**BHP Birth Outcomes Surveillance Study**

**Short title: Tsepamo**

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All questions concerning this protocol, including issues regarding:

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# Glossary

AE adverse events

ART antiretroviral therapy or treatment

BHP Botswana-Harvard AIDS Initiative Partnership

CBV Combivir

CK creatine kinase

CNS central nervous system

CRF case report form

DTG dolutegravir

DAIDS Division of AIDS

EFV efavirenz

FDA Food and Drug Administration

IRB institutional review board

3TC lamivudine

LMP last menstrual period

LPV lopinavir

PMTCT prevention of mother-to-child transmission

MTCT mother to child transmission

NNRTI nonnucleoside reverse transcriptase inhibitor

NRTI nucleoside reverse transcriptase inhibitor

NTD neural tube defect

NVP nevirapine

PCR polymerase chain reaction

PI protease inhibitor

PTD pre-term delivery

RCT randomized clinical trial

RT reverse transcriptase

SGA small-for-gestational age

TAF tenofovir alafenamide

TB tuberculosis

TDF tenofovir disoproxil fumarate

ZDV zidovudine

**TABLE OF CONTENTS**

[Study Team Roster 2](#_Toc135219786)

[Glossary 7](#_Toc135219787)

[1. STUDY OVERVIEW 10](#_Toc135219788)

[1.1 Study Schema 10](#_Toc135219789)

[1.2 Objectives 10](#_Toc135219790)

[2. BACKGROUND 12](#_Toc135219791)

[2.1. Adverse Birth Outcomes and ART 12](#_Toc135219792)

[2.2 Need for Accelerated Research in Pregnancy 13](#_Toc135219793)

[2.3 DTG Exposure in Pregnancy 13](#_Toc135219794)

[2.4 Legacy Regimens and Adverse Birth Outcomes 13](#_Toc135219795)

[2.5 Certain Ideal Randomized Studies are Not Feasible (but can be Emulated) 13](#_Toc135219796)

[2.6 Upcoming ART Program Changes in Botswana 14](#_Toc135219797)

[2.7 Botswana is an Ideal Setting for Emulated Randomized Clinical Trial Designs 14](#_Toc135219798)

[2.8 Weight Gain and Modern ART 15](#_Toc135219799)

[3. STUDY SITES 15](#_Toc135219800)

[3.1. Maternity Surveillance Sites and Study Accrual 15](#_Toc135219801)

[4. INCLUSION / EXCLUSION CRITERIA 15](#_Toc135219802)

[4.2 Inclusion and Exclusion Criteria for follow-up of births with congenital abnormalities (CAs) 16](#_Toc135219803)

[5. STUDY PROCEDURES 16](#_Toc135219804)

[5.1. Overview of Study Design 16](#_Toc135219805)

[5.2. Maternity Staff Training 16](#_Toc135219806)

[5.3. Data Collection Process 17](#_Toc135219807)

[5.4. Data Collection for Congenital Abnormalities 18](#_Toc135219808)

[5.5. Data completeness 19](#_Toc135219809)

[5.6. Data management 19](#_Toc135219810)

[6. STATISTICAL CONSIDERATIONS 19](#_Toc135219811)

[6.1. Definitions 19](#_Toc135219812)

[6.2. Projections for ART Exposures 20](#_Toc135219813)

[6.3 Anticipated Outcomes 28](#_Toc135219814)

[7. HUMAN SUBJECTS CONSIDERATIONS 28](#_Toc135219815)

[7.1. Characteristics of study population 28](#_Toc135219816)

[7.2. Sources of research material 28](#_Toc135219817)

[7.3. Participant recruitment and study consents 28](#_Toc135219818)

[7.4. Risks and benefits 29](#_Toc135219819)

[7.5. Adherence to human subjects requirements 29](#_Toc135219820)

[7.6. Procedures for minimizing potential risks 30](#_Toc135219821)

[7.7. Participation of children, women and minorities 30](#_Toc135219822)

[7.8. Monitoring and Interim Analyses 30](#_Toc135219823)

[8.0 REFERENCES 30](#_Toc135219824)

[APPENDIX A: Completed Primary Objectives from Previous Versions of the Birth Outcomes Surveillance Protocol 35](#_Toc135219825)

[APPENDIX B: Placental Collection Sub-Study of Birth Outcomes Surveillance Study (Tsepamo) 36](#_Toc135219826)

[APPENDIX C: Summary of Protocol Changes 40](#_Toc135219827)

# STUDY OVERVIEW

## 1.1 Study Schema

DESIGN

Surveillance study of birth outcomes at maternity wards in Botswana

DURATION

12 years

SAMPLE SIZE

Up to 410,000 (+/- 10%) obstetric records will be reviewed for congenital abnormalities and for adverse birth outcomes, with up to ~ 1.6% (+/- 1%) consented for anonymous photograph of congenital abnormalities.

## 1.2 Objectives

Primary objectives of previous versions of the birth outcomes surveillance that have been completed (e.g. targeting specific historical antiretroviral drug regimens) are listed in Appendix A. Primary objectives ongoing as of version 4.0 of the protocol are listed and renumbered below.

**Primary Objectives:**

**Aim 1: To compare the incidence of stillbirth, preterm delivery, small-for-gestational-age, congenital abnormalities, and in-hospital neonatal death among infants exposed to different ART regimens from the time of conception.**Previous surveillance in Botswana has found associations between ART and adverse birth outcomes, including associations with specific ART regimens. Although we have presented initial safety data for DTG *initiated* in pregnancy, it is unknown whether DTG exposure *from conception* will be associated with adverse birth outcomes or congenital abnormalities. We will perform surveillance at up to 18 representative maternity sites throughout Botswana and capture data from births with different ART exposures from conception (e.g. DTG, TAF, CAB PrEP).

**Aim 2: To use surveillance data to emulate randomized clinical trials of interest in mother-infant populations:**

1. **To emulate a randomized clinical trial of continuous “legacy” ART vs. preconception switch to DTG/TDF/FTC, evaluating for improvement in rates of combined adverse birth outcomes, and for each individual outcome (preterm delivery, small-for-gestational age infants, stillbirth, and in-hospital neonatal death).** By enhancing the current surveillance system to capture real-time data for all ART switches, we will be able to closely approximate a randomized clinical trial of continuous ART vs. switching ART pre-conception (which is the ideal randomized trial, but not feasible to perform).The primary analysis will compare combined adverse birth outcomes among those with continuous receipt of legacy ART regimens with those who switch pre-conception to DTG/TDF/FTC. Post-conception switches will be evaluated in a separate emulation. This efficient use of enhanced surveillance data will allow us to isolate the effect of specific ART regimens on adverse birth outcomes.
2. **To emulate a randomized clinical trial of DTG/XTC/TAF vs. DTG/XTC/TDF vs. EFV/XTC/TDF for women stratified by low and high weight groups, evaluating combined severe adverse birth outcomes, maternal hypertension, and each individual birth outcome (preterm delivery, small-for-gestational age, stillbirth, and in-hospital neonatal death).** The ideal randomized clinical trial evaluating ART regimens for the entire duration of pregnancy cannot be conducted, and can only be emulated within a large database such as Tsepamo that contains ART exposure data from the time of conception. The primary analysis will compare combined adverse birth outcomes for each regimen stratified by high (≥80 kg), average (60-79kg), or low weight (<60 kg), with secondary analyses aimed at identifying weight inflection points (including <50 kg or lower) for the outcomes.

**Aim 3: To evaluate changes in weight gain and other health parameters across multiple pregnancies.** With the use of a unique maternal identifier (OMANG linkage), we will evaluate changes in HIV incidence patterns, repeat congenital abnormalities and adverse birth outcomes, and birth spacing over time for all pregnant women. For women with HIV, we will compare baseline weight and health status indicators by ART regimen at subsequent pregnancies.

**Secondary Objectives:**

1. To describe the cascade of PMTCT care in Botswana, including the proportion of women tested for HIV, the proportion started on ART, the proportion remaining on ART at the time of delivery, and the proportion receiving appropriate infant prophylaxis.
2. To compare losses in the PMTCT cascade during the surveillance period with losses to the PMTCT cascade from an historical surveillance cohort prior to the shift in national guidelines to provide universal ART in pregnancy.
3. To compare adverse birth outcomes among those starting Atripla or DTG in pregnancy with those starting other ART regimens or AZT alone in pregnancy, using an historical surveillance cohort prior to the shift in national guidelines to provide universal ART in pregnancy.
4. To determine maternal risk factors for adverse birth outcomes among HIV positive and HIV negative women in Botswana, including analyses utilizing concomitant medications or conditions, distance from delivery site or other geospatial data, temperature or other climate data, or other available covariates.
5. To determine maternal risk factors for congenital abnormalities among HIV positive and HIV negative women in Botswana.
6. To compare birth weight, length and head circumference in tenofovir (TDF) exposed vs. unexposed infants in this cohort and in comparison with an historical surveillance cohort.
7. To re-validate the Botswana birth weight for gestational age norms developed in 2011.
8. To describe the medical and obstetrical management of hypertension in pregnancy and preeclampsia.
9. To determine the incidence of opportunistic infections and other maternal complications as well as risk factors for adverse birth outcomes by CD4 cell count during pregnancy.
10. To evaluate antibody transfer by maternal HIV and ART status at the level of the placenta, in stored placental specimens previously collected for histopathology.
11. To improve CD4 and viral load surveillance, and to evaluate whether it is feasible to link infant HIV status or mortality to the Tsepamo database, by accessing electronic records.
12. To describe health outcomes of infants with major congenital abnormalities born to mothers with and without HIV in the Tsepamo study, and to identify the clinical and social needs and utilization of health services for these children.

# 2. BACKGROUND

In 2014, the Botswana-Harvard AIDS Institute Partnership (BHP) established the Birth Outcomes Surveillance study “Tsepamo” at 8 sites throughout Botswana to evaluate the safety of specific ART regimens used in pregnancy. Version 1.0 of this protocol included research objectives that focused on Atripla and its components, efavirenz in particular, compared with other ART regimens (see Appendix A). In 2018 the surveillance was expanded to 18 sites with additional funding and expanded aims (in version 2.0 of the protocol) to examine exposures to dolutegravir-based regimens. In 2021 additional financial support was granted to continue surveillance through 2026 with updated scientific objectives to answer timely scientific research questions (version 4.0 of this protocol). This ongoing and responsive surveillance study offers a unique opportunity to study the impact of the most relevant modern ART regimens on congenital abnormalities and adverse birth outcomes. A summary of changes in each protocol version is included in Appendix C.

## 2.1. Adverse Birth Outcomes and ART

Described associations between ART and certain adverse birth outcomes may have clinical impact, and differences by specific regimens (particularly nevirapine [NVP] and protease inhibitor [PI]-based ART) have been demonstrated. It is therefore important to evaluate how different antiretroviral agents used in pregnancy compare in terms of preterm delivery, small for gestational age infants, congenital abnormalities, stillbirth, and neonatal death. Although HIV infection has been associated with adverse birth outcomes since the pre-ART era, the initial data from studies in the developed world were conflicted as to the effects of ART [16-19]. This lack of clarity may have been caused by ART’s ability to *reduce* adverse birth outcomes related to advanced maternal illness, while potentially increasing adverse outcomes for other reasons. A clearer picture emerged with more widespread use of ART demonstrating concerning associations between ART and preterm delivery, small for gestational age infants, stillbirths, and preeclampsia [20-26].Our research team has conducted several birth outcomes studies that have clarified the association between ART and birth outcomes. We performed the largest study at the time of birth outcomes among HIV+ and HIV- women in Africa, evaluating over 33,000 birth outcomes at 6 maternities in Botswana between 2009-2011 [20]. Controlling for ART exposure time and CD4 cell count, we found a significant increase in preterm delivery, small for gestational age infants, and stillbirth among women receiving ART during pregnancy. The absolute risk for stillbirth among women starting ART in pregnancy was 4.7%, compared with 1.7% for those starting zidovudine (ZDV). Among women on ART prior to conception, the stillbirth risk was 6.3% -- far higher than the background risk of 2.5% among HIV- women. We also conducted the first randomized study to evaluate preterm delivery and the use of protease inhibitors (PIs), and detected a clear increase in preterm delivery among PI-treated women compared with women on NRTI-based ART [27]. Taken together, our data from Botswana strongly suggest a clinically relevant effect of ART on pregnancy outcomes.

These findings were supported by the PROMISE Study, which randomized women to receive either ZDV or two different ART regimens. The PROMISE Study identified significantly higher risk for PTD (21% vs. 13%) and SGA (23% vs. 12%) with ART as compared with ZDV*,* and also highlighted the need for further research into potential differences between specific ART combinations. The Tsepamo surveillance study is optimally designed to capture ARV exposure data and evaluate such differences.

## 2.2 Need for Accelerated Research in Pregnancy

Lack of safety data in pregnancy restricts use of newer antiretroviral treatment (ART) regimens in resource-limited settings, especially where programs rely on a single ART regimen as first-line treatment for all pregnant and non-pregnant adults. This contrasts to resource-rich settings where individualized ART decisions, close monitoring of pregnancy intentions, and contraceptive availability often allow use of ART regimens that are not yet known to be safe in pregnancy.

In 2016, Botswana became the first country in Africa to update its adult treatment guidelines (including for pregnant women) to use DTG/Tenofovir (TDF)/Emtricitabine (FTC). DTG/TDF/FTC is now a preferred initial ART regimen in most settings, but its international roll out was considerably impacted by this study’s findings in 2018 of a potential association with neural tube defects (NTDs).[39] While this concern has decreased over time following expanded surveillance,[40] it has not disappeared, and ongoing surveillance is required both for DTG and for other new antiretroviral (ARV) agents. Additionally, considerations regarding weight gain with modern ART regimens that include DTG and tenofovir alafenamide (TAF) have moved to the forefront of safety discussions in recent years, and data for the impact of weight gain on pregnancy outcomes are needed.

## 2.3 DTG Exposure in Pregnancy

When DTG-based ART was introduced there were limited available data to evaluate the relationship between DTG-based ART and adverse birth outcomes, and almost no data for birth outcomes or congenital abnormalities following pre-conception DTG exposure. The Tsepamo Study described the safety of DTG/TDF/FTC for the first 845 infants exposed to this ART regimen when it was initiated during pregnancy, as compared with Efavirenz (EFV)/TDF/FTC. Following the initial Tsepamo safety signal that identified 4 NTDs associated with the first 426 DTG exposures (0.9%) in the early DTG roll out,[41] expanded Tsepamo surveillance demonstrated a decline by July 2019 to 0.30 per 100 DTG exposures[39] and to 0.19 per 100 DTG exposures by the following year.[40] Despite this decline, some concern remains as this risk is approximately two-fold higher than for children in other exposure groups, and there are now animal data showing mice exposed to 1x dose of DTG (but not 5x dose) experienced a higher risk of NTDs.[42] Importantly, the 95% confidence limits for our DTG-exposed NTD prevalence estimate remain wide, ranging from 0.01 to 0.04 per 100 births. Ongoing surveillance will tighten our confidence intervals around this estimate, confirm whether a true difference between exposure groups can be demonstrated, and ensure that the estimate is not going back up over time.

## 2.4 Legacy Regimens and Adverse Birth Outcomes

Botswana is unique among African countries because of the longevity of its ART program and the concurrent use of multiple different ART regimens in the program. Our surveillance data were first collected starting in 2009, in an era where almost all women received NVP, zidovudine (ZDV), and lamivudine (3TC). We also have surveillance data from a period when lopinavir/ritonavir (LPV/r)-based ART was used (2009-2012); when EFV/TDF/FTC was used (2012-2016); and for DTG/TDF/FTC after June 2016.

We have been able to use the Tsepamo data to study the continued receipt of legacy ART regimens in pregnancy, and to extend our knowledge of specific antiretroviral (ARV) exposures and adverse birth outcomes.

## 2.5 Certain Ideal Randomized Studies are Not Feasible (but can be Emulated)

While a randomized trial would be the ideal study for evaluating the impact of switching ART regimens on adverse birth outcomes, it is not feasible to perform. The ideal trial would randomize eligible women on legacy ART to either continue their regimen or switch to the currently recommended regimen prior to conception, and would follow them through delivery and measure birth outcomes. Switching prior to conception maximizes the ability to discriminate between regimens throughout all of pregnancy; the greatest associations with adverse birth outcomes are among women on ART from conception.

Although we have demonstrated negative associations with low baseline weight and low weight gain in pregnancy, it is not known whether this risk factor is modifiable by supporting weight gain in pregnancy. Therefore, it remains unknown whether utilizing DTG/TAF-based ART to increase weight gain in pregnancy will reduce the risk of adverse birth outcomes. Similarly, concerns about using DTG/TAF-based ART for women with higher baseline weight remain unstudied and hypothetical. While a randomized trial would be the ideal study for evaluating the impact of each ART regimen by weight strata, it is not feasible to perform. The ideal trial would randomize eligible women to one of three regimens (DTG/TDF/XTC, DTG/TAF/XTC, or EFV/TDF/XTC) prior to conception to maximize the effect of the intervention throughout all of pregnancy.

However, because the timing of pregnancy is unpredictable, such trials would take too long and be far too costly to perform, and therefore need to be emulated. Because of our extensive surveillance system in Botswana (which can be further enhanced to target the key data needed for regimen switches), the expected ongoing rollout of new agents within the Botswana ART program, and our experience as a research team emulating similar randomized clinical trials, we believe that emulated trials are a precise and efficient way to address these important scientific questions.

## 2.6 Upcoming ART Program Changes in Botswana

Botswana is unique among national treatment programs in Africa as it often utilizes older regimens for patients stably treated while also incorporating newer agents. EFV became widely used in 2012 for all women and is still in use, and DTG was introduced in 2016. TDF with FTC or 3TC has been the preferred nucleotide/nucleoside reverse transcriptase inhibitor (NRTI) backbone since 2012. This creates an environment where different ART regimens can be compared either concurrently or with relatively small temporal differences. Periodically, formulary driven switches occur and those stably receiving older agents are switched to newer formulations (e.g., recent phase out of NVP and ZDV). We therefore have the opportunity to utilize the switch from older to newer ART regimens to determine exact rates of adverse birth outcomes before and after a policy change and then emulate a randomized clinical trial of a pre-conception “switch study” among similar (age and disease-stage matched) women. These analyses will provide strong evidence – beyond currently available epidemiologic data -- that specific ART regimens impact adverse birth outcomes, and that changing off these regimens will improve outcomes.

## 2.7 Botswana is an Ideal Setting for Emulated Randomized Clinical Trial Designs

As noted above, our team has already collaborated to simulate a randomized clinical trial comparing ART regimens across surveillance eras, and we have a track record of success using this methodology.[43, 44] The birth outcomes surveillance data from Botswana are ideally suited for this type of emulation (or for marginal structural modeling, if needed), because of the high degree of homogeneity across the sites and study eras under surveillance. The HIV epidemic in Botswana has declined slowly (26.6% to 22.7% prevalence in pregnant women from 2014-2019), the demographics of HIV-infected women have remained constant, and obstetrical practices have changed minimally. Additionally, Tsepamo is in the unique position to be able to compare outcomes to women living without HIV whose data are also collected. These factors, and the large number of events captured by the surveillance system, will allow us to identify very similar women for inclusion in the emulated trials who will be minimally shifted by date of delivery. Our years of experience collecting data at maternities in Botswana have allowed us to develop a feasible plan for enhancing the current surveillance data to guarantee successful capture of complete ART data for almost all women who deliver.

## 2.8 Weight Gain and Modern ART

The most concerning side effect of modern ART regimens that utilize DTG and TAF is excess weight gain. The ADVANCE Study in South Africa demonstrated treatment emergent obesity in nearly 1 in 5 women with initially normal BMI who were randomized to TAF/FTC/DTG at 144 weeks, compared with 0% in those randomized to TDF/FTC/EFV.[45] Randomized clinical data from the VESTED study and observational data from Tsepamo both show more gestational weight gain on DTG compared with EFV based ART.[46, 47] Concerns have been raised regarding the impact of excess weight gain in pregnancy for maternal and infant outcomes, and modeling data from the ADVANCE team predicted 15% more adverse pregnancy outcomes and 28% more adverse child health outcomes with long-term use of TAF-DTG.[45] In Tsepamo, more than one-third of women with high weight in early pregnancy had hypertension in pregnancy,[48] and the risk was highest among women on DTG.[44] However, low maternal weight and inadequate gestational weight also adversely impact pregnancy outcomes.[49] In Tsepamo, women on ART at conception with low early pregnancy weight have 60% more severe birth outcomes than women of moderate weight[48] and in VESTED,low weight gain was associated with adverse pregnancy outcomes with 8% fewer adverse pregnancy outcomes among women on TAF/FTC/DTG who also had the highest weight gain.[47, 50] Importantly, in both studies the majority of women had inadequate gestational weight gain compared with IOM standards (VESTED) and women without HIV (Tsepamo). Taken together, we believe that baseline weight and weight gain in pregnancy may mediate some of the relationship between ART and birth outcomes. We hypothesize that DTG/TAF/XTC will have greatest benefit for low weight women, and that it may also be safe to use for high weight women, but this needs to be confirmed and a variety of weight-related outcomes need to be assessed.

# 3. STUDY SITES

## 3.1. Maternity Surveillance Sites and Study Accrual

The study is conducted as a surveillance study of infant live births and stillbirths born at > 24 weeks gestation at up to 18 geographically representative hospitals in Botswana. The study operates at the two largest delivery sites in Botswana, Princess Marina Hospital (PMH) in Gaborone and Nyangabgwe Hospital (NH) in Francistown. These sites deliver approximately 12,000 women per year (~ 7,000 at PMH, ~ 5,000 at NH). Additional sites create a geographically representative national surveillance system, including: Maun General Hospital, Sekgoma Memorial Hospital in Serowe, Scottish Livingstone Hospital (SLH) in Molepolole, Selebi-Phikwe Government Hospital , Mahalapye Hospital, Ghanzi Primary Hospital (GP); Bamalete Lutheran Hospital, Palapye Primary Hospital, Deborah Retief Hospital, Kanye Seventh Day Adventist Hospital, Athlone Hospital, Bobonong Primary Hospital, Gumare Primary Hospital, Goodhope Primary Hospital, Tutume Primary Hospital, and Letlhakane Primary Hospital.

The expected number of participants in surveillance with 16-18 sites is anticipated to be 30,000-40,000 per year.

Surveillance through 2026 with 30,000-40,000 births per year will total up to 410,000 (+/- 10%), including records already surveilled since study outset.

# 4. INCLUSION / EXCLUSION CRITERIA

**4.1. Surveillance Inclusion and Exclusion Criteria**

Inclusion Criteria:

• A birth outcome at ≥24 weeks gestation that occurs in the surveillance hospital and results in the registration of either the mother or one or more infants/stillbirths at the surveillance location.

Exclusion criteria:

• A birth outcome at <24 weeks gestation.

• Infants born prior to arrival at the hospital.

The 24-week gestation cut-off accounts for the fact that women <24 weeks gestation are admitted to female medical or surgical wards rather than maternity; the difficulty assessing for NTDs among deliveries <24 weeks gestation; and other potential biases that may occur by the inclusion of early pregnancy losses (non-live births <24 weeks are considered miscarriages rather than stillbirths in Botswana). The exclusion of births that occur outside hospital grounds is similarly intended to reduce bias and maintain uniformity across all study locations.

## 4.2 Inclusion and Exclusion Criteria for Follow-up of Births with Congenital Abnormalities (CAs)

--child born with a major congenital abnormality

--mother initially signed consent for a photograph at the time of birth

# 5. STUDY PROCEDURES

## 5.1. Overview of Study Design

We conduct a cohort study of infant livebirths and stillbirths born at ≥24 weeks gestation at up to 18 geographically representative hospitals in Botswana. We extract maternal and infant data from obstetrical and outpatient records and perform active surveillance for all congenital malformations in both liveborn neonates and stillbirths. Congenital malformations are classified by medical providers, by photography, and by expert review of all available data.

## 5.2. Maternity Staff Training

There is currently inconsistent documentation of congenital malformations in Botswana and rare documentation of specific neural tube defects. Several types of NTDs can occur, with anencephaly and myelomeningocele the most common. Some fetal exposures, such as maternal diabetes, have been shown to be associated with an increase in the frequency of anencephaly, myelomeningocele and encephalocele.  By contrast, exposure to the anticonvulsant drug valproate is associated with an increase in the frequency of meningomyelocele and rarely anencephaly. Given our aim to determine the association between ARV exposure and NTDs, it is critical to appropriately classify the precise neural tube defect. This is not just a problem with NTDs as we also found that many birth injuries were classified as congenital abnormalities and many descriptions of abnormalities in the physical exam were too imprecise to be used to classify the abnormality in our pilot study. We address this problem through intensive training for maternity and pediatric staff specifically designed to improve infant surface exams in African countries. Training modules have been developed by Dr. Lewis Holmes at MGH (who also developed the WHO training video to demonstrate the surface examination of newborn infants with Dr. Melba Gomes), and use WHO-approved videos to help standardize the approach to identification and classification [52]. We provide each study site with a picture atlas (either digital or printed depending on internet access) of congenital abnormalities to aid in identification and provide feedback to the sites on their diagnostic accuracy after photographs of abnormalities are reviewed by the study team. We then monitor quarterly reported rates of congenital abnormalities stratified by site, and we re-train any site with >20% change in reported rates throughout the study period.

## 5.3. Data Collection Process

Surveillance for birth outcomes occurs by having trained staff at each maternity site review delivery records and compare these against delivery logbooks to ensure that 100% of in-hospital births are collected at each site. Data will be collected without recording identifying information in the research database (maternal Omang number is kept separately from the surveillance data) and without individual consent, as currently approved by Botswana and Harvard IRBs in our pilot surveillance. Any medical records and obstetrical records may be reviewed, but the surveillance is designed to extract the data that are included on obstetrical cards (which are the primary record of events in pregnancy and at delivery) and the outpatient cards (which are the primary record of HIV treatment) for the research database, and to separately record a unique identifier that may be used for additional data extraction from other sources (e.g., electronic medical records) and at additional timepoints. The information abstracted from obstetric records includes maternal demographics, antenatal care clinic (ANC) site (a proxy for home village) and number of ANC visits attended, medical history (including diabetes, hypertension, and other common conditions as well as medications prescribed for these conditions), laboratory values measured in pregnancy (hemoglobin and rapid plasma reagin), vital signs, pre-pregnancy weight (or weight in the first trimester), diagnoses during pregnancy, medications prescribed during pregnancy (including folate and multivitamin), obstetric history (including gravidity, parity, previous contraception, last menstrual period, and estimated gestational age), referral reason (if patient referred to health center for delivery), and delivery/birth description. Details related to COVID-19 testing and symptoms will be abstracted if available as part of the medical or obstetric or electronic records. The delivery/birth details include birth weight, Apgar, vital status of infant (liveborn, fresh stillbirth, macerated stillbirth), delivery complications, complete infant exam, congenital abnormalities, initial feeding, infant ARV prophylaxis (if applicable) and infant status at discharge. Maternal HIV status and antiretroviral (ARV) history are often available in the obstetric record. This information will be cross-checked using the outpatient clinic record (and in some cases electronic records) to ensure accuracy of HIV testing date, ART treatment regimen, ART treatment start date, weight and height at ART initiation, any ART switch/discontinuation during pregnancy, and nadir and most recent CD4 cell count and viral load.

Our research assistants rely on the maternity ward nurses to complete the obstetric cards for all women who deliver. When the cards are incomplete or have errors, they ask the nurses to talk to the mothers to complete the data on the obstetric card or resolve inconsistencies. This process improves the quality of clinical data for the nurses and doctors, and also the completeness of the surveillance. However, it is often time-consuming for the nurses. Since approval in 2018 of version 2.0 of this protocol, we therefore also may have research assistants approach women on the ward and review the inpatient file to clarify situations when there is missing or inaccurate information. This improves the information on the obstetric cards without requiring the valuable time of the nurses. Only clinical information that is currently required to be completed by all women as part of their routine obstetric or medical care is discussed, and if changes are required on the obstetric cards these are recorded. In this manner, the research assistants will be more integrated into the maternity ward activities (where many have worked for several years) and improve the quality of the clinical records with minimal disruption to the maternity nurses.

Each live born or stillborn gestation delivered is captured as an independent record in the database. Multiple gestations are noted and are able to be linked to each other as twins or triplets in the analysis, but during data collection each are a separate record (with the same maternal demographic and other details recorded). Neonatal death information is captured by our study team for all infants who die before leaving the hospital by the 28th day of life. We use the estimated gestational age at delivery from the estimate in the obstetric records. For most pregnancies in Botswana, estimated date of delivery is based on last menstrual period (LMP) alone. However, if the date is uncertain or discordant with fundal measurements, gestational age is estimated using ultrasonography.

Few laboratory results (including CD4 and viral load) are documented on the obstetric cards, and infant HIV PCR status cannot be linked to maternal information. It is unknown how often CD4 and viral load tests are performed and not recorded, and linking the HIV status of the child to maternal obstetric records will help improve our understanding of MTCT risk. We therefore will improve surveillance for all laboratory testing by searching the IPMS system for relevant results. To do this, since 2018 we have collected the Omang number and patient name and stored them in a separate encrypted form in our REDCap data capture system, along with a link to the patient ID in the Tsepamo database. This form is accessible only to specific research assistants who are performing the linkages. A dedicated research assistant will use this information, as well as the date of birth, to link to available laboratory information stored in clinical electronic records for the mother or her child (in IPMS, PIMS, or other systems). Identifiable information in the separate REDCap form will be destroyed after the approved study period. No linkage will be maintained beyond the approved study period, and no personal identifiers will ever be included in the main Tsepamo database used for analysis purposes. To improve capture of electronic data for all women in the surveillance system, and to track multiple pregnancies and exposure risks over time, we added OMANG capture universally in 2021 with version 3.0 of this protocol. This occured in an identical manner as described above for women living with HIV.

## 5.4. Data Collection for Congenital Abnormalities

Congenital abnormality information are recorded for every delivery (yes/no) as part of the surveillance data, and maternity nurses are trained and prompted to record the results of a surface examination for every delivery (active surveillance). When a congenital abnormality or a potential congenital abnormality is identified, additional detail will be obtained and recorded on a dedicated form after interviewing the maternity nurses and physicians. Consent to photograph the infant will be requested of the mother, for classification purposes by the blinded expert review. Photographs will be taken with care to avoid identifying features to the extent possible. If the infant is stillborn, a surface exam will also be conducted, either at the time of delivery by nursing staff or at the hospital morgue by study staff. All data (including photos) for potential congenital abnormalities are reviewed by an expert in congenital abnormality classification (Dr. Holmes or other) approximately every 3 months. No attempt is made to exclude potential congenital abnormalities which cannot be immediately classified at the maternity ward; unidentifiable abnormalities are recorded and may or may not be included in analyses based on later expert review.

As an exploratory aim, the study team will also use the database to house reports of potential congenital abnormalities that are identified by pediatricians or care providers in Botswana following delivery for children born at the participating surveillance sites. This additional data will not be part of the primary aims, but the study team will have the option to add post-discharge data to a “post-discharge” field that will be created for each birth record in the database (identified by maternal Omang number). This post-discharge data will serve as a registry (no accurate denominators will be possible) but it will be linkable to the pregnancy ART exposures captured in the main database. Interpretation of post-discharge data will occur with extreme caution. If novel patterns or specific abnormalities of concern emerge, these will be discussed with Dr. Lewis Holmes, and possibly a larger group of birth defects experts, to determine whether further evaluation might be warranted. As such, this registry data may serve as an early warning mechanism for severe or distinctive abnormalities.

We will follow-up health outcomes for a subset of infants with major CAs via medical record review and semi-structured interviews with caregivers. For up to 150 infants born with a major congenital abnormality whose mothers already signed a consent to have an anonymous picture taken at birth, we will review the obstetric record and medical records for the infant outcome (including deaths and hospital stay). When applicable for infants who were alive at the time of hospital discharge, we will also call caregivers to determine how the child is doing. We will not contact those with known stillborn or neonatal deaths.

The data obtained from these interviews will be added to the Tsepamo REDCap database, and maintained in a coded manner. A summary will be presented to the Botswana MoH and for scientific presentation.

## 5.5. Data Completeness

We use a double reference system (obstetric cards and maternity logbooks) to ensure that every woman who delivers at the hospital is captured in the database.

## 5.6. Data Management

A unique study number is created for every delivery at each site. If a photograph is taken to accompany a delivery record, the photograph has the study number included in the actual picture in the lower left corner, and the photograph is uploaded and associated with the record in the database. All study sites are connected to the BHP Data Centre by telephone and e-mail. Data are extracted onto Study CRFs in electronic format by study personnel and stored in a REDCap database without personal identifiers (photographs of congenital abnormalities have identifying facial features blocked out). Data checks are put in place to verify completeness of the surveillance for each site (against logbook data) and to check for data errors, using modifications of automated checks designed for previous studies. Data managers in Botswana and Boston evaluate the database and check for inconsistencies and missing data on an ongoing basis, and queries will be directed back to each site to maintain data quality throughout the course of the study.

# 6. STATISTICAL CONSIDERATIONS

## 6.1. Definitions

We will use the same definitions for birth outcomes as in our previous studies [20]: preterm delivery is delivery <37 weeks gestation; small for gestational age is below the 10th percentile of birth weight using WHO norms (or in some cases norms created from infants born to HIV-uninfected women in Botswana [53]); stillbirth is fetal death >24 weeks gestation with an Apgar score of 0. Non-live births <24 weeks are defined as miscarriages in Botswana, and are not registered through the obstetric/maternity wards; accurate assessment for NTD is not feasible for these outcomes. In-hospital neonatal death is defined as death during the same hospitalization within 28 days of a live delivery. Births must occur at the hospital site (not at home or on route) to be included in the surveillance. We will define anemia during pregnancy as a recorded hemoglobin ≤10 g/dL. Maternal hypertension in pregnancy will be defined as a systolic blood pressure measurement >140 mmHg or a diastolic measurement >90 mmHg at any visit before labor, admission to hospital for hypertension, delivery complicated by hypertension, or induction for preeclampsia. For simplicity and increased accuracy of exposure categorization (which relies on maternal report of LMP), we are using the “number of women on ART from conception” rather than “the number exposed to ART in the first 6 weeks of pregnancy,” because we expect fewer than 1% of women to start ART in the first 6 weeks of pregnancy. In the analyses, we will perform sensitivity analyses either including or excluding those with ART started in the first 6 weeks of pregnancy with those receiving it from conception (because of concerns regarding the accuracy of pregnancy dating); however, we do not expect this to change the study findings given the small numbers expected. “ART from conception” will be defined as ART started before the LMP. ART switches will also be classified as pre-conception or post-conception using LMP.

An international birth defects expert (Dr. Holmes) will use data abstracted from medical records, staff interviews, and photographs to classify malformations (blinded to maternal HIV status and ART regimen). Congenital malformations will be defined as a structural abnormality with surgical, medical or cosmetic importance. We will apply previously validated inclusion and exclusion criteria in order to decrease bias from differences in provider-specific interpretations of a physical finding [54]. Minor anomalies, birth marks, positional deformities, functional abnormalities, findings in a newborn screen, physiologic findings, findings only detected on prenatal ultrasound (and not detectable on exam at birth), and findings consistent with chromosomal or genetic abnormalities will be excluded from the analyses based on expert review. Neural tube defects will be categorized as anencephaly, encephalocele or meningomyelocele (spina bifida occulta will be excluded). Malformations will then be coded using the ICD-10.

## 6.2. Projections for ART Exposures

**6.2.1 Sample Size and Power:** With surveillance occurring at all sites, we expect to extend the Tsepamo database, yielding a likely total of up to ~410,000 (+/- 10%) deliveries from 2014-2026, but the exact number will depend on our ability to maintain surveillance at as many of the 18 potential surveillance sites as possible. Our 12-year estimate is very accurate, and is based on exact birth records in recent years at the surveillance sites. For simplicity of calculations, we assumed a constant number of annual births at the study sites. However, with brisk population growth in Botswana, we may have more births than expected, increasing our sample size and power. We believe the proportion of HIV-positive pregnant women will average to ~ 22% in the 2021-2026 time period (dropping from 26.6% to 19.5% over time); overall, HIV prevalence has stayed relatively constant in Botswana over the last decade [51]. In 2016-2017, the prevalence of HIV among pregnant women in Tsepamo was 24.2% (BHP, unpublished data, 2017) and in 2019 it was 23%. It is possible that the HIV prevalence in Botswana will start to decrease faster than anticipated between 2021-2026 from prevention interventions, but it is unlikely to decrease substantially. The expected number of HIV+ women on ART from conception, on DTG/TDF/FTC from conception, and who will switch to DTC/TDF/FTC prior to conception are detailed Figures 1 and 2.

**Figure 1. Study Population 2014-2022**

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**Figure 2. Projected study population through 2026**



**6.2.2 Data Analyses and Interpretation**

**6.2.2.1. Aim 1**. This study aim is to determine the incidence of SB, PTD, very PTD, SGA, very SGA, congenital abnormalities, and in-hospital neonatal death among infants exposed to different ART regimens from the time of conception.

This aim includes an analysis of whether DTG/TDF/FTC exposure is a risk factor for these events compared with other ART regimens (particularly EFV/TDF/FTC). Analysis for this aim will separately compare the risk of each of these seven adverse birth outcomes, and combined endpoints of any adverse outcome (SB, PTD, SGA, early neonatal death) or any severe adverse outcome (SB, very PTD, very SGA, early neonatal death) between HIV-infected women on DTG/TDF/FTC with those on EFV/TDF/FTC from conception. In addition, this study aim will determine the prevalence of major congenital abnormalities (CA) and NTDs among infants exposed to DTG-based ART vs. other ART regimens from the time of conception and vs. HIV-unexposed. Analysis will separately compare the prevalence of 1) any major congenital abnormality (CA), and 2) NTD between DTG exposure from conception and non-DTG exposure from conception, EFV from conception, DTG started during pregnancy, and HIV-unexposed. We will first describe the cohort in terms of sociodemographics, clinical and obstetrical history, and delivery site by HIV status and ART exposure. The risk of each birth outcome and the prevalence of major CA and NTD will be computed overall, by HIV status and by ART exposure at conception. For each of these groups, the risk and the prevalence will be calculated as the number of events (e.g. preterm delivery) over the number of births in that exposure group.

The 95% confidence interval around the prevalence of abnormalities in each exposure group will be calculated with the Wilson method (Agresti). Differences in prevalence between the group with exposure to DTG at conception and the other exposure groups were determined and 95% CI calculated with the Newcombe method (Newcombe).

The risk ratio (relative risk) for the comparison of DTG/TDF/FTC exposure vs. other ART regimens will be the risk in the exposed group over the risk in the comparison group (e.g. risk in the DTG group vs. risk in EFV group). For each birth outcome and each comparison of DTG/TDF/FTC exposure vs. other ART regimens, the risk ratio and 95% confidence intervals (95%CI) around the estimate for each birth outcome will be determined using log-binomial regression in unadjusted analyses and then on multivariable analyses adjusted for potential confounders. If the log-binomial model fails to converge we will instead fit a Poisson regression model with robust variance. Confounders will include variables that preceed the exposure and are thought to be associated with the exposure and the outcome, and not in the causal pathway between exposure and outcome. Potential confounders may include sociodemographic variables, birth year, gravity, maternal weight gain and co-morbidities, time on ART, delivery site and its distance to initial antenatal clinic. We will have CD4 measurements in pregnancy and before initiation of ART for approximately half of women (perhaps up to 70% with enhanced data collection measures), and thus will have limited ability to control for confounding due to HIV disease status. We will exclude antiretrovirals only given in labor (and not earlier in pregnancy). In secondary analyses, we will compare DTG/TDF/FTC with other ART regimens. We will also compare outcomes among women who were on DTG at conception with those who start DTG in pregnancy, recognizing that we may have incomplete measurement of confounders related to differences between these groups. The risk in the ART group for each outcome is based on our current data. The projected sample size for each group includes only those with ART exposure data (see Fig. 1).

Table 1 shows power to detect a risk ratio of 2.5 between exposure groups for rare events (congenital abnormalities), given an alpha of 0.05, for different levels of risk (0.03%, 0.1%, 0.2%, and 0.5%). If the risk for an abnormality is 1 per 1000 births in HIV unexposed infants, by 6 years of DTG surveillance (2016-2022) we will have 94% power to detect a 2.5 fold increase in risk for DTG-exposed infants. If we reduce the risk ratio to 2.0, we will have 75% power at the 1 per 1000 level. Thus, we will have sufficient power for comparisons at a clinically meaningful level (the ability to identify a relative risk of 2.0-2.5), but we are also not overpowered. Table 2 shows the power to detect a risk ratio of 2.5 for different major congenital abnormalities.

**Table 1.Detectable Risk Ratio and Risk Difference for Each Adverse Birth Outcome by ARV Regimen at Time of Conception: DTG/TDF/FTC (N=6182 initial starts) compared to EFV/TDF/FTC (N= 10177)**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Adverse Birth Outcome** | **Percentage of Women on**  **EFV/TDF/FTC at Conception with Adverse Outcome** | **DTG/TDF/FTC compared to EFV/TDF/FTC** | | |
| **Detectable**  **Risk Ratio** | | **Detectable**  **Risk Difference (%)** |
| **For Total Enrollment\*** | **For 24 Months of Enrollment \*\*** | **For Total**  **Enrollment\*** |
| Any | 36.4 | 1.06 | 1.09 | 2.2 |
| Any severe | 12.3 | 1.12 | 1.17 | 1.5 |
| Preterm | 21.4 | 1.09 | 1.12 | 1.9 |
| Very Preterm | 4.1 | 1.23 | 1.32 | 0.9 |
| SGA | 16.9 | 1.10 | 1.14 | 1.7 |
| Very SGA | 7.1 | 1.17 | 1.23 | 1.2 |
| Stillbirth | 2.4 | 1.31 | 1.43 | 0.7 |
| Neonatal death | 1.2 | 1.45 | 1.63 | 0.5 |

\* Estimated total enrollment: DTG/TDF/FTC N = 6182, EFV/TDF/FTC N=10177

\*\*Estimated enrollment up to 24 months of study: DTG/TDF/FTC N = 2715, EFV/TDF/FTC N=7894

**Table 2**. **Power to Detect a Risk Ratio of 2.5 for Different Major Congenital Abnormalities**

**in DTG/TDF/FTC-exposed compared to EFV/TDF/FTC-exposed and to HIV-unexposed infants**

|  |  |  |
| --- | --- | --- |
| **Risk of Congenital Abnormality** | **DTG/TDF/FTC (N=9511\*)** **vs.**  **EFV/TDF/FTC (N=10177)** | **DTG/TDF/FTC (N=9511\*)** **vs.**  **HIV-unexposed (N=110970)** |
| 1 per 3000 (0.03%) | 0.29 | 0.59 |
| 1 per 1000 (0.1%) | 0.71 | 0.94 |
| 2 per 1000 (0.2%) | 0.95 | 1.00 |
| 5 per 1000 (0.5%) | 1.00 | 1.00 |

\*includes all DTG/TDF/FTC exposures prior to the pregnancy regardless of ART switch history

\*\* Congenital abnormalities at 1 per 3000 include omphalmocele, gastroischesis; 1 per 1,000 include neural tube defects, cleft lip and palate; at 2 per 1000 include club foot, microcephaly; at 5 per 1,000 include polydactyly

The projected total number of pregnancies in the Tsepamo Birth Surveillance study from 2014 through 2026 for each exposure group are: DTG from conception (N=26,131), non-DTG ART at conception (N=10,243), EFV from conception (N=24,842), and for HIV-unexposed (N=317,742). We calculated the minimum detectable difference in prevalence for all major congenital abnormalities and for NTDs between DTG at conception vs. each other exposure group, assuming 80% power and an alpha of 0.05 calculated in Pass Version 15.0. The prevalence estimates for each outcome (all major CA, NTDs) in each comparison group (DTG conception, EFV conception, and HIV-unexposed) are based on our published results.[39] The minimum detectable difference in prevalence (80% power, alpha 0.05) between DTG at conception versus non-DTG ART at conception, EFV at conception, and HIV-unexposed are 0.28%, 0.22%, 0.15% for any major CA and 0.12%, 0.07%, and 0.07% for NTD (Table 3).

**Table 3. Minimum Detectable Difference in Prevalence of Major Congenital Abnormalities (CA) and Neural Tube Defects (NTD) by Exposure groups (80% Power, alpha=0.05)**

The analysis for follow-up of births with CAs will be descriptive, summarizing the follow-up data from up the convenience sample of up to 150 infant outcomes. Infant mortality and major morbidity will be summarized.

**6.2.2.2. Aim #2A.** For this Aim, we will emulate the target trial by using the combined dataset of our Tsepamo and Tsepamo Extension Surveillance Studies. The enrollment period for the target trial is 2014-2023, and eligibility criteria include being HIV+, non-pregnant, and on a legacy ART regimen. In a target trial, eligible women would be randomized to (1) switch from their legacy regimen to DTG/TDF/FTC while non-pregnant or (2) continue on the legacy regimen while non-pregnant. The hypothetical intervention also includes becoming pregnant during follow-up and having a pregnancy outcome at 24 weeks or later at one of the 8 delivery sites.

We will include the following prognostic factors in our models: year, time from HIV diagnosis to ART initiation (1 year or less, more than 1 year), age at baseline (<25 years, 25-30 years, ≥30 years, unknown), marital status (married, unmarried or unknown), occupation (salaried, unsalaried or unknown), education (none or primary, more than primary, unknown), parity (0, 1 or more, unknown), delivery site and its distance to initial antenatal clinic, CD4 cell count (<200, 200-349, 350-499, >/=500 cells/mm3), and maternal HIV RNA when available (<400 or >/=400 copies/mL). To assess historical bias, we will use data from the HIV-negative women and women on EFV/TDF/FTC and calculate the risk of each adverse birth outcome by calendar year. We will perform a sensitivity analysis that considers treatment switching as a time-varying exposure, in which case methods designed to handle treatment-confounder feedback (e.g. marginal structural modeling) may be necessary. We will perform further sensitivity analyses that stratify our analysis by site type (tertiary vs. district hospitals); that distinguish between the type of legacy ART (NVP- or LPV-r-based, and restricted to TDF/FTC backbone); that vary the time-frame for entry into the target study following HIV diagnosis (from 0 to 10 years); that vary the time-frame between enrollment and conception (from 1 month to 8 years); and where we perform instrumental variable analysis using the calendar year of the guideline change to DTG (2016).

The prevalence of the individual and combined endpoints are known from the 2-year Tsepamo Study data for legacy ART regimens; for EFV/TDF/FTC from conception; and for DTG/TDF/FTC when started in pregnancy [59, 60]. Among women who started DTG/TDF/FTC in pregnancy, adverse birth outcomes were nearly identical to outcomes for EFV/TDF/FTC started in pregnancy. We therefore hypothesize that adverse birth outcomes for women who start DTG/TDF/FTC from before conception will also be similar to those for EFV/TDF/FTC. We have identified significant differences in adverse birth outcomes between EFV/TDF/FTC and the legacy LPV-based and NVP-based regimens. For combined adverse birth outcomes, there was a difference of 9% (from 36% to 45%) for women on EFV/TDF/FTC vs. women on any LPV-based or NVP-based regimen. When switching from a legacy regimen to a newer ART regimen, the best result we can hope for is that risk for adverse events will drop all the way to the level of those continuously on the newer regimen. We believe this will indeed be the case, based on our hypothesis that NVP- and LPV/r-based ART exert specific adverse effects in pregnancy related to placental function or the placental vasculature [26, 59, 61-65], and that removing these exposures *prior to conception* will remove all excess risk related to these agents. Thus, using the EFV/TDF/FTC data as a guide, we believe that women who switch from a legacy regimen to DTG/TDF/FTC will have a reduction of 9% in combined adverse birth outcomes (or from 45% to 36%). In Table 2, we outline the power calculations to detect this 9% difference, as well as differences of slightly smaller magnitude (7% and 5%). Similar calculations are possible for combined severe adverse outcomes, where ~8% difference is expected, and for individual adverse birth outcomes. The stillbirth risk is the outcome of greatest public health concern for legacy regimens (particularly NVP-based), and we will have 88% power to detect a true 0.9% difference between continuing or switching legacy ART. The expected numbers available in each ART exposure group are as outlined in detail in Section 6.2.1. In the event that fewer changes from legacy regimens to DTG/TDF/FTC occur than anticipated, this will minimally affect the power calculations: power would remain identical for almost all possible risk difference comparisons listed in Table 4, with a maximal decrease of only 2-3% power (for neonatal death).

**Table 4. Power for Emulated Clinical Trial Comparisons, by Expected Absolute Risk Difference**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Adverse Birth Outcome** | ***Known risk with continuous legacy ART (N=4,838)*** | ***Risk with pre-conception switch to DTG/TDF/FTC***  ***(N=3,329)*** | ***Absolute Risk Difference*** | ***RR*** | ***Power to Detect True Difference*** |
| ***Combined*** | *45%* | *36%* | *9%* | *0.80* | *1.00* |
| *38%* | *7%* | *0.84* | *1.00* |
| *40%* | *5%* | *0.89* | *0.99* |
| ***Combined Severe*** | *20%* | *12%* | *8%* | *0.60* | *1.00* |
| *14%* | *6%* | *0.70* | *1.00* |
| *16%* | *4%* | *0.80* | *1.00* |
| ***Preterm Delivery*** | *23%* | *22%* | *1%* | *0.96* | *0.19* |
| *--* | *--* | *--* | *--* |
| *--* | *--* | *--* | *--* |
| ***Small for GA*** | *27%* | *17%* | *10%* | *0.63* | *1.00* |
| *19%* | *8%* | *0.70* | *1.00* |
| *21%* | *6%* | *0.78* | *1.00* |
| ***Stillbirth*** | *4.8%* | *2.4%* | *2.4%* | *0.50* | *1.00* |
| *2.9%* | *1.9%* | *0.60* | *0.99* |
| *3.4%* | *1.4%* | *0.71* | *0.88* |
| ***Neonatal Death*** | *2.1%* | *1.2%* | *0.9%* | *0.57* | *0.88* |
| *1.4%* | *0.7%* | *0.67* | *0.67* |
| *1.6%* | *0.5%* | *0.76* | *0.39* |

**6.2.2.3. Aim 2B**

We will emulate a conditionally randomized target trial using the combined dataset of all Tsepamo surveillance. The enrollment period for the target trials is 2014-2026 (or earlier if accrual is faster than expected), and eligibility criteria include being HIV+ and non-pregnant at the time of enrollment. In the target trial, eligible women would be conditionally randomized to EFV/TDF/XTC, DTG/TDF/XTC, or DTG/TAF/XTC. Randomization would be stratified by weight (<60kg, 60-80kg, or >80kg) at the time of enrollment. The hypothetical intervention also includes becoming pregnant 6-36 months after enrollment and having a pregnancy outcome ≥24 weeks at one of the Tsepamo delivery sites.

We will include the following prognostic factors in our models: year of ART initiation, time from HIV diagnosis to ART initiation (1 year or less, > 1 year), age at baseline (<25 years, 25-29 years, ≥30 years, unknown), marital status (married, unmarried or unknown), occupation (salaried, unsalaried or unknown), education (none or primary, more than primary, unknown), parity (0, 1 or more, unknown), delivery site and its distance to initial antenatal clinic, CD4 cell count (<200, 200-349, 350-499, ≥500 cells/mm3), and maternal HIV RNA when available (<400 or ≥400 copies/mL). To assess historical bias, we will use data from the HIV-negative women and calculate the risk of each adverse birth outcome by calendar year. We will perform further sensitivity analyses that stratify low weight as <50kg and 50-60kg; that stratify our analysis by site type (tertiary vs. district hospitals); that vary the time-frame for entry into the target study following HIV diagnosis (from 0 to 10 years); that vary the time-frame between enrollment and conception (from 1 month to 8 years); that leverage the HIV-negative population to explore temporal trends in adverse birth outcomes; and where we perform instrumental variable analysis using the calendar year of the guideline change to DTG (2016) and TAF (2021). Finally, we will consider BMI at enrollment (available for a subset of the total population) and consider underweight, normal weight, overweight, and obese categories in sensitivity analyses.

Our primary outcome will be any severe adverse birth outcome, defined as very preterm birth, very SGA, stillbirth, or neonatal death. Secondary outcomes will include a combined endpoint of stillbirth or neonatal death and maternal hypertension. Tertiary outcomes will include a combined endpoint of any adverse birth outcome, individual birth outcomes, and other maternal outcomes such as macrosomia and c-section. The prevalence of the individual and combined endpoints for adverse birth outcomes are known from the Tsepamo Study data for EFV/TDF/XTC and for DTG/TDF/XTC,[39] and differences by weight strata are under investigation.[48] We have identified significant differences in adverse birth outcomes by baseline weight, and significant differences in the effect on weight by ART regimen (DTG vs. EFV). The effect of TAF combined with DTG on weight is profound[45] but it is unstudied to date in terms of birth outcomes in women on TAF and DTG at conception. Thus, we hypothesize that weight gain from TAF will improve overall outcomes for low weight women, but could worsen overall outcomes for high weight women. For high weight women, whether TAF can be used safely is a critical question, and our definition of “adverse outcome” may need to be expanded to include macrosomia, c-sections, and maternal outcomes. Accordingly, we will create an ordinal scale for infant and maternal adverse outcomes, and include a weighted score as a secondary outcome. In Table 5, we outline the minimal detectable risk differences based on 80% power comparing DTG/TAF/XTC with EFV/TDF/XTC and with DTG/TDF/XTC for any severe adverse outcome for stillbirth or neonatal death, and for maternal hypertension, separately for each weight group. We will have 80% power to detect a risk difference in any severe adverse outcome of -3.2% comparing DTG/TAF/XTC with EFV/TDF/XTC and of -3.1% comparing DTG/TAF/XTC with DTG/TDF/XTC for the low weight group, and 80% power to detect a risk difference in any severe adverse outcome of 4.6% comparing DTG/TAF/XTC with EFV/TDF/XTC and of 4.4% comparing DTG/TAF/XTC with DTG/TDF/XTC for the high weight group. Stillbirth or neonatal death is the outcome of greatest public health concern and we will have 80% power to detect a -1.6% risk difference in DTG/TAF/XTC vs. EFV/TDF/XTC and a -1.6% risk difference in DTG/TAF/XTC vs. DTG/TDF/XTC for the low weight group, and 80% power to detect a 3.2% risk difference in DTG/TAF/XTC vs. EFV/TDF/XTC and a 3.1% risk difference in DTG/TAF/XTC vs. DTG/TDF/XTC for the high weight group. Since stillbirth or neonatal death is a rare outcome, we expect to have sufficient power to detect clinically significant risk differences for all other individual outcomes. Outcome risks by weight group for women on ART at conception were computed using existing Tsepamo data. We assume TAF roll out in Botswana will follow a similar pattern to the rollout of DTG, with few initiations in 2020-2022 and then increasing over a four-year period from 2023-2026. In the event that TAF use by 2026 is only half of what is predicted, this will modestly affect the minimal detectable risk difference calculations: in the low weight group we would have 80% power to detect risk differences of -4.3% to -4.4% for any severe adverse outcome and -2.1% to -2.2% for stillbirth or neonatal death, and in the high weight group we would have 80% power to detect risk differences of 6.4% to 6.7% for any severe adverse outcome and 4.6% to 4.8% for stillbirth or neonatal death.

**Table 5. Minimal Detectable Absolute Risk Differences Based on 80% Power for Emlated Trial Comparisons, by Weight at Enrollment (ART initiation)**



**6.2.2.4. Aim 3.** Statistical considerations for Aim #3 relate to the number of second pregnancies available for evaluation from 2018-2026 for women with HIV (collection begun in 2018) and from 2021-2026 for women without HIV (collection to begin in 2021). Data for women with HIV show 834 repeat pregnancies have occurred in ~36 months. Using this as a guide, we can anticipate ~1,400 women with HIV and ~6,300 women without HIV with repeat pregnancy data by 2026. These numbers will be sufficient for the planned analyses. For example, in our primary analysis we will evaluate median inter-pregnancy weight gain (early second pregnancy weight – early first pregnancy weight), adjusted for inter-pregnancy length among women on DTG, EFV and women without HIV. Assuming 620 women on EFV and 580 women on DTG with median weight gain in the EFV group of 1kg,[45, 48] we will have 80% power to detect a mean difference of 3.5kg in inter-pregnancy weight gain between groups. We will secondarily look at the impact of breastfeeding vs. formula feeding on inter-pregnancy weight gain, evaluate risk factors for weight gain and weight loss, and use formal mediation analyses to evaluate whether inter-pregnancy weight gain mediates adverse birth outcomes (preterm, SGA, stillbirth and neonatal death) and adverse maternal outcomes (maternal hypertension, post-partum hemorrhage, C-section).

A secondary part of this analysis will be to measure HIV incidence among women without HIV in their first pregnancy and evaluate for risk factors for HIV seroconversion between pregnancies. While these data may overestimate population incidence, we will be able to identify high-risk populations that can be targeted by the rollout of PrEP in Botswana. Additionally, we can measure trends in HIV incidence over time and potentially evaluate the impact of PrEP rollout on incidence of HIV between pregnancies.

In prior Tsepamo analyses, we have been unable to evaluate the impact of prior adverse pregnancy outcomes on risks in the current pregnancy. However with data on repeat pregnancies, we will be able to estimate the increase in risk of adverse outcomes among women who have had prior adverse outcomes and evaluate whether these risks differ by HIV status and ART regimen. This is particularly important for our outcomes of preterm delivery and congenital abnormalities. We will additionally report the impact of short inter-pregnancy length on birth outcomes.

## 6.3 Anticipated Outcomes

We expect to provide evidence that pre-conception exposure to DTG/TDF/FTC does not significantly increase the risk for adverse birth outcomes as compared with EFV/TDF/FTC, and that switching women from LPV-based or NVP-based ART regimens to DTG/TDF/FTC can significantly improve birth outcomes. This information will also provide direct scientific evidence that specific ART regimens impact birth outcomes, likely at the level of the placenta.

We also expect to provide evidence that pre-conception exposure to DTG leads to a small but significant increase in risk for NTDs that will continue to provide reassurance regarding its continued use in international ART programs. We will show that DTG/TAF improves birth outcomes for women <60kg, and is safe to use for women >80 kg (though with an increase in some non-severe adverse outcomes). We will demonstrate that baseline weight between pregnancies differs significantly by HIV status and ART exposure.

# 7. HUMAN SUBJECTS CONSIDERATIONS

**7.1. Characteristics of study population**

The study population will consist of postpartum women in Botswana, whose obstetric cards and medical records will be accessed for data extraction. The anticipated age range of study mothers will be approximately 15 to 45 years; because this is an observational surveillance study, there is no age limit for data collected.

**7.2. Sources of research material**

Research material will consist of data obtained from maternity obstetric records, maternal hospital records and newborn hospital records, from ART records if required, and from electronic records if required. Photographs of infants will also be collected when congenital abnormalities are detected.

**7.3. Participant recruitment and study consents**

**Waiver of informed consent was obtained for record surveillance:** Data abstraction will occur as outlined above. Written informed consent will not be required for data abstraction, as only existing clinical data will be collected.

**Written consent for photographs of children with suspected CAs:** A short written consent form for mothers will be used to request a photograph of children with suspected neural tube defects, for proper classification of these abnormalities, taking care to avoid identifying features in the photograph. Consent forms will be written in both Setswana and English and will be reviewed verbally with mothers by the counselors/healthcare workers. The consent process will describe the purpose of the study, and the reason for a photograph, and will highlight the mother’s freedom to decline or withdraw participation at any point without compromising her future medical care. Study investigators will prioritize participant education and informed consent procedures for the study. Participant understanding of scientific information, research methods, and human subjects rights can be enhanced through the following: 1) full, standardized disclosure of study-related information in both written and verbal formats, 2) minimizing the reading level and enhancing the visual display of written documents, 3) providing adequate time and opportunity for participants to read/hear about, reflect upon, ask questions about, and discuss the research.

**Verbal (not documented) Consent for follow-up interview of mothers or caregivers of infants born with major CAs:** Following record review, if additional information is needed from mothers or caregivers of infants born with major CAs who were alive at time of hospital discharge, we will call caregivers to determine how the child is doing. If a phone number is not found in the obstetric record, we may follow-up with the antenatal care (ANC) clinic, which is documented in the Tsepamo study. Via record review and discussion with families (if needed), we will assess longer-term survival, medical and social complications among infants born with major congenital abnormalities in Botswana.

For follow-up calls, we will first use a telephone consent script. We will request a waiver of documentation of informed consent for these telephone interviews. Caregivers who agree to proceed will be asked scripted questions about the child’s diagnosis and vital status, supports or treatment, and social impact.

**7.4. Risks and benefits**

Because this is not an interventional trial, there are few direct risks/benefits of participation.

**7.4.1. Confidentiality.** Measures to protect confidentiality of data are in place that include netbook encryption and use of BID numbers instead of personal identifiers in the main database, with limited / encrypted access to the linkage between BIDs and any personal identifiers. In order to link the obstetric data to electronic laboratory data or HIV clinical care data, we will collect personal identifiers (Omang number and name) in a separate encrypted REDCap database that is access-limited to specific research assistants only. Data for any congenital abnormalities reported to the study team within the first year of life (for exploratory analysis, if patterns emerge) can also be captured using these linkages. Personal identifiers will never be included in the main database used for analysis. Linkages and personal identifiers will be destroyed at the end of the study approval period. Likewise, confidentiality will be maintained in photographs of congenital malformations to the greatest extent possible, and no personal identifiers will be on the photographs.

The data obtained from interviews with mothers or caregivers of infants born with major CAs will be added to the Tsepamo REDCap database, and maintained in a coded manner.

**7.4.2. Emotional Pain.** Women who deliver children with congenital abnormalities may experience emotional pain, and discussing taking a photograph might exacerbate that pain for some women. Our study staff will be trained medical nurses/counselors who will assess their ability to participate in the study. Women will be supported with counseling and referred to additional services if required. No woman will be asked for consent who is deemed emotionally unfit to participate.

**7.4.3. Benefits.**

Benefits of this study include possible identification of a congenital abnormality, and the ability to help other women from the knowledge gained in the study. For women who consent to have a photograph of a child with a congenital abnormality, our consent process will clearly specify that no clinical care will be provided by the study staff, and that all care for the child will occur through the Botswana medical system.

**7.4.4 Risks/Benefits.** The risks of the study are reasonable in relation to the benefits, both of which are minimal for any individual.

**7.5. Adherence to Human Subjects Requirements**

Study data will be collected in Botswana. Adherence to human subjects regulations will primarily be the responsibility of both Harvard School of Public Health and Botswana staff. The BHP team will adhere with all requirements by:

* Developing a study protocol that is able to meet its stated research objectives, and thus reflect adequate risk-benefit ratios for human subjects.
* Specifying study procedures in the protocols that protect the rights and safety of human subjects.
* Developing an informed consent form that includes all elements of informed consent required by Federal regulations and accurately represents study requirements, risks, and benefits in language that is understandable to study participants.
* Including human subjects considerations and requirements in study training sessions.
* Monitoring adherence to protocol specifications and human subjects requirements.

This proposal will be submitted to the Institutional Review Boards (IRB) of the Harvard School of Public Health, and to the Health Research and Development Committee (HRDC) in Botswana, which reviews scientific protocols.

**7.6. Procedures for minimizing potential risks**

1) Identification and disclosure of HIV Status: Women participating in our study may experience stigma from being thought to be HIV-infected. We have spent many years working to reduce the stigma concerns among participants in in previous studies, and have had success in these efforts. We have also helped to develop support groups in the community for HIV-infected individuals. Since 1999, we have seen progress in the area of stigma.

Any information about women and infants, including and especially their HIV status, must be subject to strict confidentiality. Special care will be needed in all procedures to ensure that the study does not cause release of information.

The identity of women and infants will only be recorded in a separate encrypted database to allow linkage to electronic records, and will then be destroyed after the study is completed. Personal identifiers will not be recorded in the main database. A numeric code will be assigned to photographs to link them to extracted birth records.

**7.7. Participation of children, women and minorities**

Women and their children are included in this research plan, through information collected as part of the medical record extraction. Postpartum women will comprise 100% of those asked to consent for an anonymous photograph of their child, and only children with suspected congenital abnormalities will be photographed (stillborn or liveborn). Individuals drawn from different ethnic groups and locations in Botswana will be included, all are expected to be African.

**7.8. Monitoring and Interim Analyses**

No DSMB will be established for this study, because it is non-interventional in nature. Routine reports to the study sponsor and to IRBs will occur at least annually. No interim analyses are planned other than those described.

**7.9. Biohazard containment**

No specimens will be collected as part of this study.

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# APPENDIX A: Completed Primary Objectives from Previous Versions of the Birth Outcomes Surveillance Protocol

Background, Rationale, and Analytical Approaches for these objectives are described in the corresponding version of the protocol.

**Version 1.0 Aim 1 [Completed]: To estimate the incidence of preterm delivery, small for gestational age infants, stillbirth, congenital abnormalities and in-hospital neonatal death among infants born to HIV-infected and HIV-uninfected women in Botswana, and to determine if Atripla (efavirenz/emtricitabine/tenofovir disoproxil fumarate) use is associated with these adverse birth outcomes.** *Hypothesis: HIV infection (and ART use, which will be nearly universal in pregnancy) will be associated with preterm delivery, small for gestational age infants, stillbirth, and neonatal death; the risk for these adverse birth outcomes will be similar to previous surveillance findings. Atripla use in pregnancy will not increase the risk for preterm delivery, small for gestational age infants, congenital abnormalities, stillbirth, and neonatal death compared with other ART regimens.*

**Version 1.0 Aim 2 [Completed]: To estimate and compare the incidence of neural tube defects and other congenital abnormalities among all live-born and stillborn infants born to: A) HIV-negative women, B) HIV-infected women not receiving ART from conception, C) HIV-infected women on EFV-containing ART from conception, and D) HIV-infected women on non-EFV-containing ART from conception.** *Hypothesis: First-trimester EFV exposure from conception will not increase the estimated risk of neural tube defects compared with other ART regimens or compared with infants born to HIV-uninfected women; a 2-fold or greater risk will be excluded from the 95% confidence limits for comparisons between EFV-exposed infants and all other infants.*

# APPENDIX B: Placental Collection Sub-Study of Birth Outcomes Surveillance Study (Tsepamo)

Note: This substudy was introduced in version 2.0 of the protocol and has been completed.

**Background**

Our research team has conducted several birth outcomes studies that have clarified the association between ART and birth outcomes. We performed the largest study to date of birth outcomes among HIV+ and HIV- women in Africa, evaluating over 33,000 birth outcomes at 6 maternities in Botswana between 2009-2011[20]. Controlling for ART exposure time and CD4 cell count, we found a significant increase in preterm delivery, small for gestational age infants, and stillbirth among women receiving ART during pregnancy. The absolute risk for stillbirth among women starting ART in pregnancy was 4.7%, compared with 1.7% for those starting zidovudine (ZDV). Among women on ART prior to conception, the stillbirth risk was 6.3% -- far higher than the background risk of 2.5% among HIV- women. In an important sub-study, we evaluated placentas from 100 stillbirths at PMH, and identified chronic hypertensive changes as the primary placental pathology that led to the stillbirths among HIV+ women receiving ART HIV+ women not receiving ART were more likely to have other placental pathology [2]. These findings suggest that women on ART in pregnancy may be at higher risk for chronic hypertensive damage at the placental level. We hypothesize that shifts in immunologic regulation occur among some women receiving ART that do not allow them to tolerate the placenta/fetus in a normal manner, and that this may manifest as chronic placental insufficiency (or chronic hypertensive vascular changes). We believe this explanation offers a unifying hypothesis that can explain the higher rates of stillbirths, preterm delivery, and small-for-gestational age infants that occur among women receiving ART. However, a larger sample size is needed to fully evaluate this hypothesis and whether similar placental findings will be seen among preterm and SGA deliveries.

**Primary Objective**

To determine the etiology of preterm delivery, small for gestational age infants, and stillbirths in a subset of deliveries among HIV-infected and HIV-uninfected women. *Hypothesis: Chronic placental insufficiency will be the most common etiology of stillbirth, preterm delivery and SGA among HIV infected women on ART*

Design

Cross-sectional study of placental pathology in a subset of women delivering at Princess Marina hospital (as part of the parent study conducted at 8 maternity wards in Botswana)

Duration

Placental collection will occur for up to 48 months

Sample Size

A convenience sample of up to 220 women

**Study Procedures**

*Maternity Surveillance Sites and Study Accrual:* We will conduct this sub-study at Princess Marina Hospital (PMH) in Gaborone, one of the 8 sites in the parent study (Tsepamo). This site, which delivers approximately 7,000 women per year, will be large enough to allow us to quickly accrue 220 placentas. Based on data specific to this hospital from the Tsepamo study, we anticipate that there will be approximately 5 HIV-infected women delivering per day and 4 stillbirths, 22 preterm deliveries and 19 infants born SGA per week.

*Inclusion and Exclusion Criteria*

Inclusion Criteria:

• All women delivering on the labor and delivery ward at PMH at >24 weeks gestation will be eligible. We will use convenience sampling until we have reached our quota of placentas in each category (HIV-uninfected, HIV-infected, preterm, SGA and stillbirth).

Exclusion criteria:

• A birth outcome at < 24 weeks gestation.

• Infants born prior to arrival at the hospital.

• Inability to provide consent

The 24 week gestation cut-off accounts for the fact that women < 24 weeks gestation are admitted to female medical or surgical wards rather than maternity; the difficulty assessing for NTDs among deliveries < 24 weeks gestation; and other potential biases that may occur by the inclusion of early pregnancy losses (non-live births < 24 weeks are considered miscarriages rather than stillbirths in Botswana). The exclusion of births that occur outside hospital grounds is similarly intended to reduce bias and maintain uniformity across all study locations.

*Placental Collection:* A subset of women delivering at PMH will be asked to consent (in writing) for placental collection. Collection will occur for a convenience sample of up to 220 women, and will be requested for the following groups of women (as identified at delivery and from the obstetrical record): 1) up to 20 normal deliveries (10 HIV+ / 10 HIV-); 2) up to 80 women with preterm deliveries (40 HIV+ / 40 HIV-); up to 80 women with small for gestational age infants (40 HIV+ / 40 HIV-); up to 40 women with stillbirths (20 HIV+ / 20 HIV-).

After delivery, the midwife will place the placenta in a bucket filled with formalin that has been supplied by the study team. The bucket will remain in the delivery room. Placentas will be discarded by maternity staff in the usual manner if consent does not occur. When written consent does occur, the placenta will be linked to the mother’s anonymous obstetrical record in the main study database to obtain all demographic, ART exposure, and obstetric information. The placentas will be weighed and examined fresh using a standard template for gross descriptions and sampling, and will be trimmed of cord and membranes. Small samples of placentas will be taken. Duplicate blocks will be made of proximal and distal umbilical cord, full thickness parenchyma, and any lesions grossly appreciated. The samples will be fixed in formalin and processed at the Botswana National Health Laboratory using standard protocols into paraffin blocks. The blocks will then be transported to the Massachusetts General Hospital in Boston for routine 5 mm sectioning and H&E staining.

*Obstetric Data Collection Process* Surveillance for birth outcomes and demographic data will occur via the parent Tsepamo Study. Data for this ongoing study are collected without recording identifying information and without individual consent. Any medical records and obstetrical records may be reviewed, but the surveillance is designed to anonymously extract the data that are included on obstetrical cards (which are the primary record of events in pregnancy and at delivery) and the outpatient cards (which are the primary record of HIV treatment). The information abstracted from obstetric records includes maternal demographics, antenatal care clinic (ANC) site (a proxy for home village) and number of ANC visits attended, medical history (including diabetes, hypertension, and other common conditions as well as medications prescribed for these conditions),laboratory values measured in pregnancy (hemoglobin and rapid plasma reagin), vital signs, pre-pregnancy weight (or weight in the first trimester), diagnoses during pregnancy, medications prescribed during pregnancy (including folate and multivitamin), obstetric history (including gravity, parity, last menstrual period, and estimated gestational age), referral reason (if patient referred to health center for delivery), and delivery/birth description. The delivery/birth details include birthweight, Apgar, vital status of infant (liveborn, fresh stillbirth, macerated stillbirth), delivery complications, complete infant exam, congenital abnormalities, initial feeding, infant ARV prophylaxis (if applicable) and infant status at discharge. Maternal HIV status and antiretroviral (ARV) history are often available in the obstetric record. This information is cross-checked using the outpatient clinic record to ensure accuracy of HIV testing date, ART treatment regimen, ART treatment start date, any ART switch/discontinuation during pregnancy, and nadir and most recent CD4 cell count and viral load.

*Data management and reporting:* Placentas sampled as part of this sub-study will be linked to the matching maternal record and unique identifier in the Tsepamo database at the maternity ward, and will thereafter remain anonymous. When pathologic results become available, these results will be added to this anonymous database by the same unique identifier and will thereby be linked to the information in the Tsepamo database while retaining anonymity of the placental sample and the Tsepamo records. Results from the pathologic examination will not be linked to patient identifiers and therefore will not be available to individual participants.

**Statistical Considerations**

*Definitions:* We will use the same definitions for birth outcomes as in our previous studies[20] and parent study: preterm delivery is delivery < 37 weeks gestation; small for gestational age is below the 10th percentile of birth weight using norms created from infants born to HIV-uninfected women in Botswana [3];stillbirth is fetal death >24 weeks gestation with an Apgar score of 0. Non-live births < 24 weeks are defined as miscarriages in Botswana, and are not registered through the obstetric/maternity wards. In hospital neonatal death is defined as death during the same hospitalization within 28 days of a live delivery. Births must occur at the hospital site (not at home or on route) to be included in the surveillance. We will define anemia during pregnancy as a recorded hemoglobin ≤10 g/dL. Maternal hypertension in pregnancy will be defined as a systolic blood pressure measurement >140 mmHg or a diastolic measurement >90 mmHg at any visit before labor, admission to hospital for hypertension, delivery complicated by hypertension, or induction for preeclampsia.

Pathologic definitions will be based on expert review. Categories will include infectious, inflammatory, acute placental insufficiency, chronic placental insufficiency, hemorrhage, and unknown etiology.

*Projected Accrual:* In the parent trial, approximately 135 women deliver at PMH each week and 26% (35/week) are HIV-infected. Preterm delivery occurs in approximately 16% (22/week), SGA in approximately 14% (19/week) and stillbirth in 3% (4/week). We are able to process up to approximately 4 placentas/day in the National lab, so we should be able to collect 220 samples in 6-12 months.

Up to 220 placental samples will be collected to provide convenience sampling of each adverse birth outcome and by HIV status and this study will be primarily descriptive. The sample size was calculated based on the time required for collection and available resources, but it is anticipated to be large enough to provide the first information regarding the predominant cause of each outcome in Botswana (by HIV status) and whether an placental insufficiency is a common cause for each adverse outcome. Although statistical power will be limited for this convenience sample, the sample size is sufficient to provide valuable information. In particular, this analysis will allow for an estimate of placental insufficiency among HIV+ and HIV- women with adverse birth outcomes; will allow for a determination of whether this etiology may be over-represented in HIV+ women; and will allow us to determine whether a potential intervention to prevent placental damage (e.g., antihypertensives or progesterone) may be warranted.

*Data Analyses and Interpretation:* Data analysis will be primarily descriptive. We will report the pathologic etiology of each adverse outcome (SB, PTD, SGA) by HIV-status and ART-exposure status. We will explore differences in pathology between ART regimens as well as differences by maternal comorbidity (hypertension, anemia, diabetes, infection) and maternal demographics (age, education, employment, etc).

*Anticipated Outcomes:* We expect to identify a pathologic diagnosis in >90% of placentas sampled. We hypothesize that placental insufficiency will be the most common diagnosis among stillbirth, preterm delivery and SGA infants born to HIV-infected women on ART.

**Study Limitations and Alternate Approaches**

The main limitation of our sub-study is that we may not be able to have sufficient power to find a small difference in the etiology of adverse outcomes by ART status. However, this will be among the largest placental studies in Africa and data will be used to determine the underlying mechanism for adverse birth outcomes in this population and also to generate data to guide potential interventions to prevent adverse birth outcomes.

# APPENDIX C: Summary of Protocol Changes

**Version 1.0 dated April 3, 2014 to Version 2.0 dated March 27, 2018**

Version 2.0 of the protocol introduced updates and expanded scientific aims for the surveillance study upon receipt of additional funding (R01 HD095766).

1. Updated study team roster
2. Updated study duration (60 months to 120 months)
3. Updated sample size (93,000 to 225,000 obstetric records)
4. Added Scientific Aim “To compare the incidence of stillbirth, preterm delivery, small-for-gestational-age, congenital abnormalities, and in-hospital neonatal death among infants exposed to DTG/TDF/FTC vs. other ART regimens from the time of conception
5. Added Scientific Aim “To emulate a randomized clinical trial of continuous “legacy” ART vs. preconception switch to DTG/TDF/FTC, evaluating for improvement in rates of combined adverse birth outcomes, and for each individual outcome (preterm delivery, small-for-gestational age infants, stillbirth, and in-hospital neonatal death).
6. Expanded secondary objective #4 to include analyses utilizing concomitant medications, distance from delivery site or other geospatial data, or other available covariates.
7. Added secondary objective 10:To evaluate antibody transfer by maternal HIV and ART status at the level of the placenta, in stored placental specimens previously collected for histopathology.
8. Added secondary objective 11: To improve CD4 and viral load surveillance, and to evaluate whether it is feasible to link infant HIV status or mortality to the Tsepamo database, by accessing electronic records.
9. Updated background with updated references, and guidelines to recommend dolutegravir (DTG)-bsaed ART.
10. Added **Section 2.4** on DTG in Pregnancy
11. Added **Section 2.5** on Minimal Existing Data for DTG Exposure in Pregnancy
12. Added **Section 2.6** on Legacy Regimens and Adverse Birth Outcomes
13. Added **Section 2.7** “An Ideal Randomized ‘Switch Study’ is not feasible (but can be emulated)”
14. Added **Section 2.8** on Upcoming ART Program Changes in Botswana
15. Added **Section 2.9** “Botswana is an Ideal Setting for an Emulated Randomized Clinical Trial Design.”
16. Expanded surveillance sites from 8-11, adding 3 clinics in Gaborone (G-West, Old Naledi, and Tlokweng) (**Section 3.1**)
17. Added description of research assistants collaboration with maternity ward nurses to obtain complete records on deliveries, and the permission for research assistants to discuss completeness or accuracy of clinical records with patients, when needed (**Section 5.3**).
18. Added collection of Omang number and patient name of HIV-positive mothers to allow for linkage to related laboratory results and records (e.g. CD4 and viral load results) (**Section 5.3**)
19. Added a post-discharge field to the database to track potential congenital abnormalities identified by care providers following delivery (**Section 5.4**)
20. Updated Projections for ART Exposures over time, with additional surveillance records and estimates (**Section 6.0**), including statistical considerations, and sample size and power estimates for new scientific aims.
21. Updated sources of research material to include data obtained from maternity obstetric records and newborn hospital records, from ART records if required, and from electronic records if required
22. Updated **Section 7.4.1** “Confidentiality” and **Section 7.6** “Procedures for minimizing potential risks” to address collection of personal identifiers (Omang number and name) in a separate encrypted REDCap database.
23. Added statement that women who consent to have their child photographed will be told that no clinical care is being provided by the study (**Section 7.4.2** “Benefits”)
24. Removed interim analyses from **Section 7.8**.
25. Added Placental Collection Sub-study as **Appendix** to protocol v2.0.

**Letter of Amendment #1 (June 15, 2018) to Birth Outcomes Surveillance Protocol v2.0 dated March 27, 2018**

This LoA expanded surveillance for possible neural tube defects associated with DTG exposure from the time of conception, adding 10 sites and increasing anticipated surveillance records by approximately 30% (~250,000 in total).

**Version 2.0 dated March 27, 2018 to Version 3.0 dated July 8, 2020**

Below is a summary of changes between the BHP Birth Outcomes Surveilance Study Protocol Version 2.0 dated March 27, 2018 andVersion 3.0 dated July 8, 2020.

1. Updated Study Team Roster:

* Rebecca Luckett and Chelsea Morroni have been added as co-investigators working on existing aims of the study. Charlotte Mdluli has been added as the Data Management Lead.
* Modiegi Diseko’s dual role as Co-investigator and Study Coordinator is now reflected in the list.
* Kathleen Wirth and Erik Widenfelt have been removed as they are not working on this study.
* Study Sponsor contact has been updated to include Nahida Chakhtoura.

1. The anticipated sample size/# of obstetric records to be reviewed (now up to 250,000 +/- 10%) has been updated to correspond to the expansion of surveillance sites approved in 2018. (**Sections 1.1, 3.1, 6.6.1**). The number of approved surveillance sites has been updated. (**Sections 3.1 and 5.1**)
2. Status and timing notes have been added. The status of Study Steps and Scientific Aims is now indicated as completed or ongoing (**Sections 1.2, 1.3**). The tense of some wording has been updated throughout the protocol to reflect items completed, ongoing, or planned. Planned timeframes are indicated in **Section 2.0**.
3. Secondary objective number 4 has been clarified for more specificity on some of the “other available covariates” that are planned for inclusion in analyses. (**Section 1.3**).
4. Record review procedures in **Section 5.3** on the data collection process and **Section 7.2** on sources of research material have been clarified.
5. Corrections of typographical errors and minor editorial changes have been made throughout.

**Version 3.0 dated July 8, 2020 to Version 3.1 dated June 15, 2021**

Below is a summary of changes between the BHP Birth Outcomes Surveillance Study Protocol Version 3.0 dated July 8, 2020 andVersion 3.1 dated June 15, 2021.

1. Added collection of Omang number and patient name of all mothers (previously this was only done for HIV-positive mothers) to improve capture of electronic data for all women in the surveillance system, and to track multiple pregnancies and exposure risks over time (**Sections 4.6 and 5.3**).

**Letter of Amendment #1 (April 13, 2022) to Birth Outcomes Surveillance Protocol v3.1 dated June 15, 2021**

The purpose of this LOA is to update and expand the study’s existing scientific aims in the current context of antiretroviral treatment in Botswana. Extended funding from the original sponsor (Eunice Kennedy Shriver National Institute of Child Health and Human Development) has been awarded to continue the surveillance research through 2026. The study’s overarching aims continue to be evaluating birth and maternal health outcomes in relation to current ART regimens used in Botswana and other exposures; comparing modern ART to legacy regimens; and evaluating the effect of switches in ART regimens. The extended funding and period of support allow for these aims to be updated with current exposures and health outcomes of interest and will support the expanded application of analytical methods, including using surveillance data to emulate randomized trials.

Any future full protocol amendment will include the changes outlined in this LoA.

These changes will affect the following sections of the protocol. Added text is bolded; deletions are shown in strikethrough.

1. The Study Team Roster has been updated to include the following collaborators who will participate in the expanded aims and are supported by the additional funding.

Additional funding supports the leadership of a local Data Core, which will be directed by Coulson Kgathi at BHP.

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1. Study Duration has been updated to reflect the additional funded years of surveillance (now from 2014-2026). This will be updated in Section 1.1 Study Schema.
2. Sample size: the sample size has been updated to reflect additional birth records (from 250,000 +/- 10% to 410,000 +/- 10%) that will be included in the surveillance over a longer time period. Surveillance through 2026 with 30,000-40,000 births per year will total up to 410,000 (+/- 10%), including records already surveilled since study start. This will be updated in the study schema (Section 1.1), Section 3.1 on accrual, and Section 6.6.1 on sample size.
3. Aim 3 has been updated to include current exposures as follows.

Aim 3: To compare the incidence of stillbirth, preterm delivery, small-for-gestational-age, congenital abnormalities, and in-hospital neonatal death among infants exposed to ~~DTG/TDF/FTC vs. other~~ **different** ART regimens from the time of conception.Previous surveillance in Botswana has found associations between ART and adverse birth outcomes, including associations with specific ART regimens. Although we have ~~recently~~ presented initial safety data for DTG *initiated* in pregnancy, it is unknown whether DTG exposure *from conception* will be associated with adverse birth outcomes or congenital abnormalities. We will perform surveillance at **up to 1**8 representative maternity sites throughout Botswana and capture data for ~~up to 9511~~**from** births with **different ART** ~~DTG~~ exposure**s** from conception **(e.g. DTG, TAF, CAB PrEP)**.

1. Aim 4 makes use of surveillance data to emulate a randomized clinical trial. This aim has been expanded to include trial emulation of ART regimens stratified by low and high weight groups. The Aim has been accordingly revised to read:

Aim 4: **To use surveillance data to emulate randomized clinical trials of interest in mother-infant populations:**

1. To emulate a randomized clinical trial of continuous “legacy” ART vs. preconception switch to DTG/TDF/FTC, evaluating for improvement in rates of combined adverse birth outcomes, and for each individual outcome (preterm delivery, small-for-gestational age infants, stillbirth, and in-hospital neonatal death). *[No changes other than sub-categorizing “A”]*
2. **To emulate a randomized clinical trial of DTG/XTC/TAF vs. DTG/XTC/TDF vs. EFV/XTC/TDF for women stratified by low and high weight groups, evaluating combined severe adverse birth outcomes, maternal hypertension, and each individual birth outcome (preterm delivery, small-for-gestational age, stillbirth, and in-hospital neonatal death). The ideal randomized clinical trial evaluating ART regimens for the entire duration of pregnancy cannot be conducted, and can only be emulated within a large database such as Tsepamo that contains ART exposure data from the time of conception. The primary analysis will compare combined adverse birth outcomes for each regimen stratified by high (≥80 kg), average (60-79kg), or low weight (<60 kg), with secondary analyses aimed at identifying weight inflection points (including <50 kg or lower) for the outcomes.**
3. We have added a specific aim to evaluate changes in weight gain and other health parameters across multiple pregnancies. Aim 5 has been added as follows:

**Aim 5: To evaluate changes in weight gain and other health parameters across multiple pregnancies. With the use of a unique maternal identifier (OMANG linkage), we will evaluate changes in HIV incidence patterns, repeat congenital abnormalities and adverse birth outcomes, and birth spacing over time for all pregnant women. For women with HIV, we will compare baseline weight and health status indicators by ART regimen at subsequent pregnancies.**

1. Statistical considerations (Section 6.0) for the updated aims and increased sample size with power calculations over additional years of surveillance, are as follows:

**As part of Aim 3, this study aim will determine the prevalence of major congenital abnormalities (CA) and NTDs among infants exposed to DTG-based ART vs. other ART regimens from the time of conception and vs. HIV-unexposed. Analysis will separately compare the prevalence of 1) any major congenital abnormality (CA), and 2) NTD between DTG exposure from conception and non-DTG exposure from conception, EFV from conception, DTG started during pregnancy, and HIV-unexposed. The risk of each birth outcome and the prevalence of major CA and NTD will be computed overall, by HIV status and by ART exposure at conception. For each of these groups, the risk and the prevalence will be calculated as the number of events (e.g. preterm delivery) over the number of births in that exposure group. The 95% confidence interval around the prevalence of abnormalities in each exposure group will be calculated with the Wilson method (Agresti). Differences in prevalence between the group with exposure to DTG at conception and the other exposure groups were determined and 95% CI calculated with the Newcombe method (Newcombe).**

**The projected total number of pregnancies in the Tsepamo Birth Surveillance study from 2014 through 2026 for each exposure group are: DTG from conception (N=26,131), non-DTG ART at conception (N=10,243), EFV from conception (N=24,842), and for HIV-unexposed (N=317,742). We calculated the minimum detectable difference in prevalence for all major congenital abnormalities and for NTDs between DTG at conception vs. each other exposure group, assuming 80% power and an alpha of 0.05 calculated in Pass Version 15.0. The prevalence estimates for each outcome (all major CA, NTDs) in each comparison group (DTG conception, EFV conception, and HIV-unexposed) are based on our published results. The minimum detectable difference in prevalence (80% power, alpha 0.05) between DTG at conception versus non-DTG ART at conception, EFV at conception, and HIV-unexposed are 0.28%, 0.22%, 0.15% for any major CA and 0.12%, 0.07%, and 0.07% for NTD.**

**For Aim 4B, the enrollment period for the target trials is 2014-2026 (or earlier if accrual is faster than expected), and eligibility criteria include being HIV+ and non-pregnant at the time of enrollment. In the target trial, eligible women would be conditionally randomized to EFV/TDF/XTC, DTG/TDF/XTC, or DTG/TAF/XTC. Randomization would be stratified by weight (<60kg, 60-80kg, or >80kg) at the time of enrollment. The hypothetical intervention also includes becoming pregnant 6-36 months after enrollment and having a pregnancy outcome ≥24 weeks at one of the Tsepamo delivery sites.**

**We will include the following prognostic factors in our models: year of ART initiation, time from HIV diagnosis to ART initiation (1 year or less, > 1 year), age at baseline (<25 years, 25-29 years, ≥30 years, unknown), marital status (married, unmarried or unknown), occupation (salaried, unsalaried or unknown), education (none or primary, more than primary, unknown), parity (0, 1 or more, unknown), delivery site and its distance to initial antenatal clinic, CD4 cell count (<200, 200-349, 350-499, ≥500 cells/mm3), and maternal HIV RNA when available (<400 or ≥400 copies/mL). To assess historical bias, we will use data from the HIV-negative women and calculate the risk of each adverse birth outcome by calendar year. We will perform further sensitivity analyses that stratify low weight as <50kg and 50-60kg; that stratify our analysis by site type (tertiary vs. district hospitals); that vary the time-frame for entry into the target study following HIV diagnosis (from 0 to 10 years); that vary the time-frame between enrollment and conception (from 1 month to 8 years); that leverage the HIV-negative population to explore temporal trends in adverse birth outcomes; and where we perform instrumental variable analysis using the calendar year of the guideline change to DTG (2016) and TAF (2021). Finally, we will consider BMI at enrollment (available for a subset of the total population) and consider underweight, normal weight, overweight, and obese categories in sensitivity analyses.**

**Our primary outcome for Aim 4B will be any severe adverse birth outcome, defined as very preterm birth, very SGA, stillbirth, or neonatal death. Secondary outcomes will include a combined endpoint of stillbirth or neonatal death and maternal hypertension. Tertiary outcomes will include a combined endpoint of any adverse birth outcome, individual birth outcomes, and other maternal outcomes such as macrosomia and c-section. The prevalence of the individual and combined endpoints for adverse birth outcomes are known from the Tsepamo Study data for EFV/TDF/XTC and for DTG/TDF/XTC, and differences by weight strata are under investigation. We have identified significant differences in adverse birth outcomes by baseline weight, and significant differences in the effect on weight by ART regimen (DTG vs. EFV). The effect of TAF combined with DTG on weight is profound but it is unstudied to date in terms of birth outcomes in women on TAF and DTG at conception. Thus, we hypothesize that weight gain from TAF will improve overall outcomes for low weight women, but could worsen overall outcomes for high weight women. For high weight women, whether TAF can be used safely is a critical question, and our definition of “adverse outcome” may need to be expanded to include macrosomia, c-sections, and maternal outcomes. Accordingly, we will create an ordinal scale for infant and maternal adverse outcomes, and include a weighted score as a secondary outcome. In the table below, we outline the minimal detectable risk differences based on 80% power comparing DTG/TAF/XTC with EFV/TDF/XTC and with DTG/TDF/XTC for any severe adverse outcome for stillbirth or neonatal death, and for maternal hypertension, separately for each weight group.**

**Minimal Detectable Absolute Risk Differences Based on 80% Power for Emulated Trial Comparisons, by Weight at Enrollment (ART initiation)**



**We will have 80% power to detect a risk difference in any severe adverse outcome of -3.2% comparing DTG/TAF/XTC with EFV/TDF/XTC and of -3.1% comparing DTG/TAF/XTC with DTG/TDF/XTC for the low weight group, and 80% power to detect a risk difference in any severe adverse outcome of 4.6% comparing DTG/TAF/XTC with EFV/TDF/XTC and of 4.4% comparing DTG/TAF/XTC with DTG/TDF/XTC for the high weight group. Stillbirth or neonatal death is the outcome of greatest public health concern and we will have 80% power to detect a -1.6% risk difference in DTG/TAF/XTC vs. EFV/TDF/XTC and a -1.6% risk difference in DTG/TAF/XTC vs. DTG/TDF/XTC for the low weight group, and 80% power to detect a 3.2% risk difference in DTG/TAF/XTC vs. EFV/TDF/XTC and a 3.1% risk difference in DTG/TAF/XTC vs. DTG/TDF/XTC for the high weight group. Since stillbirth or neonatal death is a rare outcome, we expect to have sufficient power to detect clinically significant risk differences for all other individual outcomes. Outcome risks by weight group for women on ART at conception were computed using existing Tsepamo data. We assume TAF roll out in Botswana will follow a similar pattern to the rollout of DTG, with few initiations in 2020-2022 and then increasing over a four-year period from 2023-2026. In the event that TAF use by 2026 is only half of what is predicted, this will modestly affect the minimal detectable risk difference calculations: in the low weight group we would have 80% power to detect risk differences of -4.3% to -4.4% for any severe adverse outcome and -2.1% to -2.2% for stillbirth or neonatal death, and in the high weight group we would have 80% power to detect risk differences of 6.4% to 6.7% for any severe adverse outcome and 4.6% to 4.8% for stillbirth or neonatal death.**

**Statistical considerations for Aim #5 relate to the number of second pregnancies available for evaluation from 2018-2026 for women with HIV (collection begun in 2018) and from 2021-2026 for women without HIV (collection to begin in 2021). Data for women with HIV show 834 repeat pregnancies have occurred in ~36 months. Using this as a guide, we can anticipate ~1,400 women with HIV and ~6,300 women without HIV with repeat pregnancy data by 2026. These numbers will be sufficient for the planned analyses. For example, in our primary analysis we will evaluate median inter-pregnancy weight gain (early second pregnancy weight – early first pregnancy weight), adjusted for inter-pregnancy length among women on DTG, EFV and women without HIV. Assuming 620 women on EFV and 580 women on DTG with median weight gain in the EFV group of 1kg,[45, 48] we will have 80% power to detect a mean difference of 3.5kg in inter-pregnancy weight gain between groups. We will secondarily look at the impact of breastfeeding vs. formula feeding on inter-pregnancy weight gain, evaluate risk factors for weight gain and weight loss, and use formal mediation analyses to evaluate whether inter-pregnancy weight gain mediates adverse birth outcomes (preterm, SGA, stillbirth and neonatal death) and adverse maternal outcomes (maternal hypertension, post-partum hemorrhage, C-section).**

**A secondary part of this analysis will be to measure HIV incidence among women without HIV in their first pregnancy and evaluate for risk factors for HIV seroconversion between pregnancies. While these data may overestimate population incidence, we will be able to identify high-risk populations that can be targeted by the rollout of PrEP in Botswana. Additionally, we can measure trends in HIV incidence over time and potentially evaluate the impact of PrEP rollout on incidence of HIV between pregnancies.**

**In prior Tsepamo analyses, we have been unable to evaluate the impact of prior adverse pregnancy outcomes on risks in the current pregnancy. However with data on repeat pregnancies, we will be able to estimate the increase in risk of adverse outcomes among women who have had prior adverse outcomes and evaluate whether these risks differ by HIV status and ART regimen. This is particularly important for our outcomes of preterm delivery and congenital abnormalities. We will additionally report the impact of short inter-pregnancy length on birth outcomes.**

**Letter of Amendment #2 (March 10, 2023) to Birth Outcomes Surveillance Protocol v3.1 dated June 15, 2021**

The purpose of this LOA is to update the study’s existing scientific aims to provide follow-up information regarding major congenital abnormalities that are identified in the Tsepamo Study. The study’s overarching aims remain the same, but we are requesting permission to reach out to a subset of mothers who had a child with a major congenital abnormality and determine the health status of these children. This information will be valuable for clinical care decisions made in Botswana and to understand the impact of major congenital abnormalities on children and families.

Any future full protocol amendment will include the changes outlined in this LoA.

These changes will affect the following sections of the protocol.

1. The Study Team Roster has been updated to include the following collaborator who will participate in the expanded aims.

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1. A primary aim of Tsepamo is to compare the incidence of congenital abnormalities among infants exposed to different ART regimens. We plan to expand this aim with the following secondary objective:

To describe health outcomes of infants with major congenital abnormalities born to mothers with and without HIV in the Tsepamo study, and to identify the clinical and social needs and utilization of health services for these children.

The following sections of the protocol will be revised as follows:

**Inclusion and Exclusion Criteria** for follow-up of births with congenital abnormalities (CAs)

--child born with a major congenital abnormality

--mother initially signed consent for a photograph at the time of birth

**Section 5.4. Data Collection for Congenital Abnormalities and Section 7.4.1 Confidentiality**

We will follow-up health outcomes for a subset of infants with major CAs via medical record review and semi-structured interviews with caregivers. For up to 150 infants born with a major congenital abnormality whose mothers already signed a consent to have an anonymous picture taken at birth, we will review the obstetric record and medical records for the infant outcome (including deaths and hospital stay). When applicable for infants who were alive at the time of hospital discharge, we will also call caregivers to determine how the child is doing. We will not contact those with known stillborn or neonatal deaths.

The data obtained from these interviews will be added to the Tsepamo REDCap database, and maintained in a coded manner. A summary will be presented to the Botswana MoH and for scientific presentation.

**Section 7.3 Participant recruitment and study consent**

Following record review, if additional information is needed from mothers or caregivers of infants born with major CAs who were alive at time of hospital discharge, we will call caregivers to determine how the child is doing. If a phone number is not found in the obstetric record, we may follow-up with the antenatal care (ANC) clinic, which is documented in the Tsepamo study. Via record review and discussion with families (if needed), we will assess longer-term survival, medical and social complications among infants born with major congenital abnormalities in Botswana.

For follow-up calls, we will first use a telephone consent script. We will request a waiver of documentation of informed consent for these telephone interviews. Caregivers who agree to proceed will be asked scripted questions about the child’s diagnosis and vital status, supports or treatment, and social impact.

**Section 6.0 Statistical Considerations**

The analysis for this aim will be descriptive, summarizing the follow-up data from up the convenience sample of up to 150 infant outcomes. Infant mortality and major morbidity will be summarized.

**Version 3.1 dated June 15, 2021 to Version 4.0 dated May 31, 2023**

Version 4.0 of the protocol incorporates previously approved revisions (clarifications of changes approved in past versions of the protocol), and changes approved in Letters of Amendment #1 and #2 to protocol version 3.1. The protocol has been organized to describe the study’s existing scientific aims in the current context of antiretroviral treatment and other exposures in Botswana, and remove outdated, historical estimates and text.

1. **Title page:** Added “Short title: Tsepamo”
2. **Co-Investigator list:** Updated to remove Miguel Hernan who is no longer working on the study. All other updates are per changes previously approved in LoA#1 and LoA#2 to protocol version 3.1.
3. Updates to duration and sample size have been made per approved LoA#1 to protocol version 3.1 (**Study Schema, Section 1.1**, **Section 3.1** on accrual, and **Section 6.2.1** on sample size and power).
4. Historical context and estimates have been removed, or updated with current context and estimates:

* **Section 1. Study Overview, Study Steps and Section 3.2 Anticipated Study Timeline** sections removed (historical description of original study timeline, no longer relevant).
* **Section 1. Study Overview, Objectives.** Primary objectives of previous versions of the protocol that have been completed (e.g. targeting historical antiretroviral regimens) have been moved to **Appendix A**. Primary objectives ongoing as of protocol version 4.0 are renumbered accordingly. All other updates to objective descriptions are per approved LoA#1 to protocol version 3.1.
* **Section 2. Background.** A description of the scope of surveillance over time with additional grant support and updated scientific aims has been added. Descriptions of historical, completed scientific aims have been removed. Protocol revisions are briefly described. Additional text describing background and rationale for current, approved, scientific aims has been added.
* Annual delivery estimates have been removed as these are variable and were based on an older version of the protocol (**Section 3. Study Sites**)
* **Section 6. Statistical Considerations** has been updated to remove outdated estimates for past completed analyses, and add current estimates per objectives approgved in LoA#1 to protocol version 3.1.

1. LoA#2 updated the study’s existing scientific aims to collect follow-up information regarding major congenital abnormalities (CAs) that are identified at birth. Changes approved in LoA#2 were made to the protocol, including **Section 1, Secondary Objectives** (#12)**, Section 4.1 Inclusion/Exclusion Criteria for follow-up of births with CAs, Section 5.4 Data Collection for Congenital Abnormalities, Section 7.4.1 Confidentiality.**
2. Clarifications related to previously approved protocol revisions are included:

* Collection of personal identifier (maternal Omang number) is clarified (as approved in protocol version 2.0 for HIV-positive mothers, and in protocol version 3.0 for all mothers) in **Section 5.3 Data Collection Process.** Outdated mention of anonymized data has been removed. The anonymity of the photograph of neonates with suspected congenital abnormalities is clarified—the expert reviewing the photograph will not have any identifying information, and the photograph will be taken with as few identifying features as possible (**Section 5.4 Data Collection for Congenital Abnormalities, Section 7.3 Written consent for photographs of children with suspected CAs, Section 7.4.1 Confidentiality**).
* Weight and height at ART initiation are added to list of variables recorded (per approved primary objective 2A and 2B) in **Section 5.3 Data Collection Process**.
* Existing waiver of informed consent and methods for obtaining informed consent are described. A waiver of informed consent was obtained for record surveillance at the beginning of the study (version 1.0 of the protocol). A written consent form is used to obtain consent to photograph infants with suspected CAs. With LoA#2 to protocol version 3.1 a verbal consent process was approved to interview mothers or caregivers of infants born with major CAs. These consent processes are now delineated in **Section 7.3**.

1. Frequency of monitoring rates of congenital abnormalities stratified by site for training purposes changed from monthly at the beginning of the study to quarterly at an advanced stage of the study (**Section 5.2 Maternity Staff Training**).
2. **Appendix A** has been added to list completed primary objectives from previous versions of the protocol for historical reference.
3. **Appendix C** has been added to chronologically summarize protocol version changes and letters of amendment.
4. **Other Administrative Changes:**

* Page numbers and subsection headings have been added to the Table of Contents.
* The Glossary has been updated to reflect current text in the protocol.
* Minor typographical or content corrections have been made.
* Section heading numbers, Tables and Figures have been renumbered according to deletions and additions.
* The reference list has been updated according to additions and deletions to the text.