"LAMPREG TRIAL"

Active case detection and treatment of malaria in pregnancy using LAMP technology: A pragmatic randomized Multi-Center diagnostic outcomes trial

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Table of Contents

SUMMARY

Background: Malaria is one of the major public health problems in sub-Saharan Africa. It contributes significantly to maternal and fetal morbidity and mortality in affected countries.

Objective: The aim of this study is to evaluate the impact of enhanced case detection using molecular testing (LAMP) on maternal and infant morbidity and mortality in a prospective study design.

Method: A pragmatic randomized control diagnostic trial will be conducted from October 2020 until October 2021 in pregnant mothers at sites in Ethiopia. Both symptomatic and asymptomatic first and early second trimester pregnant women will be included in the study and individually randomized to either standard of care or enhanced cased detection arms using LAMP for malaria. Women (n=2583) will be enrolled during a twelve-months period encompassing the peak transmission seasons and then followed until delivery. In the standard of care arm, venous blood sample will be collected from each study participant and the presence of *Plasmodium* infection will be diagnosed by microscopy in symptomatic patients. Pregnant women who test positive for malaria will be referred and treated for malaria with quinine or artemisinin combination therapies (ACTs) as per national guidelines. In the intervention arm, mothers who are symptomatic or asymptomatic will be tested by a commercially available CE-approved LAMP malaria test and microscopy/RDT for malaria at each clinic visit and treated if positive by any test. Pregnant mothers who require treatment will be referred and treated with either quinine or artemisinin combination therapy (ACTs) as per national guidelines. The primary outcome is the proportion of deliveries with low birth weight based on WHO definition, with secondary outcomes of:(i)absolute birth weight; (ii) maternal hemoglobin;(ii) neonatal hemoglobin at birth;(iv) neonatal mortality; (v) stillbirth; and (vi) prematurity in each arm of the study.

Result Dissemination plan: The results of this study will be presented at national and international scientific conferences. It will also be published in a reputable journal.

Work plan and budget: This study will be conducted from October 2020 to October 2021. The study is funded by Grand Challenges Canada, FIND, AHRI, University of Calgary, with support from test manufacturers (Human Diagnostics, Wiesbaden, Germany and Meridien Diagnostics, Cincinatti, USA)

Key words: Malaria, LAMP, Pregnancy

1. INTRODUCTION

1.1. Background

In 2017 about 219 million cases of malaria occurred worldwide and approximately 435,000 people died of the disease globally according to the World Health Organization. The sub-Saharan region experienced around 92% of these deaths [1]. Mortality is concentrated around several high-risk groups, including pregnant women and infants. In Africa, 30 million women living in malaria-endemic areas become pregnant each year. Up to 200,000 newborn deaths each year result from malaria in pregnancy. Malaria infection has an adverse impact on pregnancy in endemic countries. Diagnosis of malaria in pregnancy is limited by microscopy and RDT both with poor sensitivity. Malaria, particularly due to *P. falciparum*, in pregnant women increases the risk of maternal death, miscarriage, stillbirth and neonatal death. In areas of seasonal malaria transmission, pregnant women are three times more likely to suffer from severe malaria as compared to non-pregnant counterparts. The World Health Organization (WHO) recommends prompt diagnosis and appropriate treatment of malaria. However, diagnostics are lacking in detecting low-level infections typically found in pregnant women because malaria parasites are sequestered in the placenta and not present in the peripheral blood in large quantities.

It is well documented that sequestration in the placenta leads to adverse outcome for the mother and infant especially related to anemia and birth weight [2,3]. Both anemia and low birth weight are predictors of poor outcomes in infancy for the child. Malaria in pregnancy therefore affects not only the current generation but the next, hobbling cognitive development and thus economic productivity. In other parts of sub-Saharan Africa, the issue is so profound mothers are exposed to antimalarials during pregnancy without testing to prevent malaria. But this comes at a cost since empiric use of anti-malarial drugs contributes to resistance and may lead to side effects.

The National Strategy to eliminate malaria in Ethiopia and other sub-Saharan countries in the next 10 years will demand highly sensitive diagnostics in patients with such low-level infections. Our group has validated a DNA based test for malaria called "LAMP" and shown up to 30% greater detection of malaria in pregnant women who are symptomatic and even greater than 90% detection when screening “asymptomatic” women. We are now actively seeking to scale up this heath care

technology to determine the true impact across Ethiopia with key partners. Meaningful impact will be measured by determining health outcomes for mother and child, especially reduction in infant anemia and improvement of birth weight.

1.2. Literature Review

There are many different types of tests available for malaria. Microscopy of stained blood smear and rapid diagnostic techniques (RDTs) are the most widely used laboratory methods. Molecular tests for malaria such as polymerase chain reaction (PCR) are highly sensitive but not feasible in resource-limited settings due to high capital equipment costs, laboratory infrastructure needs, reagent supply chain, and specialized training. The process of the PCR is also time-consuming resulting in delays in reporting the results to clinicians which, in turn, compromises timely management of patients. Traditionally, laboratory diagnosis has relied on the identification of parasites in a peripheral blood film using either Giemsa, Wright, or Field stain[4,5]. Microscopy is an accurate tool but requires well-trained laboratory technicians. In experienced hands, the limit of detection is about 50 parasites/μL[5]. Rapid diagnostic tests (RDTs) are alternatively used for the diagnosis of malaria in all health facilities or through rural health extension and outreach. RDTs are easier to perform and used for screening of malaria in remote areas where electricity and other resources are limited [6,7]. However, microscopy and RDTs cannot reliably detect lower-density parasitemia (<100 parasites/μL)[5]. As most adverse outcomes caused by malaria are because of wrong, late, or unavailable diagnosis, there is a need to find a new alternative diagnostic tool for field diagnosis of malarial infection [8].

Loop-mediated isothermal amplification (LAMP) is a potential point of care test (POCT) that provides an alternative to microscopy and RDTs [9–11]. It is a molecular method, which in comparison to PCR is cheaper, simpler, and faster, overcoming three major disadvantages of PCR. The LAMP test is a nucleic acid amplification method that relies on a strand-displacement DNA polymerase which also retains reverse transcriptase activity. The principal merit of this method is that no denaturation of the DNA template is required, and thus, the LAMP reaction can be

conducted under isothermal condition [12]. It is low cost, requires no electricity, provides rapid results and can be performed by minimally trained health workers [13]. Studies have found that LAMP has a comparable sensitivity and specificity to PCR, and is superior to microscopy and RDTs [14]. The method can detect parasitaemia as few as 1 parasite/μl of blood or lower, below the detection limit of microscopy or RDTs [12,15]. This is particularly important in pregnant women, where sequestration of parasites in the placenta is associated with low level parasitemia in peripheral blood [3]. This would lead to cases of chronic malaria going undetected. Although microscopy is considered as a gold-standard method, several nucleic acid amplification techniques have been evaluated for the diagnosis of malaria in the general population and also in pregnant women. Rantala and colleagues (2010) compared a real-time PCR assay and conventional microscopy for the detection of *P. falciparum* in Malawi. Of the 475 women, *P. falciparum* was detected in 11 (2.3%) by microscopy and in 51 (10.7%) patients by real-time PCR, thus questioning the accuracy of microscopy in this population.

Our group conducted a cross-sectional study in North Gondar, Ethiopia in 2014 to evaluate the use of LAMP in combination of a non-instrumented nucleic acid amplification (NINA) heater for the diagnosis of malaria. Using nested PCR as reference, the sensitivity and specificity of the primary NINA-LAMP assay were 96.8% (83.2% - 99.5%) and 84.3% (71.4% - 92.9%), respectively for detection of *Plasmodium* genus. Microscopy demonstrated sensitivity and specificity of 93.6% (78.5% - 99.0%) and 98.0% (89.5% - 99.7%), respectively for the detection of *Plasmodium* parasites. Post-hoc repeat NINA-LAMP analysis showed improvement in diagnostic accuracy, which was comparable to nested PCR performance and superior to microscopy for detection at both the *Plasmodium* genus level and *P. falciparum* parasites[10]. Recently, our group also showed the usefulness of LAMP for the diagnosis of malaria in pregnant women with 100% sensitivity achieved in a cohort of 87 women diagnosed with malaria [9]. What remains to be demonstrated is that gains in sensitivity with LAMP translate to improved outcomes for mother and child. Malaria contributes very significantly to maternal anemia and fetal mortality [16]. Pregnant women are three times more likely to suffer from severe malaria compared to the non-pregnant control population. Pregnant women infected with malaria usually have more severe symptoms and outcomes, with higher rates of miscarriage, intrauterine demise, premature delivery, low-birth-weight neonates, and neonatal death. Chronic non-fatal infections also lead to complications with

the main problems being maternal anemia, intra-uterine growth retardation [2] and low birth weight.

In sub-Saharan Africa, anemia reportedly accounts for about 20% of all maternal deaths [17]. It is estimated that in areas where malaria is endemic, around 19% of infant low birth weights are due to malaria and 6% of infant deaths are due to low birth weights caused by malaria [18]. Low birth weight is thought to be the single biggest risk factor for neonatal and infant mortality [16].These complications are so commonplace and severe that most countries in sub-Saharan offer intermittent presumptive therapy (IPT) to pregnant women, but resistance rates to the drug used (sulfadoxine-pyrimethamine) are very high and compliance with the full 3 doses during pregnancy poor. Other interventions include insecticide treated nets (ITN) and effective educational outreach programs [19].

Low-level parasitemia also have a role in disease in the non-pregnant population. Recent studies have pushed for asymptomatic malaria to be termed chronic malaria instead, with a view that even low level parasitemia can lead to severe health and economic consequences[20]. Additionally, as malaria incidence continues to fall, asymptomatic carriers with a low level parasitemia become more important in reducing transmission. They are thought to contribute to the malaria reservoir which leads to ongoing transmission by mosquitoes. Identifying and treating this group will be essential if the fight to eradicate malaria is to succeed.

An accurate parasite-based diagnosis of malaria is essential for proper treatment of the individual patient. Reliably excluding the diagnosis is equally valuable, because this will guide the clinician to consider an alternative diagnosis, which can be lifesaving [21]. A correct diagnosis is also important for public health, because avoidance of inappropriate antimalarial treatment will reduce costs and helps prevent the spread of drug resistance.

2. SIGNIFICANCE OF THE STUDY

Malaria in pregnancy often results in high degree of morbidity and mortality of the pregnant mother and the fetus. Early and accurate diagnosis of subclinical infections will be critical to malaria elimination and specifically the goals of the World Health Organization to reduce the

burden of disease by 90% before 2030. This goal can only be achieved using highly sensitive methods such as LAMP that are capable of detecting subclinical infections with very low parasitemia. Currently both Giemsa stained blood film microscopy and RDT are the only laboratory methods that are used to diagnose malaria both in pregnant mothers and the general population. This leaves a big gap in the detection of low-level infections and asymptomatic malaria due to the documented lack of sensitivity of the aforementioned methods. This, in turn, predisposes pregnant mothers to malaria-related complications that endangers the life of the mother and the fetus. In this study, we propose that the use of a highly sensitive LAMP technique will enable us to detect more asymptomatic *Plasmodium* infections in pregnant women. This consequently results in early treatment of the pregnant mothers and may avert maternal and fetal morbidity and mortality. This study is of particular importance in Ethiopia where IPT is not used for pregnant women and therefore accurate screening is paramount.

3. PILOT DATA

At the time of writing, the phase 1 proof of concept retrospective study is complete. Phase 1 data are shown in Table 1 and 2 demonstrating the superior performance of LAMP technology compared to traditional methods. In particular, LAMP was able to detect up to 30% more malaria infected women than traditional methods using passive case detection (i.e., only symptomatic women).

Table 1: Demographic and clinical features of pregnant women enrolled in the phase 1 retrospective evaluation of LAMP (commercial) versus traditional malaria diagnostic methods.

Variable	Frequency*	
	Positive N (%)	Negative N (%)
Age 18-24	3 (13.0)	20 (87.0)
25-30	5 (10.9)	41 (89.1)
31-35	1 (7.1)	13 (92.9)
36+	1 (25.0)	3 (75.0)
Previous malaria history		
Yes	5 (11.4)	39 (88.6)
No	5 (11.6)	38 (88.4)
Trimester		
First	2 (13.3)	13 (86.7)
Second	5 (13.9)	31 (86.1)
Third	3 (8.3)	33 (91.7)
Gravidity		
Primigravidae	2 (9.1)	20 (90.9)
Multigravidae	8 (12.3)	57 (87.7)

* As determined by PCR.

Table 2: Performance of LAMP compared to microscopy and RDT in symptomatic pregnant women (N=87, Gondar, NW Ethiopia), phase 1 retrospective study.

Method	Sensitivity % (95%CI)	Specificity % (95%CI)
Microscopy	90 (66.3-100)	98.7 (96.5-100)
RDT	70 (33.8–100)	97.4 (92.9–100)
LAMP	100 (100)	93.5 (86.5-100)

In phase 2 proof of concept, we compared ELUCIDx and a commercial LAMP assay to traditional diagnosis (RDT and microscopy) in both asymptomatic and symptomatic pregnant women in a

prospective fashion at three antenatal clinics. Our most complete data to date is from Jawi Health Centre in Amhara Region during the 2018 peak transmission season. In this study population, 28 of 34 pregnant women (mean age 24.2 years) who presented to the antenatal clinic were asymptomatic, 26 of 34 were in the first trimester, and 13 of 34 were primigravida. Malaria positivity amongst asymptomatic women was 3/34 (8.8%) by microscopy, 1/34 (2.9%) by RDT, 12/34 (35.3%) by commercial LAMP, and 12/34 (35.3%) by ELUCIDx, and 11/34 (32.3%) by qPCR. The sensitivity of microscopy in pregnant women in this study population was 25.0% (95% CI 5.49 - 57.19%), RDT 8.33% (0.21 - 38.48)), commercial LAMP 81.82% (48.22 - 97.72), ELUCIDx 100.00% (73.54 -100.00) and qPCR 91.67% (61.52 - 99.79) using a consensus gold standard. These data suggest that ultrasensitive diagnostics like LAMP are able to detect malaria far more frequently than traditional diagnostic methods when coupled to active case detection of symptomatic and asymptomatic pregnant women. The marked decrease in sensitivity for microscopy and RDT in phase 2 as compared to phase 1 is directly linked to the low level of infection found in asymptomatic pregnant women. We predict that greater detection of malaria in pregnancy with LAMP-based active case detection will lead to better critical outcomes such as LBW in pregnancy in the full-scale LAMPREG trial.

Table 3: Performance of LAMP compared to microscopy and rapid diagnostic tests (RDT) in asymptomatic and symptomatic pregnant women (N=34, Jawi, Amhara Region), phase 2 proof of concept prospective study with active case detection.

Method	Sensitivity % (95%CI)	Specificity % (95%CI)
Microscopy	25.00 (5.49 - 57.19)	100.00(84.56 - 100.00)
RDT	8.33 (0.21 - 38.48)	100.00(84.56 - 100.00)
Meridian LAMP (competitor)	81.82 (48.22 - 97.72)	86.96 (66.41 - 97.22)
ELUCIDx LAMP	100.00(73.54 -100.00)	100.00 (84.56 - 100.00)
qPCR	91.67 (61.52 - 99.79)	100.00 (84.56 - 100.00)

In additional preliminary data from Gojeb, Southwest Ethiopia, our study team has shown that treating pregnant women positive by LAMP has benefits in terms of fetal hemoglobin and birth weight, with the former reaching statistical significance (n=200, $p<0.05$). This is important data which requires confirmation in a full-scale trial across several epidemiological settings in Ethiopia.

Table 4: Outcome measures in pregnant women (n=200) randomized to LAMP versus standard of care microscopy/RDT in Gojeb, Ethiopia.

Outcome measure	**LAMP Arm Mean (n=150)**	**SOC Arm Mean (n=50)**	**p Value (t test)**
Birth Weight (g)	3059	2965	0.1478
Mat Hgb 1st visit (g/dL)	12.11	12.01	0.4981
Mat Hgb 2nd visit (g/dL)	11.68	11.63	0.7211
Mat Hgb 3rd visit (g/dL)	11.33	11.24	0.4606
Mat Hgb at birth (g/dL)	11.51	11.55	0.771
Fet Hgb at birth (g/dL)	13.1	12.84	0.04286

4. OBJECTIVES

4.1. General objective

To assess the impact of LAMP in the diagnosis of malaria in pregnancy and its potential role in reducing mortality and morbidity attributable to malaria. We hypothesize that the additional sensitivity of LAMP coupled with active case detection in detecting malaria in pregnancy will result in additional cases being identified and treated.

4.2. Specific objectives

1. To evaluate the impact of LAMP versus microscopy/RDT for the detection of malaria in pregnant mothers in terms maternal and infant morbidity and mortality.
2. To evaluate the impact of enhanced case detection of malaria in pregnancy by screening asymptomatic mothers at each antenatal visit until delivery.
3. To determine the impact of treating LAMP-positive asymptomatic and symptomatic pregnant women as compared to standard of care. Treatment is per national guidelines.

5. MATERIALS AND METHODS

5.1. Study area

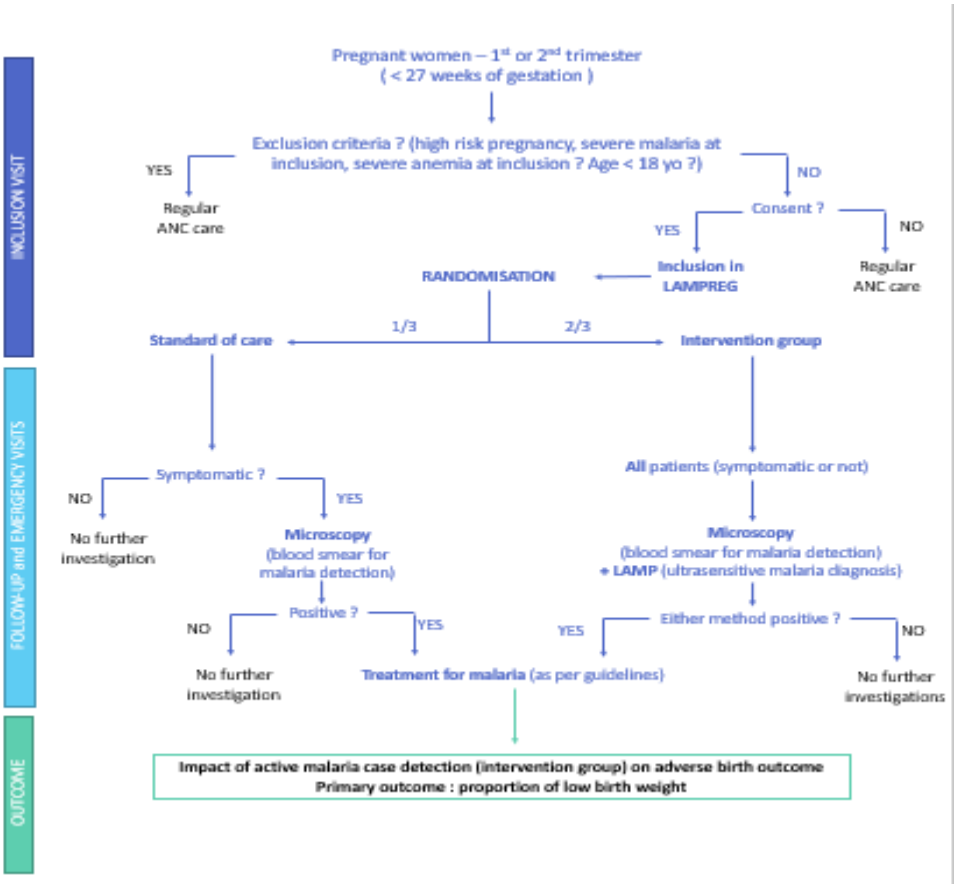
The study will be conducted at sites across several sites in Ethiopia to obtain sufficient enrolment and spanning different transmission settings based on epidemiological data provided by the Federal Ministry of Health. The study sites selected are Chisabay, Hamusit and Andasa Health centers; northwest Ethiopia, from southwest Ethiopia; Bonga area (Uffa Health Center and Bonga (GebreTsadik Shawo) General Hospital) and one site from Gambela (Lare Health center); in which some are sites where preliminary data were gathered. In Ethiopia, malaria is characterized by its seasonality where the peak transmission season is from October to December with a second peak in June. *P. falciparum* and *P. vivax* are the predominant species in the area [22]. Residents often live in non-substantive accommodation and despite a scale up in preventative measures in 2005 including ITN distribution, they are at risk of malaria.

5.2. Study design and period

The study is prospective diagnostic study of malaria in pregnant women. The goal is to determine whether: (i) LAMP provides a clinically measurable benefit compared to current first line diagnostic test of Giemsa-stained microscopy and whether (ii) enhanced case detection of asymptomatic mothers with LAMP has added value in terms of outcomes. We hypothesize that addition of LAMP to one arm will be of greater benefit than microscopy alone due to additional LAMP sensitivity. We further hypothesize that enhanced case detection by screening asymptomatic mothers at each antenatal visit will be of additional value in treating malaria. Both

symptomatic and asymptomatic first and second trimester mothers will be included in the study and individually randomized to one of two arms: standard of care or enhanced cased detection arms using LAMP for malaria. Mothers will be enrolled during a twelve-months period from October 2020 to October 2021and then followed until 28 days after delivery. Given the rate of pregnant mothers at the locations, we anticipate that the required minimum of 2583 mothers will be enrolled in the study during the study period. In the first standard of care arm, venous blood sample will be collected from each study participant and the presence of *Plasmodium* infection will be diagnosed by microscopy in symptomatic patients. Pregnant women who test positive for malaria will be referred and treated for malaria with quinine or ACTs as per national guidelines. In the second (test) arm, mothers whether symptomatic or asymptomatic will be tested by a commercially available CE-approved LAMP malaria test (Meridien Biosciences, Illumigene Malaria M kit, Cincinatti, USA) and Human Diagnostics LoopAMP (Wiesbaden, Germany) at each clinic visit in addition to RDT/microscopy. The commercial LAMP tests can distinguish *P. falciparum* and *P. vivax* and treatment will be given according to national guidelines. The purpose of doing all tests in the intervention arm is to determine how many additional cases LAMP identified. The primary outcome is the proportion of deliveries with low birth weight, with secondary outcomes of: (i) absolute birth weight; (ii) maternal hemoglobin; (ii) neonatal hemoglobin at birth (*Standard Hgb testing from peripheral blood or finger prick)*; (iv) neonatal mortality; (v) stillbirth; and (vi) prematurity in each of the two arms. Additionally, signs of current or past *Plasmodium* sequestration in the placenta will be evaluated.

Figure 1: Flowchart summarizing randomized trial



There will be a total of at least five antenatal visits (mandatory). Emergencies (medical consultation outside the planned visit) will be recorded for each included woman. Visit 1 and 2 may be combined for mothers who present in the second trimester. The following table summarizes the procedures to be conducted at each visit.

Table 5: Actions to be taken at each visit to the health center by a pregnant mother

Visit #	Time frame	Aim	Action for the study
1	Visit at health care center	Inclusion	Enrolment and randomisation
2	26 weeks gestation	Follow up	Malaria testing as per inclusion arm (2mL venous blood on EDTA) +ANC regular care Maternal Hgb
3	32 weeks gestation	Follow up	Malaria testing as per inclusion arm (2mL venous blood on EDTA) +ANC regular care Maternal Hgb
4	36-38 weeks gestation	Follow up	Malaria testing as per inclusion arm (2mL venous blood on EDTA) +ANC regular care Maternal Hgb
5	Delivery	Delivery visit	Foetal Hb Maternal Hb Malaria testing Birth weight + size Weeks of pregnancy - preterm? Placenta blood Death
6	28 days after birth	Néonatal assessment visit	Newborn weight/ hemoglobin/ death/ other

5.3. Population

5.3.1. Study population

The study population will comprise pregnant women 18 years of age and above, in the first and second trimester, both symptomatic and asymptomatic, attending antenatal clinics during the study period. Gestational age will be determined using the mother's last normal menstrual period and by

measuring symphysis fundal height. Women will be assisted to determine their last normal menstruation with detailed calendar including local events reminder. Those who don't remember their last normal menstruation will not be included in the study. As described in the WHO guidelines, in high-transmission settings, where levels of acquired immunity tend to be high, *P. falciparum* infection is usually asymptomatic in pregnancy. Yet, parasites may be present in the placenta and contribute to maternal anemia even in the absence of documented peripheral parasitemia. Both maternal anemia and placental parasitemia can lead to low birth weight, which is an important contributor to infant mortality. In high-transmission settings, the adverse effects of *P. falciparum* infection in pregnancy are most pronounced for women in their first pregnancy. In low-transmission settings women of reproductive age have relatively little acquired immunity to malaria, malaria in pregnancy is associated with anemia, an increased risk of severe malaria, and it may lead to spontaneous abortion, stillbirth, prematurity and low birth weight. We hypothesize that both symptomatic and asymptomatic cases of malaria occur in pregnant women. Patients who are positive by LAMP and negative by microscopy will be consented for treatment based on the evidence that LAMP has greater sensitivity.

5.4. Inclusion and exclusion criteria

Pregnant women in their first or second trimester (until 28 weeks of pregnancy) presenting to the health centers will be enrolled in the study and randomized to one of two arms as above. Informed written consent will be obtained from participating women based on the level of literacy. Pregnant women in the third trimester, those mothers with severe malaria, those who are taking or have taken anti-malaria medication three weeks prior to study commencement, those with severe anemia with hemoglobin of less than 8 g/dL, pregnant mothers who are severely unwell and mothers who are classified as having high risk pregnancy will be excluded from the study. Women who do not remember the date of their last menstruation will be excluded.

5.5. Variables

5.5.1. Primary outcome

- ✓ Proportion with low birth weight (<2500gm)

5.5.2. Independent variables

- ✓ Maternal hemoglobin during pregnancy and at delivery

- ✓ Fetal hemoglobin
- ✓ Actual birth weight (gm)
- ✓ Stillbirths
- ✓ Premature births
- ✓ Neonatal death

5.5.3 Other endpoints

Performance of LAMP, RDT, Microscopy, compared to qRT-PCR (gold standard) including sensitivity, specificity, PPV, NPV. Signs of *Plasmodium* infection: current (with presence of asexual parasites in placental blood), or past infection (presence of malaria pigment).

5.6. Sample size and sampling techniques

The sample size calculation for this study is based on detecting a difference in the proportion of deliveries with a low birth weight (LBW), which was felt to be the most clinically important outcome variable. The prevalence of deliveries with a LBW in Ethiopia was estimated to be 17.3%. An alpha of 0.05 was selected with an allocation ratio of 3:1 in favor of the enhanced case detection arm. The study will be powered to detect an absolute difference in the proportion of deliveries with a LBW of 5%, so a proportion in the enhanced case detection arm of 12.3% compared to 17.3% in the standard of care arm. Using a continuity correction and an attrition rate of 20%, a total sample size of 2583 is required to achieve 80% power.

5.7. Data collection and laboratory methods

5.7.1. Socio-demographic and clinical data

Socio-demographic characteristics and clinical data will be collected using interview-based questionnaire. Questionnaire will be developed in English and translated to Amharic and then translated back to English to maintain its consistency.

Written informed consent will be obtained from study participants and data collected will be anonymized. Data will only be used for the purpose of this trial. Participants who test positive for malaria will be referred for medical consultation in the ante-natal clinic to ensure appropriate

treatment. LAMP testing has been shown to be superior in sensitivity and specificity to Giemsa microscopy and RDTs.

The participants will then be followed up for the duration of their pregnancy and their hemoglobin will be measured when they attend for delivery. The fetal birth weight will be recorded as an additional end point and any complications will be documented.

5.7.2. Blood sample collection and processing

For microscopy, two milliliters of venous blood will be collected from each study participant using EDTA anticoagulant test tube. Soon after venous blood collection, a drop of blood will be taken for RDT test and another two separate drops of blood will be placed on frosted microscope slides to prepare thin and thick blood films. For LAMP, two milliliters of venous blood will be collected from each study participant using EDTA anticoagulant test tube. LAMP will be performed at the respective health centers based on the manufacturer's recommendation (Meridian Diagnostics, and Human Diagnostics). Dried blood spot (DBS) samples will also be collected from all individuals by using two drops of blood placed on a Whatmann filter paper. DBS will be stored in AHRI for further testing if required. DBS will also be sent to the University of Calgary, Canada (cf MTA) for ultrasensitive malaria technique (qRT PCR) and parasitic whole genome sequencing. For placental infection assessment, placental blood will be collected on anticoagulant after delivery.

5.7.3. Malaria microscopy and RDT

Blood film will be air-dried at room temperature and the thin blood film will be fixed with absolute methanol. Then, blood film will be stained with 10% Giemsa solution for 10 minutes and examined by experienced laboratory technician. The presence of *Plasmodium* will be ruled out if no parasites are observed after examination at least 100 microscopic fields with hundred (100x) objective. Parasitemia will be estimated in thick film by counting the number of asexual parasites along with 200 white blood cells (WBC) or 500 WBC if the parasite count is less than 10 parasites per 200 WBC. A total of 8,000/µl white blood cells count will be considered for the determination of parasitemia. At delivery, placental blood will be collected with anticoagulant and immediately dried as a smear on a microscope slide. Placental blood smear will be air dried then stained using

10% Giemsa (as for regular peripheric blood samples). Presence of asexual parasites or malaria pigment will be recorded.

5.7.4. Loop-mediated isothermal amplification (LAMP)

Since patients testing positive by LAMP will be treated for malaria, a CE-marked test (approved for human use in Europe) will be used to diagnose patients. We will use the Meridien Biosciences, Illumigene Malaria M kit (Cincinatti, USA) and Eiken LoopAMP Malaria (Human Diagnostics, Wiesbaden, Germany). The test will be conducted exactly according to manufacturer's recommendations. The tests are both run to detect *P. falciparum* and *P. vivax*. A third investigational LAMP test (ELUCIDx, Calgary, Canada) will be run only for data gathering and method validation purposes.

5.8. Statistical Analysis Plan

Raw data will be entered in SPSS version 20 software and analyzed using STATA version 13. Mothers with twins will be excluded from the analysis of birth outcomes. The sensitivity, specificity and predictive values will be calculated for microscopy and LAMP versus a gold standard of PCR. Statistical analysis of epidemiological variables and malaria positivity will be determined using univariate binomial regression. Variables found to be statistically significant with initial analysis will then characterized through multivariate analysis regression analysis. Risk ratios for primary outcome variables for intervention against standard of care arm will be calculated. Subgroup analysis will be done for potential confounders, and to compare positivity by LAMP and microscopy in the intervention arm.

5.9. Data analysis and interpretation

Raw data will be entered in SPSS version 20 software. The sensitivity, specificity, predictive values and kappa coefficient will be determined using SISA online statistical software.

5.10. Benefits and beneficiaries of the proposed study

Based on previous studies performed by our group, molecular diagnostics such as LAMP is now considered imperative in the malaria elimination plan for Ethiopia. Our group conducted a community survey of asymptomatic residents of Gambella, Ethiopia using LAMP and confirmed that over 50% of infected are missed by microscopy and RDT in this high transmission setting. These so-called asymptomatic individuals were anemic (lower hemoglobin) compared to uninfected persons in this survey suggesting there is a consequence to parasite carriage. Malaria in pregnancy has been shown to lead to maternal anemia, intra-uterine growth restriction and reduced fetal birth weight. In sub-Saharan Africa, anemia reportedly accounts for about 20% of all maternal deaths [17] and low fetal birth weight is thought to be the single biggest risk factor for neonatal and infant mortality. We aim to demonstrate that improved detection and then treatment of low level parasitemia translates into a clinical benefit for both mother and child. This proof of clinical benefit may lead to the increased use of LAMP testing in the resource limited setting and pave the way for more research in the area. LAMP methodology technology transfer will occur in the course of this study. Staff from the local study sites will be trained in LAMP technology. The study aims to show the benefits of active case detection using a reliable highly sensitive malaria point of care diagnostic test. In the long term, we hope that the technology and protocol will be considered at the national level and implemented in the local laboratories. However, this will depend on funding from FMOH and support from multilateral agencies.

5.11. Dissemination of findings

The results of this study will be presented at national and international scientific conferences. The result of the study will be communicated to the Ministry of Health of Ethiopia and the regional health bureaus where the study will be conducted. Dissemination workshop will be done after the completion of the study. Study findings will also be submitted to internationally reputable scientific journals.

5.12. Ethical Considerations

Ethical clearance will be obtained from Institutional Research Ethics Board (IRB) of the Armauer Hansen Research Institute in Ethiopia, the Conjoint Health Research Ethics Board (CHREB)

University of Calgary, and from National Ethical Review Committee of Ethiopia. Consultation and permission to conduct this study will also be obtained from regional Health Offices as appropriate.

In each LAMPREG study center, medical officer will be informed of the study and trained on study inclusion procedure, including informed consent collection. Only trained staff will be allowed to proceed to inclusion. All eligible women consulting for ANC in a LAMPREG study center will be made aware of the study by the trained medical officers. Women interested in the study will be informed of the protocol by the medical officer as per Protocol Information Sheet. The fact that women agreeing or not to participate in the study will not impact the quality of the care received will be emphasized. Medical officers will be vigilant to give eligible women enough time and a safe space to decide whether they agree or not to participate in the LAMPREG study. No coercion or undue influence will be imposed on women. Written informed consent will be obtained from every study participant by the trained medical officer, as per Informed Consent sheet. For women who are illiterate, particular attention will be given in ensuring that each point of the protocol and consent form are well understood. For these women, signature on the consent sheet will be by either initials or inked fingerprint. Medical officers will inform women that they can withdraw their participation from the study at any time, and that doing so will not affect the medical care provided to them. All women will be free to be accompanied or not by a person of their choice during each visit.

To ensure confidentiality about participants information, anonymous typing will be used, and any personal identifier of participants will not be written. Samples will be identified solely with patient inclusion number. Information for the LAMPREG study will be collected at the time of the study and directly anonymized. Discussions between pregnant women and medical staff regarding study protocol, inclusion, randomization, study withdrawn and follow up visit will be held in a confidential space to ensure that women are not subject to social or staff pressure.
In case of adverse events during the study, women will be referred to a hospital and the event will be notified to study investigator. Contact of study investigators are available to women through inclusion forms. Women having any concerns regarding study procedure will be welcome to ask precisions to medical officers at any time during the LAMPREG trial. Study investigators will monitor adverse events from each study site on a monthly basis and take appropriate measures if needed. Since the LAMPREG trial is integrated with regular ANC visit, women participation in the study will not be noticeable by other community members, ensuring confidentiality.

The LAMPREG trial intervention arm consists of asymptomatic women screening for malaria during each ANC visit with highly sensitive techniques. The major added risks are due to the blood sampling. All material used will be single-used sterile needles. Medical staff will be vigilant on communication and will be explaining the steps of the study to the participants. Women tested positive for malaria will be administered treatment according to national guidelines pending on the gestational age. Before treatment administration, women will be explained the results of the LAMP test and the potential benefits of treatment.

At delivery, women are particularly vulnerable. The LAMPREG trial staff will respect women privacy during delivery. LAMPREG data collection and sampling will not affect the care given to the women and the newborn during delivery, and absolute priority will be given to the mother and child health and safety. For placenta blood collection, study staff will remind women about the procedure before sampling, and women will be allowed to withdraw consent.

Pregnant women who do not consent to participate in the study will be tested for malaria using standard Giemsa microscopy, ensuring that they receive prompt treatment, as per Ethiopian national guidelines. Pregnant women who are severely unwell requiring admission to the health center for monitoring or referred to a hospital will be excluded from the study. Only CE-marked commercially available diagnostic tests for LAMP (Meridien Biosciences, Illumigene Malaria M kit, Cincinatti, USA) will be used in this study. These kits are able to identify both *P. falciparum* and *P. vivax* to the species level. Treatment will be according to national guidelines for symptomatic and asymptomatic women positive for *Plasmodium.* Quinine has been widely used for MiP treatment. The main adverse effect of quinine are cinchonism (with tinnitus (38%-85%), vomiting, dizziness, nausea and anorexia), quinine related hypoglycemia (17%) and QT prolongation (1%) [23]. Abortion risk is considered as very low for quinine [24] at the normal dosage.

Artemisinin derivatives (in combination with partner drug such as lumefantrine) showed similar profiles in terms of stillbirth, congenital abnormalities and risk of miscarriage in women exposed to ACT during pregnancy compared to standard intermittent preventive treatment (IPT) for malaria [23]. Despite animal preclinical studies highlighting a risk of embryotoxicity and teratogenicity (musculoskeletal/cardiovascular defects) [25], a recent meta-analysis study did not show a difference in pregnant women treated with ACT compared to quinine, even in the embryo sensitive

period (6-12 weeks of gestational age) [26]. Two studies report ECG monitoring of pregnant women treated by artemether lumefantrine [27,28] and showed a QT prolongation in 2% of treated women. Several studies have reported the safety of artemether lumefantrine during the first trimester of pregnancy in 1693 women, and no increase in miscarriage or congenital abnormalities where observed compared to standard of care [29–32]. Recently, the PREGACT trial compared four artesunate combined therapy in the second and third trimester of pregnancy in women positive for *P. falciparum* infection regardless of symptoms. No differences were observed in terms of treatment associated negative birth outcomes [33].

Regarding chloroquine, a cohort of 169 pregnant women treated by chloroquine in non-malaria endemic area did not show newborn abnormalities compared to placebo [34]. Recently, a study conducted in Malawi to evaluate the use of chloroquine for intermittent preventive treatment did not show any adverse effect in the 600 treated women compared to the standard of care IPT (sulfadoxine pyrimethamine) [35].

In regards of the recent literature and publications related to quinine, artemether lumefantrine and chloroquine during pregnancy, there is no major risk for treatment of women presenting asymptomatic *Plasmodium* spp infection. Importantly, *Plasmodium* spp infection, even asymptomatic and submicroscopic, has impact on birth outcomes.

Asymptomatic malaria is the presence of *Plasmodium* spp infection in peripheral blood in the absence of any symptom of the disease. During pregnancy, peripheral parasitemia are particularly low due to sequestration of infected erythrocytes in the placenta villosities [36,37]. The sequestration of the parasite in the placenta originates the negative birth outcomes associated with MiP : low birth weight, maternal anemia, abortion and stillbirth [38]. Estimation recall that 20% of stillbirth and 11% of all newborn death are direct consequences of MiP [39]. Both *P. falciparum* and *P. vivax* induce adverse pregnancy outcome. *P. falciparum* pathogenicity in MiP is well described [37,40] while the pathophysiology of *P. vivax* infection during pregnancy needs yet to be elucidated [41,42].

It is clearly demonstrated that pregnant women presenting asymptomatic or submicroscopic *Plasmodium* infection present more anemia than uninfected women [43]. A recent longitudinal study combining *Plasmodium* peripheral detection and placenta histology at delivery showed that

even women with only submicroscopic infection have an increased risk of placental malaria compared to women without any parasitemia [44].

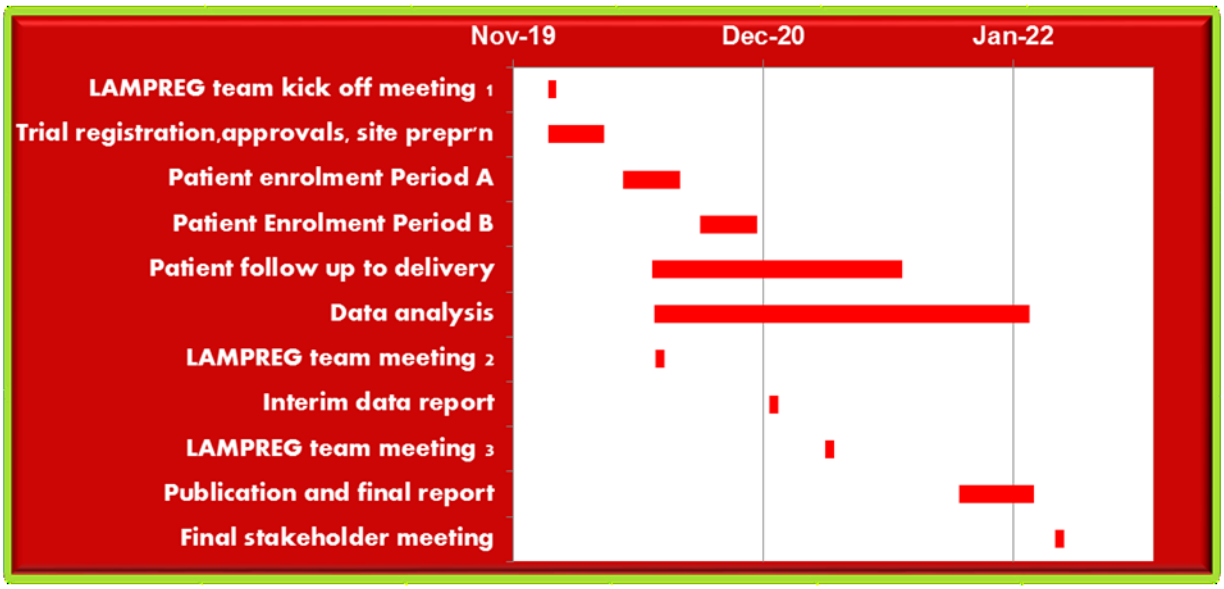
For the above-mentioned reason, *Plasmodium* infection, even asymptomatic, present a risk for the mother health and fetal development.

Expectant mother safety is a priority during the LAMPREG trial. After *Plasmodium* spp infection diagnostic, final treatment choice will be at the discretion of the physician assessing the patient. Any pre-existing condition will be taken in consideration.

For any adverse event, women will be given the contact of the local study coordinator. Community Health Worker will be sensitive to the study. At the following ANC visit, women will be encouraged to report any side effect or concern to the study staff. Each local study site coordinator will summarize any adverse event on a monthly basis and transmit the corresponding information to the national coordinator. Proper pharmacovigilance notification will be operated to the health authorities if necessary.

The LAMPREG clinical trial aims to evaluate the impact of active detection of both sub microscopic symptomatic MiP and asymptomatic (microscopic and submicroscopic) MiP. In this trial, even asymptomatic and submicroscopic MiP will be treated for *Plasmodium* spp infection as if they were uncomplicated MiP cases. The proposed used drugs fall under the drugs approved by Ethiopia Food, Medicine and Health Care Administration and Control Authority and have been proven safe during pregnancy. Importantly, the benefits of the treatment overpass the risk due to treatment induction. Information sheet and informed consent were modified to provide information regarding the treatment risk. Importantly, drugs prescription will be at the discretion of the physician assessing the women, and women safety will be the main priority of the medical staff in charge of the study.

6.0. WORK PLAN


Nov-19
Dec-20
Jan-22
LAMPREG team kick off meeting 1
Trial registration,approvals, site prepr'n
Patient enrolment Period A
Patient Enrolment Period B
Patient follow up to delivery
Data analysis
LAMPREG team meeting 2
Interim data report
LAMPREG team meeting 3
Publication and final report
Final stakeholder meeting

7.0 English Version of Questionnaire

Patient inclusion questionnaire

This questionnaire records both socio-demographic and clinical characteristics. For each question please give the answer carefully and clearly. The participant's name is not included in the questionnaire and their information will be kept completely anonymous and confidential. Their answers will be kept only by the study investigators and will not be distributed to anyone else.

Socio-demographic characteristics

1.1. ID no. of participant: _____

1.2. Age: _____ (Years)

1.3. Address:

_____

1.4. Are you currently pregnant? A. Yes B. No

1.5. How many weeks pregnant are you? _____

1.6. How many pregnancies have you had before this one? _____

1.7. Have you had any miscarriages or stillbirths before? A. Yes B. No

1.8. If yes, how many? _____

1.9. Do you spend most your time indoor or outdoor?

A. Yes B. No

1.10. Has your house undergone indoor residual spraying in the last 3 months?

A. Yes B. No

1.11. Do you sleep under insecticide treated nets?

A. Yes B. No

1.12. Has anyone in your household been diagnosed with malaria in the last month?

A. Yes B. No

1.13. What is your level of education?

None Primary Secondary Post-secondary

Clinical characteristics

1. Does the participant have any of the following symptoms or signs?

Fever	A. Yes B. No
Chills	A. Yes B. No
Sweats	A. Yes B. No
Headache	A. Yes B. No
Muscle pains	A. Yes B. No
Nausea	A. Yes B. No
Vomiting	A. Yes B. No
General malaise	A. Yes B. No
Diarrhea	A. Yes B. No

2. Does the participant have an axillary temperature greater than 37.5 degrees Celsius?

A. Yes B. No

3. Does the participant have any signs of severe malaria?

A. Yes B. No

4. Has the participant taken any anti-malarial medication in the last three weeks?

A. Yes B. No

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