolonged periods, as in the treatment of toxoplasmosis, folic acid deficiency can be avoided by administration of folinic acid.
Pregnancy, nursing mothers: In animal experiments embryotoxic and teratogenic effects were abolished by administration of folic acid in the form of leucovorin. Prophylactic and therapeutic administration of Gamsidar to pregnant women led to no embryotoxic effects. Nevertheless, Gamsidar should be used during pregnancy only if there is a compelling indication for its use and only after a consideration of the expected benefits in relation to the potential risk to the foetus. Women of child bearing age should be advised to take contraceptive measures during treatment with Gamsidar and for three months after the last dose. As Pyrimethamine and sulfadoxine are excreted in breast milk: nursing mothers should either not take Gamsidar or cease breast feeding.

Undesirable effects
Provided the recommended dosage is observed, Gamsidar is generally well tolerated. As with other products containing sulfonamides and/or pyrimethamine, the following side effects and hypersensitivity reactions can occur:
Effects on the skin: Drug rash, pruritus, urticaria, photosensitization, and mild hair loss have been observed. These effects are generally mild and resolve spontaneously after discontinuation of the product. Rarely, particularly in hypersensitive patients, severe, potentially life-threatening skin reactions such as erythema multiforme, Stevens-Johnson syndrome, and Lyell's syndrome can occur.
Gastrointestinal side effects: Feelings of fullness, nausea, more rarely vomiting, diarrhoea, and stomatitis can occur. Isolated cases of a transient rise in hepatic enzyme levels, hepatitis, and liver cell damage have been observed in temporal association with the use of Gamsidar.
Hematologic changes: Rare instances of generally asymptomatic thrombocytopenia, megaloblastic

Metrozole Injection 0.5% (500mg): A colourless to pale yellow solution without any visible particles

Pharmacological Action:
Metronidazole penetrates the cell membrane unchanged, but once inside the cell the nitro-group is reduced in the redox condition prevalent in the anaerobic cell. The reduced product is known to damage DNA causing eventual death of the organism.

Metronidazole, a nitroimidazole, has anti-protozoal activity against *Trichomonas vagina lis*, and other protozoa including *Entamoeba histolytica*, and *Giardia Lamblia*. It does not affect the acidophilic flora of the vagina and it has no effect on candida species.
Metronidazole has bactericidal activity against obligate anaerobic bacteria whether gram negative or gram positive bacilli or cocci, including *Bacteroides fragilis:* It does not interfere with the activity of anti-bacterial agents which are active against a variety of aerobes and facultative anaerobes. Metronidazole is essentially inactive against aerobic and micro aerophilic bacteria.

Pharmacokinetics and Bio pharmaceutics
Metronidazole is very well absorbed orally, with 250mg and 500mg producing peak plasma concentrations of 5 and 12mcg/ml, respectively at 1-2 hours in adults. Vd is 0.6-0.8L/kg (i.e. about equal to total body water), 10-20% protein bound, wide distribution with therapeutic levels in many tissues, including abscesses, bile, bone and Cerebro spinal fluid (CSF). Metronidazole is extensively metabolized in the liver by hydroxylation, oxidation and glucuronide formation. 44-80% is excreted in the urine in 24hrs, about 8-20% as unchanged drug.

Half life (t1/2) is 6-8 hours in adults, it is not increased with impaired renal function but is prolonged variably in severe hepatic impairment.

Indications:
- Oral treatment of urogenital trichomoniasis, all forms of amoebiasis, acute ulcerative gingivitis (Vincent's infections), giardiasis and acute pericoronitis.

- Treatment of infections due to anaerobes particularly B.fragilis and other species of Bacteroides. Treatment of other infections for which metronidazole is effective such as fusobacteria, clostridia, eubacteria and anaerobic streptococci.

- Metronidazole has been successfully used in the treatment of septicemia, bacteraemia, thrombophlebitis, brain abscess, pelvic cellulitis and post-operative wound infections from which one or more of these anaerobes have been isolated.

- Prevention of post operative infections due to anaerobic bacteria, particularly species of bacteroides and anaerobic streptococci.

- given before or after surgery;
- given alone before or after appendicectomy;
- given in association with appropriate antibacterial agents (e.g., kanamycin) before and after colonic surgery.

Contraindications:
Metronidazole should not be given during the first trimester of pregnancy, although there is no direct evidence of teratogenicity in humans or animals.

METRONIDAZOLE TABLETS

METRONIDAZOLE SUSPENSION

METRONIDAZOLE INJECTION

Registration Number

Metronidazole Tablets 200mg: 009/059

Metronidazole Tablets 250mg: TBA

Metronidazole tablets 500mg: TBA

Metronidazole Suspension 200mg: TBA

Metronidazole Injection 0.5%: TBA

Pharmacological Classification

Anti infective / anti protozoal

Category of Distribution

Prescription Only Medicine (POM)

Proprietary Name

Metronidazole Tablets 200mg

Metronidazole Tablets 250mg

Metronidazole Tablets 500mg

Metrozole Suspension 200mg/5ml

Metrozole Injection I.V 0.5%

Composition:

Metronidazole Tablets 200mg: Each tablet contains Metronidazole BP 200mg.
Metronidazole Tablets 250mg: Each tablet contains Metronidazole BP 250mg.
Metronidazole Tablets 500mg: Each tablet contains Metronidazole BP 500mg
Metrozole Suspension 200mg/5ml: Each 5ml of suspension contains Metronidazole 200mg as metronidazole Benzoate BP
Metrozole Injection I.V 0.5%: Each 100ml vial contains Metronidazole BP 0.5% (500mg)

Identification:
Metronidazole Tablets 200mg: A white circular biconvex bevel edged tablets embossed with 'g' on one side and plain on the other side.
Metronidazole Tablets 250mg: A white circular biconvex tablets embossed with 'g' on one side and plain on the other side.
Metrozole Suspension 200mg: A white to off white homogeneous suspension

Symptoms of Overdosage and particulars of its treatment:
There is no known specific treatment of gross overdose of metronidazole but early lavage is recommended. Generally supportive management should be instituted.

Presentation:
Metronidazole tablets 200mg : Packed in plastic containers in 1000's
Metronidazole tablets 250mg : Packed in plastic containers in 1000's
Metronidazole suspension 200mg : 100ml amber glass bottle with ROPP cap
Metronidazole injection 0.5% (500mg): 100ml clear glass vials

Storage Instructions:
Store at room temperature below 30°C in a dry place. Protect from light.

NAME AND BUSINESS ADDRESS OF APPLICANT:

Baxy Pharmaceuticals Manufacturing Company Limited

P.O. Box 70286, Zambia Road

Ndola - Zambia

Date of Publication:

Nov. 2013

P-ALAXIN™

Dihydroartemisinin 40 mg + Piperaquine Phosphate 320 mg Tablets

COMPOSITION :
Each film coated tablet contains :

Dihydroartemisinin	40 mg
Piperaquine Phosphate	320 mg
Excipients	q.s.

Colour : Brilliant blue, Indigo carmine & Titanium dioxide

PHARMACOLOGY & PHARMACOKINETICS :

Mode of action of Dihydroartemisinin :

Dihydroartemisinin mainly interferes with the membrane structures of trophozoites (erythrocytic asexual forms), i.e. whorled food vacuole membrane, distended mitochondria, swollen nuclear membranes, dissociation of ribosomes from endoplasmic reticulum leading to cytoplasmic vacuolization and autophagocytosis. In addition, biochemical depression of protein synthesis and nucleic acid synthesis are exhibited.

Upon oral administration Dihydroartemisin is rapidly absorbed and maximum blood concentration attained 1 hour afterwards, with a half-life of about 4 hours. It is widely distributed in the liver, kidneys and bile. Approximately 80% is excreted through the urine and feces within 24 hrs after administration. It is metabolized to two inactive metabolites, deoxydihydroartemisinin and dihydroxydihydroartemisinin.

Mode of action of Piperaquine Phosphate :

Experimental results show that PQP interferes with physiological function of the food vacuole membrane of the trophozoites leading to autophagocytosis of the parasites. It has no marked effect on the ring forms, immature or mature schizonts and the male or female gametocytes.

Upon oral administration about 80-90% is absorbed within 24 hrs. It is widely distributed in the body mainly in the liver, kidneys, lungs and spleen. About 25%of the total dose is partitioned in the liver within 6 hrs of intake. Elimination is very slow with the half life of about 9.4 days. It is excreted through bile by hepatoenteral circulation.

INDICATIONS :

Treatment of clinical attacks of Malaria caused by P. falciparum, P. Vivax and P. malariae

ADMINISTRATION AND DOSAGE :

Oral administration :

Patient should follow doctor's instruction. The recommended dosages are in the following table.

Weight (Age group)	5-9.9 Kgs (6 months-1 Year)	10-19.9 Kgs (≥ 2-9 Years)	20-39.9 Kgs (≥ 10-14 Years)	≥ 40 Kgs (≥ 15 Years)
Day 1	0.5 Tablet	1 Tablet	2 Tablets	3 Tablets
Day 2	0.5 Tablet	1 Tablet	2 Tablets	3 Tablets
Day 3	0.5 Tablet	1 Tablet	2 Tablets	3 Tablets
Total	1.5 Tablets	3 Tablets	6 Tablets	9 Tablets

ADVERSE REACTIONS :

1. Nausea or vomiting may occur occasionally with incidence of less than 6%
2. No noticeable side effect of Dihydroartemisinin is reported. The Dihydroartemisinin would, for certain individuals, bring effects of greater or lesser severity : for example, a reversible reduction in reticulocyte counts.
3. Possible side-effect of PQP include mild dizziness, vertigo, headache, nausea, vomiting and abdominal discomfort. Reversible leucopenia was infrequently reported; dysonea and palpitations were also reported but not further specified.

PRECAUTIONS :

1. Do not exceed the stated dosage.
2. Lactation : excretion of P-ALAXIN™ TABLETS through breast milk has not been established.

CONTRAINDICATIONS :

The product is not recommended for use in women during the first 3 months of pregnancy.

PRESENTATION :

9 Tablets per blister pack.

STORAGE :

Preserve in light-resistant containers. Store below 30°C.
Keep out of reach of children.

DATE OF PUBLICATION/REVIEW : 16/04/2015

Mfd. by : **BLISS GVS PHARMA LTD.**
Dewan Udyog Nagar, Aliyali, Palghar, Maharashtra - 401 404, INDIA.
Regd. Off. : 102, Hyde Park, Saki Vihar Road, Andheri (E), Mumbai - 400 072, INDIA.

TROPICAL DISEASES RESEARCH CENTRE (TDRC)

DEPARTMENT OF BIOMEDICAL SCIENCES

TECHNICAL STANDARD OPERATING PROCEDURES (SOP – T-MOL BIOL – 4.00)

Title of SOP	**Procedure for Malaria qPCR**
Version number of SOP	**1.0**
Type of SOP	**TECHNICAL**

Approved by: Name, Signature & Date:	Mr. Sydney Mwanza	Designation: Scientific Officer
Authorized by: Name, Signature & Date:	Dr Justin Chileshe	Designation: A/ Head of Biomedical Sciences.

Address:
6th & 7th floor Ndola Central Hospital
Corner of Nkana and Broadway Roads,
P.O. Box 71769, NDOLA, ZAMBIA

Website: www.tdrc.org.zm

PROTOCOL TITLE	Procedure for Malaria PCR (Detection of Cytochrome B)		
WRITTEN BY	Mr. Jay Sikalima	**Signature:**	**Date:**
REVIEWED BY	Mr. Sydney Mwanza	Signature:	Date:
	Dr. S. Baboolal	Signature:	Date:

REVISED BY	SIGNATURE	REVISED DATE	REASON FOR REVISION

PURPOSE

The purpose of this document is to provide guidelines for performance of Malaria qPCR in the Molecular Biology Laboratory.

SCOPE

This SOP will be used at the Molecular Biology Laboratory at TDRC.

RESPONSIBILITY

The Laboratory personnel in the Molecular Biology Laboratory are responsible to carry out the procedure. The Chief Laboratory Technologist is ultimately responsible to ensure that all staff follow this procedure.

DEFINITIONS

NA

ABBREVIATIONS

TDRC	Tropical Diseases Research Centre
SOP	Standard Operating Procedure
DNA	Deoxyribonucleic acid
PCR	Polymerase Chain Reaction
PBS	Phosphate Buffered Saline
QC	Quality Control
dH_2O	Distilled water
PPE	Personal Protective Equipment
MSDS	Material Safety Data Sheet
ID	Identification
N/A	Not Applicable
qPCR	Quantitative Polymerase Chain Reaction

1. PRINCIPLE

2. EQUIPMENT SUPPLIES AND REAGENTS

Equipment	Supplies	Reagents
Microcentrifuge to hold 1.5ml tubes	Scissors	70% Ethanol
Water bath @100^{o}C	1.5ml centrifuge tubes	Distilled water
Autoclave	Micropipette and tips	Phosphate buffered Saline (PBS) - autoclaved
Real Time Thermocycler	Fine tip permanent marker	SYBR® Green PCR Master Mix (Applied Biosystems; Life Technologies Ref # 4309155)
Balance and weigh paper	2 inch small gauge needle	3D7 genomic DNA standards (10ng/ul to 10E-6ng/ul serial dilutions) for *Pf* assay
Vortex	PCR tubes (0.1ml)	Nuclease free water
	Pipette of various sizes	Positive and negative controls
	Tips of various sizes	Primers with the desired sequences
	Flask for preparing gel	
	Paraffin wax paper	
	Nitrile gloves	
	96-well PCR plate (Applied Biosystems; Fast)	
	PCR plate rack	
	Optical adhesive film	
	Fine tipped permanent marker	

3. SPECIMENS

- Blood spots collected on Filter paper

4. QUALITY CONTROL

- All reagents used must be within the expiry dates.
- Perform and document QC on all new lots of reagents.

5. SAFETY PROCEDURE

- Use appropriate PPE when handling blood spots and reagents (gloves and laboratory coat).
- Read the MSDS for working with and disposing of reagents (saponin and chelex).

6. STEP BY STEP PROCEDURE

11.1 Reagent Preparation

The following reagents are prepared prior to beginning the extraction procedure.

a. 1 x PBS
   - Dissolve 1 PBS tablet in 1L of distilled water
   - Label with name of reagent, concentration (1%), date prepared, expiry date, lot number and name of person who prepared reagents.
   - Autoclave for 15 minutes at 120^{o}C.

b. 1 x PBS with 0.1% Saponin weight/volume
   - Weigh out 0.05g saponin
   - Measure out 50ml 1xPBS
   - Dissolve saponin in PBS
   - Label with the following:
     - Name and concentration of reagent
     - Date prepared
     - Batch number
     - Expiry date
     - Name of person who prepared the reagent
     - Storage condition
   - Store reagent at room temperature for up to 2 weeks

c. dH_2O with 2% chelex weight/volume
   - Weigh out 0.05g chelex
   - Measure out 2.5L dH_2O
   - Dissolve chelex in dH2O
   - Label as above
   - Use up reagent as it cannot be stored. Discard any unused reagent.

11.2 Procedure

- For each specimen label two sets of 1.5ml tubes with specimen ID. One tube will be used for the extraction process and the second to store the extracted DNA.
- Cut out one full dried blood spot (from filter paper) and place it into the respective labeled tube.
- Wipe scissors between each specimen with 70% alcohol to prevent cross contamination.
- Add 1.0ml of the 1xPBS/saponin solution to each tube ensuring that the pipette tip does not touch the tube or blood spot.

- Ensure that the blood spot is submerged into the liquid.
- Incubate specimen tubes at room temperature for 10 minutes to allow elution of the blood.
- Centrifuge specimen tubes for 2 minutes at 14,000rpm.
- Discard the supernatant (liquid) using a Pasteur pipette. Pipette should be cleaned after each specimen by washing with 2 sets of water, then 1 set 70% Ethanol and then with water.
- Add 1.0ml of 1xPBS to each tube.
- Centrifuge specimen tubes for 2 minutes at 14,000rpm.
- Discard the supernatant (liquid) using a Pasteur pipette that has been cleaned as above.
- Add 150µl of dH_2O/Chelex solution to each tube.
- Add 50µl dH_2O to each tube.
- Close the specimen tubes and pierce a hole in each cap with a needle.
- Incubate the specimen tubes at 100^oC in a heating block for 8 minutes.
- Centrifuge the specimen tubes at 14,000rpm for 1 minute.
- Transfer the supernatant from tube one to the respectively labeled tube 2.
- Store specimens (DNA Extracts) at -20^oC until tested.

11.3 PREPARATION OF MASTER MIX

- The Master Mix is prepared in the Master Mix room ensuring that all precautions are taken to ensure that there is no DNA contamination.
- The working area should be sterilized before each experiment
- The Laboratory Scientist will begin by making a reaction mix sufficient for the number of samples to be run. For a full 96-well plate the following calculation should be used:

sample type	actual number	duplicate number
Participant samples	30	60
DBS controls*	6	12
Standards (3D7 g-DNA)	8	16
no template control	1	2
	45	90

- The duplicate total should be used to determine the volumes of the PCR reagents required.
- * 1000para/ul, 100para/ul, 10para/ul, 1para/ul, 0.1para/ul and uninfected RBC DBS spots that were extracted together with the participant samples to be run.
- *P. falciparum* q-PCR reaction mix preparation:

	x 1 sample	x total sample number
2x SYBR Green PCR mix	10µL	
Molecular grade Water	7µL	
Pf forward primer 100µM	0.5µL	
Pf reverse primer 100µM	0.5µL	
	18.0µL	

11.4 Loading the 96-well PCR plate

A map of how samples will be loaded onto the PCR plate should be indicated in the lab book using the template print out provided. Pipette 18 µL of the reaction mix into all wells that will contain samples. Add 2 µL of DNA sample into the respective wells according to the plate map changing tips each time. This gives a total reaction volume of 20µL.

The Laboratory Scientist will take all precautions to avoid contaminating the reaction mix or cross-contaminating the individual samples whilst loading the plate.

Seal the loaded plate securely with the adhesive film and centrifuge at 2000rpm for 5 minutes. Load the PCR plate into the real time q-PCR thermocycler (AB Biosystems Fast).

Setting up the q-PCR experiment on thermocycler

Open '7500 ABS software' on desktop and login as 'Guest'. In the tool bar select 'open' → 'Desktop' → 'SYBR Green q-PCR template' → open file.

Review and/or edit the experiment name, properties, plate map, thermoprofile, reaction set up and define standards according to the experiment. Thermoprofile must be as follows: 95°C for 10 minutes followed by 55 cycles of 95°C for 10 seconds and 55°C for 1 minute [collect data on hold at this step]. Select 'step and hold' for melt profile with default program of 95°C for 15 secs, followed by 60°C for 1 minute and 95° for 15 secs

Complete the 'reaction mix calculations' according to the experiment

	Starting Conc.	Final Conc.
SYBR Green PCR Master Mix	2 x	1 x
*Pf*cytb F & R primer 100µM	0.1 nmol/ul	500nm

*Pm*cytb F & R primer 100µM	0.1 nmol/ul	200nm

In the tool bar select 'save' → 'save as' → 'desktop' → 'SYBR Green results'→ 'save' →Click 'Start Run' button. After the run is complete, label and store the PCR plate at 4°C.

5. **Analyzing the results**

Amplification plot: displays amplification of all samples in the selected wells. The plot can be viewed as either linear or log 10. To analyze the experiment, review the following parameters,

1. ΔRn versus Cycle. Check that baseline and threshold values are set correctly
2. Rn versus Cycle. Identify and examine irregular amplification
3. Ct* versus Well. Locate outliers

*Ct = Threshold cycle; The PCR cycle number at which the fluorescent level crosses the threshold. A Ct value >8 indicates that there is too much template in the reaction while a Ct value >35 indicates a low amount of target in the reaction

Automatically calculate baseline and threshold values based on the assumption that the data exhibit a typical amplification plot. However, experimental error can cause atypical amplification resulting in incorrect baseline and threshold values calculated by the software.

! Examine the amplification plot and review the automatically assigned baseline and threshold values for each well after experiment is complete. Manually change values if they are set incorrectly and always make a note of the manually-set threshold used to make calls for an experiment in the laboratory book.

Standard Curve: An optimal quantitation experiment should have a standard curve with values close to the following,

Slope -3.3 Efficiency 100% (> 90%) R^2 (Corr. Coefficient) > 0.99 Ct values >8 and <35

If the experiment results have significant deviation from these values, troubleshoot by:

i) Omitting wells from analysis (outliers) ii) Rerun the experiment

Melt Plot: Shows the melting temperature (Tm) profile of the q-PCR amplicon in a sample.

The Tm of unknown samples that show amplification must be the same as the Tm (±0.5 °C) given by the standards (i.e. 3D7 g-DNA) in order for these samples to be called 'positive'. Melt temperatures that are significantly different from that of the standards should not be called 'positive' even if they have amplification.

! Plates with samples having amplification but aberrant Tm must be run on 4% agarose gel to check band size of amplicon in comparison to band size of the standards.

7. REPORTING

7.2 Record results in worksheet.

7.3 Open the saved image in the “paint” program on the computer. Use the “insert text” feature to number the wells (e.g. 1, 2, 3 etc.)

7.4 Have the worksheet signed by an authorized reviewer before proceeding to the next step.

7.5 Sign into the google group page and post the image of the gel in the “Files” section. In the “discussion” section create a text entry that lists the well number and the corresponding sample ID.

8. PROCEDURAL NOTES

NA

9. WASTE MANAGEMENT

- All waste should be placed in the designated waste container and clearly labelled Biohazard waste bags for incineration.

10. RELATED DOCUMENTS

- DNA preparation worksheet
- PCR worksheet

11. REFERENCES

12. APPENDICES

NA

AMENDMENT RECORD

NAME	**DATE**	**SUMMARY OF CHANGES**

ACKNOWLEDGEMENT OF READING AND UNDERSTANDING THIS SOP BY USERS

NAME	SIGNATURE	DATE
Mr. Edson Mumba		
Mr. Jay Sikaima		
Mr. Kenny Situtu		

Appendix 9.5 SOP for *Plasmodium falciparum DHFR* and DHPS Genotyping

INTRODUCTION

Description and purpose of the SOP
Plamodium falciparum antifolate drug resistance-conferring polymorphisms can be detected using simple PCR and restriction enzyme digestion

A nested PCR strategy is used, in which the dihydrofolate reductase-thymidylate synthetase (DHFR-TS) and dihydropteroate synthetase (DHPS) domains spanning the drug resistance-conferring mutations are amplified. The nested PCR product is then used as substrate for allele-specific restriction enzyme assays that target restriction sites created or destroyed by the mutations. The ensuing restriction fragment length polymorphism (RFLP) is analyzed using simple agarose electrophoresis and visualized under UV transillumination.

The purpose of this SOP is to genotype *P. falciparum* dry blood spot (DBS) field samples for DHFR and DHPS polymorphisms.

II DEFINITIONS

Definitions of key terms used in the SOP (if necessary)

III FORMS or APPENDICES

List of forms or appendices associated with the SOP
Appendix 1. Oligonucleotide primer sequences
Appendix 2. Recipe for glycerol loading buffer (6X).

IV MATERIALS

Remember to fill in an audit sheet for each reagent whenever a new batch is opened.

Reagents

- *Taq* polymerase (store at -20ºC) with appropriate buffer
- $MgCl_2$ stock solution (often provided by enzyme supplier, usually at 25 mM)
- dNTP mix: a working solution with a concentration of 10 mM each of dATP, dTTP, dCTP and dGTP (store at -20ºC)
- Oligonucleotide primers (see Appendix 1 for sequences): a working stock concentration of 10 µM for each primer (store at -20ºC)

- Gel Loading Buffer (6X) (see Appendix 2 for recipe)
- 10X TBE buffer:
    - 1 M Tris
    - 1 M boric acid
    - 50 mM EDTA
- 100 bp DNA Ladder
- Agaroses:
    - Ultrapure agarose
    - high resolution agaroses (such as NuSieve or MetaPhor, Cambrex)
- Ethidium bromide stock solution (10 mg/ml)
    - CAUTION: This chemical is toxic and mutagenic. Wear gloves and use only in a chemical fume hood. Store in the dark at 4ºC.
- Double distilled water

Equipment *(refer to instrument manual for each piece of equipment for use)*

- 10 µl, 200 µl, 1000 µl micropipettes and tips
- thermal cycler
- horizontal gel electrophoresis system
- photographic equipment and UV transilluminator
- refrigerator (4ºC) and freezer (-20ºC) for reagent storage
- 250 ml Erlenmeyer flask
- microcentrifuge

V PROCEDURES

V.1 Extracting DNA from Dry Blood Spot Samples *(by Trained Laboratory Scientist)*

Use the Chelex method as follows.

1. Insert the dried sample strip into the mouth of a sterile, pre-labelled 1.5 ml eppendorf tube by making a right angular fold at the blood-free base.
2. Using a clean pair of scissors cut the blood-free section of the sample strip and allow strip to drop into the eppendorf tube.
3. Wipe the pair of scissors with a fresh cotton wool swab of 70% ethanol and flame on a bunsen burner between cuttings to preclude cross-contamination of samples.
4. After loading sample strips into Eppendorf tubes, add 1 ml of autoclaved 1X phosphate buffered saline (PBS)/1% (w/v) saponin solution to each tube and leave at room temperature for 20 minutes.
5. Spin the sample tubes at top speed (about 15 000 revolutions per minute (rpm)) in a microcentrifuge for 2 minutes.

6. Aspirate and discard supernatant and any debris, leaving the sample strips in the tube. Add 1 ml of 1X PBS to each sample tube and repeat the 2-minute centrifugation.
7. Aspirate and discard supernatant and any debris, leaving the sample strips in the tube. Add another 1 ml of 1X PBS to each sample tube and repeat the 2-minute centrifugation.
8. Discard supernatant again, retaining the sample strips in the tubes. To each sample tube add 150 μl of autoclaved double distilled water and 50 μl of 20% w/v chelex resin suspension in autoclaved double distilled water.
9. Close sample tube and pierce a fine hole in the lid using a hot 23G hypodermic needle.
10. Flame the needle between sample tubes to avoid cross contamination. Boil the sample strip in the chelex resin for 8 minutes on a heat block (Alternatively the tubes could be floated on boiling water in a water bath. The fine hole introduced in the lids of the sample tubes should prevent them from popping open during the boiling step).
11. After boiling, spin the sample tubes at maximum speed in a microcentrifuge for 1 minute.
12. Transfer supernatant from this spin in 100 μl duplicate aliquots to storage vials and keep at 4°C pending use in PCR assays (-20°C for longer storage periods).

V.2 Setting up the first amplification reaction *(by Trained Laboratory Scientist)*

1. Calculate the total volume of reaction mixture required for the samples (include a laboratory standard clone as positive control, and one blank filter paper extract and PCR water as negative controls). Multiply (total number of reactions + 1 (for pipetting error)) by 25 µL volume per reaction to determine this total reaction mixture volume. From this, the number and size of microcentrifuge tubes required to hold the master-mix can be known.
2. For each 25 µL reaction, calculate the volume of:
    a. buffer (final concentration 1X)
    b. $MgCl_2$ (final concentration 1.5 mM)
    c. dNTPs (final concentration 200 µM each)
    d. separate primer pairs M1-M5, F-M4, M3-F/ (DHFR), R2-R/, J-K/, K-K/, L-L/ (DHPS), final concentration 0.25 µM each
    e. *Taq* polymerase (final amount concentration 1 unit per reaction)
    f. water to make up the total volume, less 2 µl for template
3. Calculate the total volume of each reagent to be added into the reaction master-mix by multiplying the volume for single reaction by (total number of reactions + 1)
4. Add into an autoclaved microcentrifuge tube, the correct volume to constitute master-mix, for each of the following:
    a. Water
    b. Oligonucleotide primers
    c. $MgCl_2$,

d. dNTPs
e. buffer
f. Taq polymerase

5. Close the microcentrifuge and mix by inverting the tube a few times, followed by a short vortex pulse.
6. Aliquot 23 µL of the reaction mixture per 0.2 ml PCR tube, each pre-labelled with the respective ID number for each sample.
7. Add 2 µL of the corresponding DNA template, positive control, negative control extract and PCR water to each PCR tube.
8. Tightly close lids for the reaction tube and place in the cycler
9. Run the PCR program for the reaction.

V.3 Setting up the Secondary Amplification Reactions *(Laboratory Scientist)*

1. For each of the secondary primer pairs for DHFR (F-M4, M3-F/) and DHPS (J-K/, K-K/, L-L/), repeat steps 1 – 9 as above. Two and three sets of reactions, respectively, will now be prepared for DHFR and DHPS, since each primer pair is run separately
2. For each sample, pipette 2 µL of product from the primary reaction to separate reaction tubes containing the respective primer sets for DHFR and DHPS (above).
3. Place the reaction tubes in the thermal cycler and run the PCR reaction.

V.4 Setting up Annealing Cycle Parameters (*Laboratory Scientist*)

For both primary and secondary reactions, use the following temperature cycle program, which must be set up on the thermal cycler:

Stage 1 (1 cycle):

- Initial denaturation at 94ºC for 2 minutes

Stage 2 (25 cycles):

- Denaturation at 94ºC for 45 seconds
- Annealing at 43.4ºC for 45 seconds
- Extension at 65ºC for 1 minute

Stage 3 (1 cycle):

- Final extension at 65ºC for 2 minutes

The risks of contamination are enormously increased when nested PCR is performed. Thus the transfer of the product of the first amplification reaction to the second amplification reaction mixture should be performed with extreme care. Use of barrier tips is important to avoid aerosols that can cause such carry-over contamination. Separate tips must be used

per sample. Separate rooms or workstations must be used for extracting DNA (see V.1), setting up the PCR reactions and electrophoresis of the PCR product (see V.5 below)

V.5 Restriction Enzyme Digestion

Set up restriction digest assays following the manufacturer's instructions. Use 4µl of amplicon as substrate in the reaction mix. For samples showing faint bands for the nested PCR product, use 6-8µl.

Enzymes for analyzing each codon are as shown in Table 1 –Table 2.

Table 1 RFLP analysis of DHFR polymorphisms

Substrate	Codon	Enzyme	RFLP	Residue
M3-F/	DHFR 16	NlaIII	93bp, 376bp	Ala
			146bp, 376bp	Val
	DHFR 51	Tsp509I	120bp, 154bp	Asn
			120bp, 218bp	Ile
	DHFR 108	AluI	196bp, 326bp	Ser
			522bp (no cut)	Other
		BsrI	190bp, 322bp	Asn
			522bp	Other
		BstNI	196bp, 326bp	Thr
			522bp	Other
F-M4	DHFR 59	XmnI	137bp, 189bp	Cys
			137bp, 163bp	Arg
	DHFR 108	AluI	119bp, 180bp	Ser
			299bp	Other
		BsrI	146bp, 180bp	Asn
			326bp	Other
		BstNI	145bp, 181bp	Thr
			326bp	Other

Table 1 RFLP analysis of DHPS polymorphisms

Substrate	Codon	Enzyme	RFLP	Residue
K-K/	436	MnlI	121bp, 278bp	Ser
			121bp, 317bp	Other
		MspA1I	406bp	Ala
			438bp (no cut)	Other
	437	MwoI	387bp	Ala
			419bp	Other
		AvaII	404bp	Gly
			438bp	Other
	540	FokI	85bp, 320bp	Glu
			405bp	Other (Lys)
J-K/	436	*Hind*III	406bp	Phe
			438bp	Other
		*Hha*I	406bp	Ala
			438bp	Other
L-L/	581	BstUI	105bp	Ala
			138bp	Other
		BslI	128bp	Gly
			161bp (no cut)	Other
	613	MwoI	128bp	Ala
			161bp	Other
		BsaWI	131bp	Ser
			161bp	Other
		AgeI	128bp	Thr
			161bp	Other

V.6 Analysis of the PCR product (*Laboratory Scientist*)

1. Determine the volume of gel required to accommodate combs with sufficient wells to run the samples

2. Weigh appropriate amount of agarose in Elernmeyer flask sufficient to make 2% (w/v) in 0.5X TBE buffer.

3. Add the 0.5X TBE buffer to make the require gel volume and microwave suspension to the boil till all agarose has dissolved to a clear.

4. Place the flask on benchtop and let the solution cool till no steam can be seen escaping from the mouth of flask (about 70ºC)

5. Add 5 µl of 10mg/ml ethidium bromide solution per 100 ml of gel (making 0.5 µg/ml final concentration) and swirl to uniform solution.

6. Pour into appropriate gel casting tray pre-mounted with clean gel combs and leave to set for about 40 minutes.

7. Carefully pull-out combs and place gel in electrophoresis tank.

8. Add sufficient 0.5X TBE buffer, also containing 5 µl (10 mg/ml) ethidium bromide solution per 100 ml of buffer) to just cover the gel

9. Pipette 5 µL of glycerol loading buffer into PCR product for each sample and mix.

10. Load 1.5 µl 100 bp DNA ladder into the centre lane

11. Load sample amplicon (13 µl) individually in the flanking lanes, and separately for each allelic family

12. Migrate samples in electrophoresis tank at 100V

13. Visualize and capture gel images on UV transilluminator. Use image analysis software to size bands for each of the *P. falciparum* DHFR and DHPS primer sets and the respective RFLP from restriction enzyme digestions.

14. Dispose of gel and gloves into special ethidium bromide waste containers. Rinse the gel tray and combs with water and dry before putting away.

DHFR and DHPS Oligonucleotide Primer Sequences

- **Primary reaction:**
  - **DHFR**
    - M1: 5'-TTTATGATGGAACAAGTCTGC-3'
    - M5: 5'-AGTATATACATCGCTAACAGA-3'

  - **DHPS**
    - R2: 5'-AACCTAAACGTGCTGTTCAA-3'
    - R/: 5'-AATTGTGTGATTTGTCCACAA-3'

- **Secondary reaction:**

  DHFR

  - M3: 5'- TTTATGATGGAACAAGTCTGCGACGTT -3'
  - F/: 5'- AAATTCTTGATAAACAACGGAACCTttTA -3'

  - F: 5'- GAAATGTAATTCCCTAGATATGgAATATT -3'
  - M4: 5'- TTAATTTCCCAAGTAAAACTATTAGAgCTTC -3'

  DHPS

  - *K: 5'- TGCTAG*TGTTATAGATATAGGatGAGcATC -3'
  - K/: 5'- CTATAACGAGGTATTgCATTTAATgCAAGAA -3'

  - J: 5'- TGCTAGTGTTATAGATATAGGTGGAGAAagC -3'
  - K/: 5'- CTATAACGAGGTATTgCATTTAATgCAAGAA -3□

  - L: 5'- ATAGGATACTATTTGATATTGGAccAGGATTcG -3'
  - L/: 5'- TATTACAACATTTTGATCATTCgcGCAAccGG -3'

Appendix 2: Preparation of 6X Glycerol Loading Buffer

- Dissolve 25mg bromophenol blue and 25 mg xylene cyanol FF
- Add 3 ml glycerol
- Make up to 10 ml with distilled water
- Store at 4ºC

References

Mkulama MA, Chishimba S, Sikalima J, Rouse P, Thuma PE, Mharakurwa S: Escalating Plasmodium falciparum antifolate drug resistance mutations in Macha, rural Zambia. *Malar J* 2008, 7:87.

Duraisingh MT, Curtis J, Warhurst DC: *Plasmodium falciparum*: detection of polymorphisms in the dihydrofolate reductase and dihydropteroate synthetase genes by PCR and restriction digestion. *Exp Parasitol* 1998, 89:1-8.

Mharakurwa S, Simoloka C, Thuma PE, Shiff CJ, Sullivan DJ: PCR detection of Plasmodium falciparum in human urine and saliva samples. *Malar J* 2006, 5:103.

Appendix 2.5a. Treponema pallidum hemagglutination Assay

	Name and Position	**Signature**	**Date**
Authored by			
Reviewed by			
Approved by	Dr Mike Chaponda **Principal Investigator**		

CONTENTS

ABBREVIATIONS

CI	Chief Investigator
Co-PI	Co-Principal Investigator
PI	Principal Investigator
RPR	Rapid Plasma Reagin
SOP	Standard Operating Procedure
TPHA	Treponema Pallidum Haemagglutination Assay

1. PURPOSE

This document describes the procedure for Syphilis testing using Treponema pallidum haemagglunation test (TPHA) for participants in the ASPIRE trial. IMMUTREP TPHA is a specific, sensitive passive hemagglutination test for the detection of antibodies to Treponema pallidum present in the sample serum or plasma.

2. ALLOWABLE EXCEPTIONS

This SOP is meant to be followed without deviation. However, it is an allowable exception to follow procedures specified in a protocol that may deviate from this SOP.

3. PERSONNEL RESPONSIBLE

This SOP applies to the Chief Investigator, Trial Manager, Principle Investigators (PI) and Coinvestigators, Coordinators, Site Managers, Research Nurses and other relevant staff of the ASPIRE study.

4. MATERIALS

4.1 Pipette, size P200

4.2 Pipette tips

4.3 Centrifuge machine

4.4 Treponema pallidum haemagglutination test kit

4.5 Microtiter plate

4.6 Timer

5. PROCEDURES

5.1 Allow samples and reagents to reach room temperature and ensure that samples and all reagents are fully resuspended before use. Samples do not require any pretreatment.

5.2 Each test requires 4 wells of a microtitre plate.

5.3 Dispense Diluent into the microtitration plate as follows:

- 5.3.1 25µl in rows 1, 3 & 4 and 100µl in row 2.
- 5.3.2 Dispense 25µl of each sample into a well in row 1.
- 5.3.3 Mix well and transfer 25µl from row 1 to row 2.
- 5.3.4 Mix well and transfer 25µl from row 2 to row 3.
- 5.3.5 Mix well and discard 25µl from row 3.
- 5.3.6 Mix well and discard 25µl from row 4 Transfer.
- 5.3.7 25µl from row 2 to row 4.

5.4 Add 75µl of well mixed Control Cells to row 3.

5.5 Add 75µl of well mixed Test Cells to row 4. Tap plate gently to mix.

5.6 The final dilutions in row 3 and 4 are 1/80.

5.7 Cover and let stand at room temperature for 45 to 60 minutes (alternatively the plates can be left overnight).

5.8 Examine for agglutination patterns.

5.9 Note: Kit controls are prediluted and should be added directly into individual wells in row 3 and 4 (no diluent required).

5.10 Results and Interpretation

5.10.1 Kit controls or known level value samples should be tested with each test run.

5.10.2 The kit negative control should give a negative result after 45 minutes.

5.10.3 The kit positive control should give a positive result after 45 minutes. If levels of controls or users known samples do not give expected results, test results must be considered invalid.

5.10.4 Screening Procedure Agglutinated cells form an even layer over the bottom of the well.

5.10.5 Non-agglutinated cells form a compact button in the centre of the well. Weakly agglutinated cells form a characteristic ring pattern.

5.10.6 Agglutination of the Test Cells but not the Control Cells indicates the presence of specific antibody to *T.pallidum*.

5.10.7 Absence of agglutination indicates that antibody is below the limit of detection of the system.

5.10.8 Do not use the Control Cell pattern as an indication of a negative result since they give a more compact button of cells.

5.10.9 Agglutination of the Control Cells as well as the Test Cells indicates the presence of anti-cell antibody. In this event the test is not valid and should be repeated.

6. TRAINING

6.1 Each staff member receives or has direct access to applicable Standard Operating Procedures (SOPs).

6.2 New staff are trained on applicable SOPs prior to their participation in the study specific procedures and being entered in the delegation log.

6.3 Staff members whose duties fall within this SOP scope are retrained within 7 days of the approval of each SOP revision.

7. ATTACHMENTS

7.1 N/A

8. REFERENCES

N/A

Document Control Section Tracking SOP review

Purpose: The log records review dates of this SOP and the status of the review. The Tracking Changes and Version Control Log are completed to detail status of the review.

When: The SOP is reviewed every year or more often when necessary.

By whom: The SOP is reviewed by staff directly following the SOP. The review process is overseen by PIs/designee and reviewed by review team.

Supersedes version number	Date & New version number	Reason for review/change	Name of reviewer

Appendix 9.6. Syphilis *Treponema Pallidum* Haemagglutination Test

Atlas Medical CE

TPHA TEST KIT

For the detection of antibodies to T.pallidum in human Serum using micro haemagglutination.

IVD *For In-Vitro diagnostic and professional use only*

Store at 2° to 8° C

INTENDED USE

TPHA test kit is designed for the detection of antibodies to *Treponema pallidum* (IgG and IgM antibodies) in human serum or plasma based on the principle of passive haemagglutination.

INTRODUCTION

Syphilis is a venereal disease caused by the spirochaete micro-organism *Treponema pallidum*. As this organism cannot be cultured on artificial media the diagnosis of syphilis depends on the correlation of clinical data with the specific antibody demonstrated by serological tests. Serological screening tests for syphilis using cardiolipin and lecithin as antigens are simple to perform but biological false positive (BFP) reactions occur frequently because the tests use non-treponemal antigens.

The TPI and FTA-ABS tests utilize pathogenic *Treponema pallidum* as the antigen but these tests present some difficulties for routine serodiagnosis. The TPI test requires living pathogenic *T. Pallidum* and the FTA-ABS test requires a flourescence microscope. Both tests require a high level of expertise.

TPHA test kit has been shown to be a convenient and specific test for the diagnosis of treponemal infection, having specificity similar to that of the TPI test and sensitivity comparable to that of the FTA-ABS test. It requires minimum laboratory equipment and is very simple to perform.

TPHA reagents are used to detect human serum antibody to *T. pallidum* by means of an indirect haemagglutination (IHA) method. Preserved avian erythrocytes are coated with antigenic components of pathogenic *T. pallidum* (Nichol's strain). These Test Cells agglutinate in the presence of specific antibodies to *T.pallidum,* and show characteristic patterns in microtitration plates. Any non-specific reactions occurring are detected using the Control Cells, which are avian erythrocytes not coated with *T. pallidum* antigens. Non-specific reactions may also be absorbed out using these Control Cells. Z

Antibodies to non-pathogenic treponemes are absorbed by an extract of Reiter's treponemes, included in the cell suspension. Test results are

obtained in 45-60 minutes and the cell agglutination patterns are both easily read and long lasting.

The test sample is diluted in absorbing diluent to remove possible cross-reacting heterophile antibody and to remove, block, or absorb potentially cross-reacting. Nonpathogenic treponemal antibodies.

MATERIALS

MATERIALS PROVIDED

- Test cells; preserved avian erythrocytes sensitised with *T.pallidum* antigen.
- Control cells; preserved avian erythrocyte.
- Diluent.
- Positive control serum; (prediluted 1:20), Use neat. This will give an equivalent titer of 1/640:/2560 in the quantitative test.
- Negative control serum; (prediluted 1:20), Use neat.
- Package Insert.

MATERIALS NEEDED BUT NOT PROVIDED

- Accurate pipettes for delivering 10:25:75 and 190 microlitres.
- U-Well microtitration plates.

PRECAUTIONS

The reagents and controls contain 0.1% sodium azide as a preservative. Avoid ingestion and contact with skin or mucus membrane. Normal laboratory precautions should be maintained while handling test reagents.

REAGENTS HANDELING

- All the reagents must be allowed to reach room temperature before use.
- Do not freeze any of the reagents.
- Do not use heamolysed, contaminated or lipaemic serum or plasma for testing as this will adversely affect the results.

REAGENTS STORAGE

- The kit should be stored at 2-8º C in an upright position at all times.
- Under these conditions, kit performance characteristics will be maintained for at least 15 or 18 months from date of manufacture. See expiry date on k
- The reagents in each kit have been standardized to produce the proper reaction and reagents should not be interchanged with those from other batches.

SAMPLE PREPARATION

- The test is designed for use with serum only.

Plasma samples should not be used.

- The samples should be free from haemolysis and contamination.
- Serum samples may be stored at 2-8° C if a preservative is added prior to storage.
- For long term storage sera should be stored at -20° C Strictly avoid contaminating any of the reagents or serum dilutions with saliva. This will cause confusing patterns similar to positive results with specimens which should be negative.

PROCEDURES

QUALITATIVE METHOD

Each sample requires 3 wells of a microtitration plate.

1. Add 190µl of diluent to Well 1.
2. Add 10µlserum to Well 1. (Sample dilution 1:20).
3. Using a micropipette, mix contents of Well 1 and transfer 25µl to Wells 2 & 3.
4. Ensure that the Test and Control Cells are thoroughly resuspended. Add 75µlof control cells to Well 2. Add 75µl of Test Cells to Well 3.
5. Tap the plate gently to mix the contents thoroughly.
6. Incubate 45-60 minutes at room temperature.
7. Caution! Keep the plate away from heat, direct sunlight and any source of vibration.
8. Read results. Results are stable for 24hrs if the plate is covered and the above precautions are observed.

NOTE

Kit controls can be run in parallel and are diluted and ready for use.

QUANTITATIVE TEST

Each sample requires 8 Wells of a microtitration plate, Labeled A through to H.

1. Add 25µl of diluent to Wells B to H inclusive.
2. Transfer 25µlof 1:20 serum dilution from screening test to Wells A and B.
3. Take 25µl of diluted serum from Well B and serially dilute from Wells B to H inclusive in 25µl aliquots, discarding 25µl of diluted serum from Well H.
4. Ensure that the Test Cells are thoroughly resuspended. Add 75µl of Test cells to wells A to H inclusive. This will give a dilution of serum of 1/ 80 in well A through 1/ 10240 Well H.
5. Shake the plate gently to mix the contents thoroughly.
6. Incubatefor45-60 minutes at room temperature.
7. Caution! Keep the plate away from heat, direct sunlight and any source of vibration.

8. Read results. Results are stable for 24hrs. if the plate is covered and the above precautions are observed.

RESULTS

RESULTS	TEST CELLS	CONTROL CELLS
Strong Positive	Full cell pattern covering the bottom of the well.	No agglutination tight button
Weak Positive	Cell pattern covers approx. 1/3 of well bottom	No agglutination tight button
Indeterminate	Cell pattern shows a distinctly open center	No agglutination tight button
Negative	Cells settled to a compact bottom, typically with a small clear center.	No agglutination tight button
Non-specific *	Positive reaction	Positive reaction

Non-specific absorption *

1. Add 10µl to a small tube then add 190µl of Control Cells. Mix well and stand for 30 minutes.

2. Centrifuge for 15 minutes at 1000 rpm and test the supernatant by the qualitative method.

Note

If the result is repeatedly non-specific the sample should be tested by another method eg. Reagin or FTA-ABS.

Although TPHA test is highly specific, **false positive results** have been known to occur in patients suffering from leprosy, infectious mononucleosis and connective tissue disorders. For confirmation FTA-ABS test should be used.

INTERPRETATION OF RESULTS

Strong positive reactions may show some folding at the edge of the cell mat. When the Test well is positive, the Control well should be observed.

The Control cells should settle to a compact button. They should not be used as a comparison for Non-Reactive serum patterns since the Control Cells will give a more compact pattern than the Test Cells.

Weak positive may show partially not full cell pattern cover the well bottom

INVALID may show Agglutination in the Control well indicates the presence of non-specific agglutinins in the sample. A serum that gives this result may be absorbed using the Control Cells as detailed under Non-specific absorption.

1. **INDETERMINATE**A may show a doubtful reaction with Test Cells This result review. Genitourin Med. 1992; 68: 413-9.

	Catalogue Number		Temperature limit
	In Vitro diagnostic medical device		Caution
	Contain suffic fo s ient r <n> tests and Relative size		Consult instructions for use (IFU)
	Batch code		Manufacturer
	Fragile, handle with care		Use-by date
	Manufacturer fax number		Do not use if package is damaged
	Manufacturer telephone number		Date of Manufacture
	Kee fro p away m sunlight		Keep dry

ATLAS MEDICAL
Ludwig-Erhard Ring 3
15827 Blankenfelde-Mahlow
Germany
Tel: +49 - 33708 – 3550 30
Email: Info@atlas-medical.com
PPI1457A01
Rev A (02.09.2019

may indicate a low level of antibody in early primary syphilis or yaws. This sample should be first retested in the qualitative test then a further sample and/or another confirmation test (FTA-ABS) to complete the profile of the test serum.

Negative may show cells settled as a dot at the bottom of the well

PERFORMANCE

SENSITIVITY

With clinical samples when compared to FTA-ABS and/or clinical diagnosis was 99.7% (298/299)

SPECIFICITY

With clinical samples was 99.3% (301/303).

CROSS REACTIVITY

Reactive results may indicate an active or successfully treated infection. The following have all been shown not to interfere with the test results (10 clinical samples of each)

- Rheumatoid Factor.
- Post Hepatitis B vaccination.
- Genital Herpes.
- Leptospirosis.
- EBV Infection.
- SLE.
- Lyme's Disease.

REFRENCES

2. Rathlev T. - Haemagglutination tests utilizing antigens from pathogenic and apathogenic Treponema pallidum WHO/VDT/RES 1965; 77: 65.

3. Tomizawa T, Kasamatsu S. - Haemagglutination tests for diagnosis of syphilis. A preliminary report. Japan. J. Med. Sci. Biol. 19, 305-308, 1966.

4. Rathlev T. - Haemagglutination test utilizing pathogenic Treponema pallidum for the serodiagnosis of syphilis. Br J Vener Dis 1967; 43: 181-5.

5. Tomizawa T. Kasamatsu S. Yamaya S. - Usefulness of the haemagglutination test using Treponema pallidum antigen (TPHA) for the serodiagnosis of syphilis. Jap J Med Sci Biol 1969; 22: 341-50.

6. Sequeira P, J, L. Eldridge A, E. – Treponemal Haemagglutination test. Br J Vener Dis 1973; 49: 242-8.

7. Larsen S.A., Hambie E.A., et coll., Specificity, sensitivity, and reproducibility among the fluorescent treponemal antibody absorption test, the micro hemagglutination assay for Treponema pallidum antibodies, and the hemagglutination treponemal test for syphilis. J. Clin. Microbiol., 1981; 14: 441 – 445.

8. Houng H. - Syphilis: new diagnostic directions. Intern. J. STD and AIDS 1992; 3: 391-413.

9. Sluis J.J. Van Der. - Laboratory Techniques in the diagnosis of syphilis: a

Appendix 9.7 Diagnosis of Bacterial Vaginosis

Effects of metronidazole plus intermittent preventive treatment of malaria in pregnancy on birth outcomes: a randomised controlled trial in Zambia

SOP TITLE: Diagnosis of Bacterial Vaginosis	**VERSION:1.0**
SOP NUMBER: ASP-LAB-13	**Effective date: 10th Dec 2019**

.

	Name and Position	Signature	Date
Authored by			
Reviewed by			
Approved by	Dr. Mike Chaponda **Principal Investigator**		

ABBREVIATIONS

BV Bacterial vaginosis

SOP Standard operating procedure

1. PURPOSE

This document describes the procedures to be followed when diagnosing bacterial vaginosis in the ASPIRE study to ensure that diagnosis is conducted and results recorded in a standardized way.

2. ALLOWABLE EXCEPTIONS

This SOP is meant to be followed without deviation. However, it is an allowable exception to follow procedures specified in a protocol that may deviate from this SOP.

3. PERSONNEL RESPONSIBLE

This SOP applies to the Chief Investigator, Trial Manager, Principle Investigators (PI) and Co-investigators, Coordinators, Site Managers, Research Nurses and other relevant staff of the ASPIRE study.

4. INSTRUMENTATION AND MATERIALS

4.1 Microscope

4.2 Crystal violet

4.3 Iodine solution

4.4 Alcohol/acetone solution

4.5 Safranin

4.6 Blotting paper

5. SPECIMEN STORAGE AND HANDLING

5.1 A smears prepared for the diagnosis of BV should be left to air dry.

5.2 Air dried smears shall be neatly packed in slide boxes and transported to St. Paul's Hospital laboratory.

5.3 The BV smear will be heat fixed before being Gram stained.

5.4 The ideal BV smear should upon visual inspection cover at least two-thirds of the surface area of a properly labeled glass slide.

5.5 The slide should be properly labeled including the date of collection, using an indelible marker that will not wash off during the decolourisation step of the Gram staining procedure.

5.6 The BV smears should be packaged so as to protect the glass slides from breaking enroute.

6. PROCEDURE FOR GRAM STAINING AND MICROSCOPIC EXAMINATION

6.1 CRITERIA FOR REJECTION

6.1.1 Inadequately, unlabeled, or illegibly labeled slides.

6.1.2 Broken slides.

6.1.3 Slides lacking specimen inoculum.

6.1.4 Slides containing < 2 epithelial cells per oil powered field; indicates sample collected from wrong site; cervical, not vaginal.

6.1.5 Slides containing only cellular debris.

6.2. PROCEDURE FOR GRAM STAIN

6.2.1 The smear shall be heat fixed over a flame.

6.2.2 The slide shall be flooded with crystal violet - 20 sec.

6.2.3 The smear shall be washed with distilled water - 2 sec.

6.2.4 The slide shall then be flooded with Gram iodine - 1 min.

6.2.5 Decolourisation shall be performed by tilting the slide and drop by drop rinsing with 95% ethanol until ethanol runs clear - about 10 to 20 sec.

6.2.6 The slide shall be washed with distilled water - 2 sec.

6.2.7 The slide shall then be flooded with safranin - 20 sec.

6.2.8 Finally, the slide shall be washed with distilled water - 2 sec and blotted dry.

6.3 SLIDE READING

The slides shall first be scanned using a low power objective to locate any clusters of epithelial cells. The flora in these areas should be noted. Using the oil immersion lens (x1000) 10 to 20 representative fields shall be examined to observe cell morphology and Gram reaction.

The BV score for Gram staining will be calculated by Nugent's method (1991). Briefly, the average number of lactobacilliary morphotypes per oil immersion field will be quantified. These organisms are usually filamentous, gram positive rods of varying length that often form chains, but occasionally, they may stain gram negative.
The average number of *Gardnerella* spp. and anaerobic gram negative rods shall also be quantified. These may appear as small, gram variable pleomorphic coccobacilli.
Finally, the amount of *Mobiluncus* morphotypes present shall be quantified. They are often thin, wipy, eyelash-like faintly staining curved gram negative rods. Alternatively, they may be much smaller "banana-like" forms with pointed ends. Occasionally, they may stain gram positive.
Results will be classified based on the Nugent criteria (256) and recorded in the appropriate result record form.

Nugent score = the sum of the score for each bacterial morphotype listed in Table 1(Note: number of organisms seen/100X objective.

Table 1: Guide for laboratory examination of vaginal smear and the determination of Nugent score.

lactobacilli	score	Gardinerella/ bacteroides	score	Curved gram-negative bacilli	score	Sum=*N-score
30 or >	0	0	0	0	0	0
5-30	1	<1	1	<1	1	3
1-4	2	1-4	2	1-4	1	5
<1	3	5-30	3	5-30	2	8
0	4	30 or >	4	30 or >	2	10

*Interpretation of Nugent score		
If N score is	AND	Then report
0-3		Smear not consistent with BV
4-6	Clue cells not present	
4-6	Clue cells are present	Smear consistent with BV
≥7		

SAFETY PRECAUTIONS

7.1 The technologist should wear gloves, laboratory coat and safety glasses while carrying out the staining procedure.

7.2 All Gram stain reagents should be properly handled and stored in accordance with safety regulations.

7.3 The alcohol/acetone decolorization solution should be stored in a safety cabinet designed to hold flammable liquids.

7. QUALITY ASSURANCE

8.1 For quality control (5%) randomly selected slides will be read by a second observer. Any disagreements in the results will be settled by a third observer.

8. TRAINING

9.1 Each staff member shall receive or will have direct access to applicable Standard Operating Procedures (SOPs).

9.2 Designated staff member shall review the applicable SOPs at least every 3 months (quarterly).

9.3 All SOP training shall be documented and tracked and stored in the research office.

9.4 New staff shall be trained on applicable SOPs prior to their participation in the study specific procedures and being entered in the delegation log.

9.5 Staff members whose duties fall within this SOP scope shall be retrained within 7 days of the approval of each SOP revision.

9. ATTACHMENTS

10.1 N/A

10. REFERENCES

Document Control Section Tracking SOP review

Purpose: The log records review dates of this SOP and the status of the review. The Tracking Changes and Version Control Log are completed to detail status of the review.

When: The SOP is reviewed every year or more often when necessary.

By whom: The SOP is reviewed by staff directly following the SOP. The review process is overseen by PIs/designee and reviewed by review team.

Supersedes version number	Date & New version number	Reason for review/change	Name of reviewer

Appendix 9.8. A multiplex PCR assay for the detection of Chlamydia trachomatis, Neisseria gonorrhoeae, and Trichomonas vaginalis

TROPICAL DISEASES RESEARCH CENTRE (TDRC)

DEPARTMENT OF BIOMEDICAL SCIENCES

TECHNICAL STANDARD OPERATING PROCEDURES (SOP – T-MOLB – 1.01)

Title of SOP	**A multiplex PCR assay for the detection of Chlamydia trachomatis, Neisseria gonorrhoeae, and Trichomonas vaginalis**
Version number of SOP	**1.0**
Type of SOP	**TECHNICAL**

Approved by: Name, Signature & Date:	Mr. Sydney Mwanza	Designation: HoU
Authorized by: Name, Signature & Date:	Dr. Justin Chileshe	Designation: HoD

Address:
6th & 7th floor Ndola Central Hospital
Corner of Nkana and Broadway Roads,
P.O. Box 71769, NDOLA, ZAMBIA
Website: www.tdrc.org.zm

PURPOSE

The purpose of this document is to provide guidelines for the detection of Chlamydia trachomatis, Neisseria gonorrhoeae, and Trichomonas vaginalis using a multiplex PCR.

SCOPE

This SOP will be used in the molecular Biology laboratory.

RESPONSIBILITY

The Laboratory Scientist and Technologists are responsible to carry out the procedure. The Chief Laboratory Technologist is ultimately responsible to ensure that all staff follow this procedure.

DEFINITIONS

NA

ABBREVIATIONS

TDRC – Tropical Diseases Research Centre

SOP – Standard Operating Procedure

PPE – Personal Protective Equipment

PCR – Polymerase Chain Reaction

N/A – Not Applicable

13. PRINCIPLE

The multi-template multiplex PCR principle relies on the amplification of different templates in a single reaction. Meaning, it amplifies various templates present in a single sample using different primer sets. Each set of primers is unique to each template. And therefore can amplify the target/complementary sequence (template) only. Multi-template multiplex PCR is used in microbial genetics, pathogen identification and detection of microbes. Here, it quantifies, amplifies and identifies microbes/pathogens present in a sample.

14. EQUIPMENT SUPPLIES AND REAGENTS

Equipment	Supplies	Reagents
Abbott 7500 Fast real-time PCR machine	PCR plates	**Extraction Kit: Quick-DNA™ Miniprep Kit:** Genomic lysis buffer DNA pre-wash buffer g-DNA wash buffer DNA elution buffer Zymo-spin™ IICR columns Collection tubes
Micro-centrifuge	Clean micro-centrifuge tubes	Internal control (IC2M)
vortex	Pipette tips: 1000ul,200ul,20ul,10ul	Primers
	Pipettes	70% alcohol
	Sharps box	
	Cold block	
	Pen/marker/pencil	

15. SPECIMENS

- Vaginal swabs

16. QUALITY CONTROL

- Ensure that the specimen containers are labelled with a unique identifier, visit number and date of collection.

17. SAFETY PROCEDURE

- Follow all standard guidelines and universal precautions for working with bio-hazardous materials.
- All vaginal swabs must be regarded as potentially infectious.
- All sharps should be placed in a puncture-resistant, leak proof, sharps disposal container.
- Report all injuries involving sharps to the Laboratory Supervisor and Safety Officer.
- Use appropriate PPE when during DNA extraction and PCR (gloves and laboratory coat).
- Observe good personal hygiene (e.g., wash hands after removing PPE and before leaving the work area).
- Clean up spills with an appropriate disinfectant (1-10 % bleach).
- Decontaminate all instruments, materials and workstation used with an appropriate disinfectant (70% alcohol).
- **DO NOT HOLD SPECIMENS DIRECTLY IN YOUR HAND.**

18. STEP BY STEP PROCEDURE

11.1 DNA Extraction– Vaginal swab

- Add 500ul of **Genomic Lysis Buffer** to the vaginal swab.
- Vortex for 4-6 seconds.
- Allow to stand at room temperature for 10minutes.
- Transfer the mixture to **Zymo-Spin IICR Column** pre-labelled with sample ID number in a collection. Centrifuge at 12,000 rpm for one minute.
- **Discard** the collection tube with the flow through. Transfer the Zymo-Spin™ IICR Column to a new collection tube
- Add 200ul of **DNA Pre-Wash Buffer to** the spin column. Centrifuge at 12,000 rpm for one minute.

- Add 500ul of **g-DNA Wash Buffer** to the spin column. Centrifuge at 12,000rpm for one minute.
- Transfer the spin column to a clean micro-centrifuge tube labelled with the sample ID number.
- Add ≥50ul of **DNA Elution Buffer** to the spin column.
- Incubate for 5 minutes at room temperature and then centrifuge at 14,000rpm for 30seconds to elute the DNA.
- The eluted DNA can be used immediately for molecular application or stored at ≤ 20ºC for future use

PCR STAGE

- Remove samples from the freezer to thaw as Master-mix is been prepared.
- For samples, prepare 25 µl PCR reactions consisting of 1× SsoFastTM EvaGreen® Supermix and10 µl extracted DNA (this should theoretically contain ~2.5 pg IC2M, 100 nM forward and reverse CT primers, 175 nM forward and reverse NG primers, 125 nM forward and reverse TV primers and 150 nM IC2M forward and reverse primers. For the limit of detection studies, DNA extracted from a known copy number of each target was spiked into the PCR reaction, in addition to 2.5 pg IC2M
- PCR amplification was performed on the Abbott 7500Fast Real-Time PCR machine using a touchdown PCR protocol. that includes:
    - 5 minute hot start (95 °C)
    - 2 cycles of 5 second denaturation (95 °C)
    - 30 second annealing/extension at 70 °C,
    - 2 cycles of 5 at 95 °C and 30 s at 67 °C,
    - 2 cycles of 5 s at 95 °C and 30 s at 65 °C,
    - 2 cycles of 5 s at 95 °C and 30 s at 63 °C, and finally
    - 32 cycles of 5 s at 95 °C and 30 s at 60 °C.
    - A melt protocol was also included from 60 -95 °C at 0.2 °C/s.

19. INTERPRETATION OF RESULTS

- Based on the reference protocol (Experimental and Molecular Pathology 98 (2015) 214–218), the following are melt curves for the 3 organisms:
    - TV at 78 °C,
    - NG at 82.3 °C,
    - CT at 85.7 °C,
    - IC2M at 92 °C

20. REPORTING

NA

21. PROCEDURAL NOTES

NA

22. WASTE MANAGEMENT

- Discard leaking or contaminated specimens in a biohazard bag and decontaminate by either autoclaving or incineration.
- Discard contaminated gloves and other wastes in a biohazard bag and decontaminate by either autoclaving or incineration.

23. RELATED DOCUMENTS

- Specimen request forms
- Specimen listing – for specimens collecting on the field

24. REFERENCES

- Experimental and Molecular Pathology 98 (2015) 214–218
- Equipment manual for ABS 7500 fast

25. APPENDICES

NA

AMENDMENT RECORD

NAME	**DATE**	**SUMMARY OF CHANGES**

Appendix 9.9. SOP for Placental Histopathology Evaluation

ASPIRE PLACENTAL HISTOPATHOLOGY EVALUATION

STANDARD OPERATING PROCEDURES

SPECIMEN RECEPTION AND ACCESSIONING

Purpose

To provide criteria for reception, registration and handling of placenta biopsy specimens and ensure that the biopsies received are correctly identified before registration by manual methods in the designated register.

To accession the received placenta biopsy specimens by giving them laboratory numbers that will uniquely identify each specimen and maintained in a designated register.

Principle

The placenta biopsy specimens are recognized and identified upon their arrival in the laboratory, by a simple visual inspection performed by laboratory personnel.

The samples are then accepted according to the rejection criteria of the laboratory and registered. Specimen registration proves that a specimen has been received in the laboratory.

Materials, Equipment, Reagents and PPEs

Materials and Supplies
1. Specimen collection forms
2. Specimen register
3. Specimen correction book

Equipment
1. Fume cabinet or
2. Domestic fan

Special protective equipment
1. Examination gloves
2. Face mask
3. Lab coat
4. Goggles

Reagents
Ideal fixative (Formalin)

1.4 Specimen

Specimen
1. Placenta biopsies submitted for pathological review.
2. Biopsies must be accompanied by specimen collection forms

Specimen Collection Forms.

Specimen Collection Forms
1. Designated research number
2. Health facility
3. Age and Sex,
4. Location of the Patient
5. Date and Time of Collection
3. Name and Contact of the specimen collector

Specimen container

Specimen Collection Forms
1. Designated research number
2. Health facility
3. Age and Sex,
4. Location of the Patient
5. Date and Time of Collection
4. Name and Contact of the specimen collector

Special safety precautions

Safety precautions
General safety precautions as described in the local including Universal Precautions must be adhered to.

Procedure Step by step

Procedure
1. Specimens are received by the designated research laboratory technician.
2. The specimen container and specimen collection form be complete.
3. Make sure that specimen is immersed in adequate fixative.
4. If insufficient fixative is insufficient, additional fixative should be added.
5. The containers should be sealed to avoid spillage and loss of fixative.
6. Details on specimen collection form and specimen container should match.
7. The specimen is accessioned by giving them a laboratory number.
8. Check the last recorded number in the register and continue from there.
9. Assign the unique lab number to the specimen and the collection form.
10. The same assigned details must be entered in the register
11. Enter the request form details in the specimen register before the grossing.
12. There should be sufficient space available to store surgical specimens.
13. Specimens with their forms should be taken to the grossing bench.

Quality control.

Quality control
Samples for pathological review should be placed in the appropriate fixative immediately after removal from body.

Calculation of Results/Reference range/Test Interpretation

Quality control
Fixatives penetrate slowly approximately 1mm per hour.

SPECIMEN ACCEPTANCE AND REJECTION CRITERIA

Purpose

To ensure proper identification and documentation of problematic placenta biopsy specimens received in the laboratory so as to avoid processing of specimens that may compromise the quality of research result.

Principle

The placenta biopsies are singled out upon their arrival by the research laboratory technician by way of a simple visual inspection. Suitable samples are accepted and problematic samples are rejected according to the Acceptance/Rejection criteria of the laboratory.

Materials, Equipment, Reagents and PPEs

Materials and Supplies
1 Registration Book
2 Occurrence Log
3 Stationary

Equipment
1. Empty specimen containers
2. Fume hood
3. Fan

Special protective equipment
5. Examination gloves
6. Face mask
7. Lab coat
8. Goggles

Reagents
Ideal fixative (Formalin)

Specimen

Specimen
Placenta biopsies that fail set standard of tissue acceptance criteria

Special safety precautions

Safety precautions
General safety precautions as described in the local including Universal Precautions must be adhered to.

Procedure Step by step

Criteria for Rejecting Placenta Biopsies and Entering them in the Rejection Log Book
1. Placenta biopsies without labels on the container and collection form 2. Contradicting details on specimen container and collection form 3. Autolyzed specimens should be reviewed by the research Pathologist. 4. Leaking specimens with soiled sample collection forms.
Criteria for accepting placenta biopsies for Processing after Documentation and Corrective Action
1. Unautolyzed Placenta biopsies without a fixative OR in insufficient fixative **2.** Placenta biopsies without collection form. A copy should be generated. **3.** Collection forms with inadequate details have to be verified by collector. **4.** Leaking specimen containers should be corrected. **5.** Soiled collection forms should be dried.

Quality control.

Quality control
1. All occurrences should be logged in the occurrence/rejection log and follow-up action done immediately. 2. All corrective action should be done and properly documented

Calculation of Results/Reference range/Test Interpretation

Quality control
The laboratory's objective is to have less than 0.05% rejection rate.

SPECIMEN HANDLING FOR GROSSING

Purpose

To provide specific guidelines on how to handle placenta biopsies prior to grossing.

Principle

To macroscopically inspect placenta biopsy specimens using naked eyes in order to obtain diagnostic information while being processed for further microscopic examination

Materials, Equipment, Reagents and PPEs

Materials and Supplies
1. Embedding Cassettes
2. Occurrence Log blank form
3. Pencil/cassette marker
4. Cassette container
5. Grossing SOPs and Templates
6. Request Forms

Equipment
1. Grossing tools
2. Grossing board
3. Fan or fume extractor

Special protective equipment
1. Examination gloves
2. Face mask
3. Lab coat
4. Goggles
5. Aprons

Reagents
1. 10% NBF
2. Ethyl Alcohol
3. Running water

Specimen

Specimen
Formalin fixed placenta biopsy specimens

Special safety precautions

Safety precautions
1. All placenta biopsy specimens are considered highly infectious at all times.
2. All precautions must be considered to avoid inhaling the fixative.
3. Specimen collection form should be clean of blood and fixative at all times
4. General safety precautions as described in the local including Universal Precautions must be adhered to.

Criteria for Rejecting Placenta Biopsies and Entering them in the Rejection Log Book

1. Registered specimens either by LIS or manual method with respective request forms are moved from the registration workstation to the grossing bench.
2. Arrange the specimens in ascending order of registration ID and request forms should be arranged accordingly.
3. Ensure that all the reagent and consumables are available and the grossing kit is clean and disinfected. The place should be free of clutter.
4. The specimen container should remain sealed so that drying or other specimen damage cannot occur.
5. Identity of every specimen is maintained at all times during the gross examination steps. The specimen containers should be compared with the request form upon grossing to ensure the correct specimen for the right request form.
6. Labelling of cassettes should be done as each specimen is grossed to avoid mislabeling.
7. The case specimen number if there are multiple specimens for the same patient case are labeled alphabetically as A, B, C or numerically 1, 2, 3 etc. after the accessioning number on the cassettes respectively e.g. ZH01062009-A or ZH01062009-1.
8. Ensure that specific specimens follow the usage of the standard templates e.g. Breast Template, Colon Template
9. Ensure that there is complete adherence to the Grossing SOPs for each specimen type.
10. Sample size must be thin (3-4 mm) enough to ensure adequate fixation and processing of the tissue and small enough to fit in the cassette and allow space for processing fluids to enter the cassette on all sides.
11. Bloody or friable tissues should be wrapped so that the tissue sample is contained within the cassette to avoid cross contamination with other samples.
12. The number of biopsies or cores should be limited to enable proper embedding; all samples must lie flat in the cassette and within the same plane.
13. The number of cassettes per sample and number of pieces per cassettes should be recorded
14. All grossing details (description) must be written on the request form with drawings were applicable. Drawings and photographs can help to indicate the margins in complex specimens.

15. Gross descriptions include;
    . Anatomical source
    . Number of fragments
    . Dimensions and weight
    . Colour and consistency including visible lesions if present
    . Type of biopsy
16. Specialized embedding instructions/ processing or staining proceedures should be documented.
17. Record all NCEs observed during the grossing session in the NCE form and/or occurrence log.
18. Arrange the cassettes for processing appropriately and load in the tissue processing basket (Refer to tissue processing).
19. For bone and other mineralized tissues, refer to the Decalcification SOP (Document ID).
20. Ensure periodic review of specimens undergoing decalcification to avoid over decalcification.
21. The grossing bench should be cleaned by the personnel handling the grossing session

3.1 Quality Control

1. Ensure all reagents used are QC validated as per reagent quality control.
2. A xylene/ alcohol resistant marker, pen or pencil must be used to label the cassettes

REFERENCES

1 Bancroft J.D. Stevens, A. Theory and Practice of Histological Techniques. **Churchill, Livingstone, London, 1982.**

1 Bancroft, J.D.; Cook, H.C. Manual of Histological Techniques, **Churchill, Livingstone, London, 1984.**

2 **Bancroft JD, Gamble M,** Theory and Practice of Histological Techniques. **Churchill, Livingstone, London, 2008.**

3 Cook H.C.; Manual Histological Demonstration Techniques; **Butterworth's, 1974.**

4 Ministry of Health Standard operating Procedure for level III Hospitals (2008 Revision), Lusaka, Zambia.

Confidential

Screening

Screening Number (same number recorded on the screening registry)	_____ (should be as follow:e.g. 9XABCD, where 9 identifies the ID as a screening ID; X is the site identifier (1=Kabuta, 2=Kafutuma, 3=Kashikishi, 4=Nchelenge) ABCD are the subsequent participant numbers during screening, starting from 0001)
Initials of the person conducting the screening and entering the data	_____
Inform consent given?	◯ Yes ◯ No (If not, do NOT proceed with the screening)
Date and time informed consent is signed:	_____
Date of visit:	_____
Scan and upload the ANC card to record the HIV status and other useful information	(Please do not take photo of the identifier (cover the name))
INCLUSION CRITERIA	
1.Able to give consent or assent	◯ Yes ◯ No
2. Being HIV negative	◯ Yes ◯ No
3. Resident in the study area	◯ Yes ◯ No
4. Willingness to adhere to study visit procedures	◯ Yes ◯ No
5. Willingness to deliver at the trial facility	◯ Yes ◯ No
6. Not having a known cardiac ailment	◯ Yes ◯ No
7.Not having taken SP (Fansidar) during the current pregnancy	◯ Yes ◯ No
8.Not having a known allergy or contraindication to any of the study drugs	◯ Yes ◯ No

9. Not currently participating on another trial	◯ Yes ◯ No
10. Having Hb at enrollment >= 7g/dL	◯ Yes ◯ No
Ultrasound	
10. Carrying a single pregnancy	◯ Yes ◯ No
11.Severe malformations or non-viable pregnancy observed by ultrasound?	◯ Yes ◯ No
11b. If yes, type of malformation:	_____
12.Ultrasound gestational age (in weeks and days)- e.g. 12.3 for 12 weeks and 3 days	_____
12.1 Gestational age at enrollment_WEEKS only (for instance 21 for 21 weeks):	_____ (no decimal places)
12.2 Gestational age at enrollment_ADDITIONAL DAYS only (for instance 2 for 2 days- the value should be between 0 and 6)	_____ (Integer between 0 and 6)
12.b Photo of the ultrasound result:	(Please take a photo of the ultrasound results)
13. Between 16-28 gestational weeks? (both inclusive measured by sonography)	◯ Yes ◯ No (between 16.0 weeks and 28.0 weeks)
Eligibility:	
The woman is eligible	
The woman is NOT eligible	
Comments on eligibility:	_____
Signature of the person who entered the data:	_____
Name of the person who entered the data:	_____

Enrollment Baseline Data

Screening Number
(same number recorded on the screening registry)

_____
(should be as follow:e.g. 9XABCD, where 9 identifies the ID as a screening ID; X is the site identifier (1=Kabuta, 2=Kafutuma, 3=Kashikishi, 4=Nchelenge) ABCD are the subsequent participant numbers during screening, starting from 0001)

1.1.1 Date of visit

_____

1.1.2 Initials of the staff member doing the enrolment

_____

1.1.3 Was a copy of the consent form provided to the woman?

- ◯ Yes
- ◯ No

(Answer must be YES, please give a copy if not previously provided)

1.1.4Eligible to enrol?

- ◯ Yes
- ◯ No

1.1.5Scan the QR code on the drug envelop

_____
(Scan the QR code of the drug envelop)

1.1.6Scan the QR code on the Woman Study ID

_____
(Scan the QR code in order to register the ID associated with the screening number)

The QR codes are matching, please proceed with the allocation of the current ID and enrolment

The QR codes are NOT matching, please DO NOT proceed to allocate this ID. Please inform the PI and CO-PIs

1.2.1 What is your age ?

_____
((collect from ANC card))

1.2.2 What is your marital status?

- ◯ single
- ◯ married or living with a partner
- ◯ separated or divorced
- ◯ widow

1.2.3 What is the name of your village ?

_____

3. Behavioural data_malaria

1.3.0 Do you have one or more bed nets in the house?	◯ Yes ◯ No
1.3.1 Do you use a bed net at home?	◯ Yes ◯ No
1.3.2 Did you use the bed net the last night you slept at home?	◯ Yes ◯ No
1.3.3 Is the bed net impregnated (treated with insecticide)?	◯ Yes ◯ No ◯ Don't know
1.3.4 Where did you get the bed net from?	◯ ANC ◯ Market/shop ◯ NGO ◯ Other ◯ Don't know
If other, specify:	_____
1.3.5 Do you live in a house that was sprayed for malaria control in the last 12 months?	◯ yes ◯ no ◯ don't know

4.Pregnancy related questions

1.4.1 Is this your first pregnancy?	◯ Yes ◯ No
1.4.2 If not how many previous pregnancies have you had?	◯ 1 ◯ 2 ◯ 3 ◯ 4 ◯ 5 ◯ 6 ◯ 7 ◯ 8 ◯ 9 ◯ 10 ◯ 11 ◯ 12 ◯ 13 ◯ 14 ◯ 15 ◯ more than 15 ((All previous pregnancies resulting in live births, still births, abortions and miscarriages))

Appendix 9.11. Ethical Approval Letter

TROPICAL DISEASES
Tel/Fax +260212 615444
P O Box 71769
tdrc-ethics@tdrc.org.zm
NDOLA, ZAMBIA

RESEARCH CENTRE

TDRC ETHICS REVIEW COMMITTEE
IRB REGISTRATION NUMBER : 00002911
FWA NUMBER : 00003729

TRC/C4/07/2019

7th August 2019
Dr. Mike Chaponda
Tropical Diseases Research Centre
NDOLA

Dear Dr. Chaponda,

RE: Request for review and approval of a study entitled "Effects of metronidazole plus intermittent preventive treatment of malaria in pregnancy on birth outcomes: a randomized controlled trial in Zambia (ASPIRE Trial) Final Version 0.5 May 20 2019; STC 2019/2

On behalf of the Chairperson of the TDRC Ethics Review Committee (ERC), I wish to inform you that the Committee reviewed the responses to the queries it earlier raised and is satisfied with the responses and amendments to the protocol. Therefore, ethical approval has been granted based on the following conditions;

You are required to submit progress reports to the ERC twice a year. The Committee shall not provide renewal of ethical clearance for on-going projects in absence of progress reports.

You are now required to submit your protocol to the National Health Research Authority for final approval following the link: **https://www.nhra.org.zm** and submit a final report to the ERC Secretariat at the end of the study.

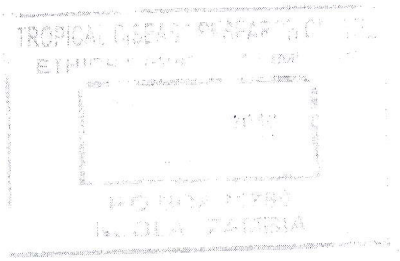
Should there be any protocol modifications or amendments, you are required to notify the ERC and submit protocol amendments for approval.

This approval is valid for the period 7th August 2019 to 7th August 2020

The Committee wishes you success in the execution of the study.

Yours faithfully
TROPICAL DISEASES RESEARCH CENTRE

Edna Mwale. Simbayi
SECRETARY – TDRC Ethics Review Committee
CC: Chairperson – TDRC Ethics Review Committee



Appendix 9.12. Zambia Medicines Regulatory Authority Approval

All correspondence should be addressed to the Director General

In reply, please quote

DMS/7/9/22/CT/091

ZAMBIA MEDICINES REGULATORY AUTHORITY

3rd December, 2019

Dr Mike Chaponda,
Tropical Disease Research Centre,
6th/7th Floors, Main Building,
Ndola Teaching Hospital,
Ndola

Email: ChapondaM@tdrc.org.zm; mikechaponda@yahoo.com
Phone: +260966849622

Dear Sir,

RE: EFFECTS OF METRONIDAZOLE PLUS INTERMITTENT PREVENTIVE TREATMENT OF MALARIA IN PREGNANCY ON BIRTH OUTCOMES: A RANDOMIZED CONTROLLED TRIAL IN ZAMBIA (ASPIRE)

Reference is made to your application to conduct the above stated clinical study.

We wish to advise that we have completed our review of the submissions and are pleased to inform you that the Zambia Medicines Regulatory Authority (ZAMRA) considered the additional information and the corresponding evaluation reports and based on the submitted information, approved the conduct of the above mentioned study with clinical trial number and protocol version as indicated below:

No.	Name of Clinical Trial	ZAMRA Clinical Trial Application No.	Protocol Number	Protocol version
1	Effects of Metronidazole Plus Intermittent Preventive Treatment Of Malaria In Pregnancy On Birth Outcomes: A Randomized Controlled Trial In Zambia (Aspire)	CT091/19	TDRC-STC/2019/2	Version 1.0 Dated 29-Nov-2019

Head Office
Plot No: 6903, TuletekaRoad/ Off Makishi Road
P.O. Box 31890 Lusaka, ZAMBIA
Tel: +260 211 220429, Telefax: +260 211 238458
E-mail: pharmacy@zamra.co.zm

Ndola Office
No. 41 Kafironda Drive, Itawa
P.O. Box 70876
Telefax: +260 212 610522
Website: www.zamra.co.zm

Report Adverse Reactions to:
Pharmacovigilance Unit, Lusaka
Tel: +260 211 220088 / 220096 / 220109
E-mail:pharmacy@zamra.co.zm

We wish to advise that you are required to provide periodic updates on the study and report any adverse events that may occur during the study. Furthermore, ZAMRA will carry out clinical trial site inspections as may be deemed necessary.

Should you have any questions, please do not hesitate to contact our Secretariat.

Yours faithfully,
for/**Zambia Medicines Regulatory Authority**

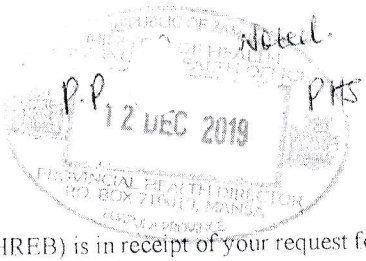
B C Mwale (Mrs)
DIRECTOR – GENERAL

Appendix 9.13. National Health Research Authority Approval

NATIONAL HEALTH RESEARCH AUTHORITY
Paediatric Centre of Excellence, University Teaching Hospital, P.O. Box 30075, LUSAKA
Tell: +260211 250309 | Email: nhrasec@gmail.com | www.nhra.org.zm

Date: 2nd December 2019

Ref No:.....
The Principal Investigator
Dr. Mike Chaponda
Tropical Disease Research Centre
P.O Box 71769
Lusaka, Zambia



Noted.
PHS - FYI.

Re: Request for Research Approval

The National Health Research Ethics Board (NHREB) is in receipt of your request for authority to conduct research titled **"Effects of Metronidazole Plus Intermittent Preventive Treatment of Malaria in Pregnancy on Birth Outcomes: A Randomised Controlled Trial in Zambia".**

I wish to inform you that following submission of your request to the Board, the review of the protocol and in view of the ethical clearance, the Board has no objection to your study on condition that:

1. The relevant Provincial and District Medical Officers where the study is being conducted are fully appraised;
2. Progress updates are provided to NHRA quarterly from the date of commencement of the study;
3. The final study report is cleared by the NHRA before any publication or dissemination within or outside the country;
4. After clearance for publication or dissemination by the NHRA, the final study report is shared with all relevant Provincial and District Directors of Health where the study was being conducted, and all key respondents.

Yours sincerely,

Prof. Patrick Mfuso[illegible]
Chairperson
National Health Research Ethics Board

Number of mutations	DHFR Codons 51-59-108-164	DHPS Codons 431-436-437-540-581-613	Zambia number of haplotypes (%)	Zambia mixed infections (%)	Zambia total (%)
2+2	**I**-C-**N**-I	I-S-**G**-**E**-A-A		4 (9.5)	4 (3.6)
2+1	**I**-C-**N**-I	I-S-A-**E**-A-A		1 (2.4)	1 (0.9)
2+2	N-**R**-**N**-I	I-S-**G**-**E**-A-A		1 (2.4)	1 (0.9)
3+1	**I**-**R**-**N**-I	I-S-A-**E**-A-A	2 (2.9)	6 (14.3)	8 (7.1)
3+1	**I**-**R**-**N**-I	I-S-**G**-K-A-A	13 (18.6)	5 (11.9)	18 (16.1)
1+1	N-C-**N**-I	I-S-A-**E**-A-A	1 (1.4)		1 (0.9)
3+1	**I**-**R**-**N**-I	I-S-A-E-**G**-A	1 (1.4)	1 (2.4)	2 (1.8)
Quintuple	**I**-**R**-**N**-I	I-S-**G**-**E**-A-A	47 (67.1)	19 (45.2)	66 (58.9)
Sextuple	**I**-**R**-**N**-I	I-S-**G**-**E**-**G**-A	6 (8.6)	5 (11.9)	11 (9.8)
		total	70	42	112