The Tshilo Dikotla Study: Metabolic Outcomes of Children HIV/ARV-exposed uninfected (MOCHA)

Study Protocol (Version 5.0)

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A prospective cohort study with a nested randomized component investigating the early longitudinal metabolic effects of *in utero* HIV/antiretrovirals among HIV-exposed (HEU) children compared to HIV-unexposed uninfected (HUU) children in Botswana.

A Collaboration Between:
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All questions concerning this protocol including issues about daily operations, laboratory tests, forms and data analysis should be directed to the study's Principal Investigator, Dr. Jennifer Jao via email at jennifer.jao@northwestern.edu.

Glossary of Abbreviations

3TC	Lamivudine
AAs	Amino Acids
AAAs	Aromatic Amino Acids
ACs	Acylcarnitines
ACTG	AIDS Clinical Trials Group
ADP	Adenosine diphosphate
AGA	Appropriate-for-Gestational Age
ARVs	Antiretrovirals
ATP	Adenosine triphosphate
AZT	Zidovudine
BCAAs	Branched Chain Amino Acids
BHP	Botswana Harvard AIDS Institute Partnership
cART	combination Antiretroviral Therapy
CBMCs	Cord Blood Mononuclear Cells
ECAR	Extracellular Acidification Rate
EDC	Electronic Data Capture and Management System
EFV	Efavirenz
FTC	Emcitritabine
GA	Gestational Age
GDM	Gestational Diabetes Mellitus
GEE	Generalized Estimating Equation
HEU	HIV-Exposed Uninfected
HIV+	HIV-infected
HIV-	HIV-uninfected
HOMA-IR	Homeostatic Model Assessment- Insulin Resistance
HRDC	Health Research Development Committee
HTC	HIV Testing and Counseling
HUU	HIV-Unexposed Uninfected
IMPAACT	International Maternal Pediatric Adolescent AIDS Clinical Trials
IUGR	Intrauterine Growth Restriction
LAZ	Length-for-Age Z score
LC-MS/MS	Liquid Chromatography Mass Spectrometry
mtDNA	mitochondrial DNA
MGH	Massachusetts General Hospital
MOH	Ministry of Health
NRTIs	Nucleoside Reverse Transcriptase Inhibitors
NVP	Nevirapine
OCR	Oxygen Consumption Rate
OGTT	Oral Glucose Tolerance Test
OXPHOS	Oxidative phosphorylation
PBMCs	Peripheral Blood Mononuclear Cells
PC	Principal Component
PCA	Principal Component Analysis
PHACS	Pediatric HIV/AIDS Cohort Study
PMH	Princess Marina Hospital
PMTCT	Prevention of Mother-To-Child Transmission of HIV
SGA	Small-for-Gestational Age
T2DM	Type 2 Diabetes Mellitus
TDF	Tenofovir
QUICKI	Quantitative Insulin Sensitivity Check Index
WAZ	Weight-for-Age Z score
WLZ	Weight-for-Length Z score

1.0 Study Overview

1.1 Study Schema

1.1.1 Design

This study is a prospective cohort study with a nested randomized component of HIV-infected (HIV+) and -uninfected (HIV-) pregnant woman/child dyads in Botswana which will take place in Gaborone, Botswana at Botswana-Harvard AIDS Institute Partnership's (BHPs) clinical research facilities. A total of 300 HIV+ pregnant woman/fetus dyads on cART and 150 HIV- pregnant woman/fetus dyads will be evaluated for insulin sensitivity and followed through the child's 3rd birthday. Amongst HEU infants we will randomize participants at birth 1:1 with 150 to receive neonatal AZT prophylaxis and 150 to receive neonatal NVP prophylaxis. We will use targeted metabolomics to assess the role of intermediary metabolites in insulin resistance and directly assess mitochondrial function using Seahorse XF96e technology. At the time of study enrollment, all women must be willing to exclusively breastfeed for the infant's first 6 months of life. If *in utero* and neonatal HIV/ARV exposures are found to be associated with derangements in intermediary metabolism such that HEU infants are at increased risk for insulin resistance by 3 years of age, this would impact screening and prevention strategies for diabetes in this vulnerable population and argue for further research to identify prenatal and neonatal ARV regimens with superior PMTCT efficacy but minimal adverse metabolic consequences.

The primary endpoint of this study is insulin sensitivity assessed as: 1) pre-prandial (infants) or fasting (children) insulin, 2) Homeo-static Model Assessment – Insulin Resistance (HOMA-IR) [glucose (mg/dL) X insulin (µU/mL)/ 405].[1]

The primary objectives of this study are:

- To assess the early longitudinal metabolic effects including insulin sensitivity in HIV-exposed uninfected (HEU) children compared to HIV-unexposed uninfected (HUU) children
- To determine differences in the effects of neonatal zidovudine (AZT) vs. nevirapine (NVP) prophylaxis on early longitudinal changes in insulin sensitivity in the first 3 years of life.

The secondary objectives are to assess mechanisms underlying early longitudinal metabolic effects of *in utero* HIV/ARV exposure including specific alterations in intermediary metabolism and mitochondrial dysfunction.

1.1.2 Duration

Pregnant woman/child dyads will be enrolled antenatally [16-36 weeks gestational age (GA)] and mother/child dyads will be followed through the child's 3rd year of life. The entire study duration will be 5 years.

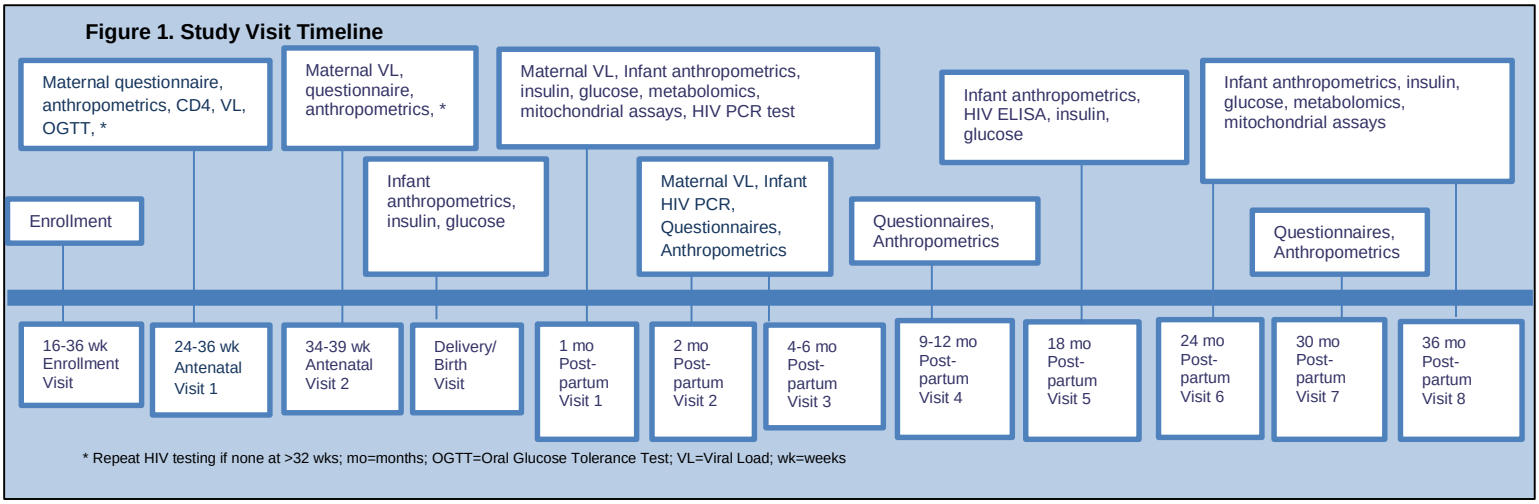
1.1.3 Samples Size

Based on previous data reporting a difference in slopes between SGA and AGA infants for insulin over time (birth to 3 years)[2,3] we anticipate a difference in slope for fasting insulin of approximately 0.48/year between the HEU and HUU infants with a standard deviation (SD) of 3.55.[4] Fixing the type I error at 0.05, and assuming an intra-class correlation of 0.20 among repeated measures,[5] a sample size of 300 HEU and 150 HUU will yield >90% power to detect a difference in insulin slopes of at least 0.48 between HEU and HUU children.

We anticipate a <1.1% mother-to-child transmission rate[6] but higher mortality rate (5%) among HEU infants in the first 12 months of life. We believe that HUU infant mortality will be lower at 2.5% in the first year of life. From experience in past mother-infant studies conducted by BHP we anticipate that the loss to follow-up rate will be 6% for HEU and HUU infants. For these reasons, we will recruit 336 HIV+ and 163 HIV- woman/child dyads, with the expectation that longitudinal data/samples will be available from at least 300 HEU and 150 HUU children.

1.2 Study Steps

The overall study design including a timeline of study visits is shown in Figure 1 below:



Study steps are detailed below.

Step 1: Maternal screening for study eligibility and possible enrollment
Location: Antenatal Clinic in a site where privacy can be offered

- Ensure that prospective women to be enrolled are between 16-36 weeks GA
- Explain the purpose of the study and eligibility criteria (see inclusion and exclusion criteria below)
- Ensure the woman is ≥ 18 years of age and has not had previously diagnosed diabetes
- Ensure the woman has a singleton gestation by ultrasound to meet inclusion criteria
- Ensure any HIV-infected woman is receiving or willing to initiate tenofovir (TDF)/ lamivudine (3TC) or emcitritabine (FTC)/ efavirenz (EFV) or TDF/3TC or FTC/Dolutegravir if HIV-infected
- Provide counseling on risk and benefits of breastfeeding versus formula feeding using a script approved by MoH and HRDC
- Determine maternal HIV status
    - A woman will be considered HIV-infected if she has evidence of a positive HIV test or, in the absence of documentation of a positive HIV test, can demonstrate that she is taking ARVs or has documentation of HIV care.
    - If a woman has evidence of a negative HIV-test on or after 32 weeks gestational age, she will be considered HIV-uninfected at study enrollment. Otherwise, she will receive HIV-testing and counseling (HTC) in accordance with Botswana national standards and the results of that test will serve as her classification status. Of note, all HIV-uninfected women must be willing to undergo HTC at all study visits during the period of breastfeeding, so monitoring for seroconversion can be achieved, for the protection of their breast fed infant.
- It is recognized that a proportion of HIV-infected women, after appropriate counseling, will elect to formula feed their infant. Since the study will require a 2:1 ratio enrollment of HIV+ vs. HIV- pregnant women arms, the primary recruiting effort will focus on recruitment of HIV-infected women who, after appropriate counseling and consideration, find breastfeeding to be the best infant feeding option for themselves and their infant.
- Provide details of the study to eligible women interested in participating, allowing the potential participant sufficient time to consider study participation and discuss her decision with any other individuals with whom she wishes to discuss participation. If interest remains, obtain written informed consent for study participation.

Step 2: Study Visit #1 – Antenatal Visit #1
Antenatal Study Visit #1 will occur at 24-36 weeks GA and the following maternal data will be collected:

1. Socio-demographics,
2. History of co-morbid conditions prior to pregnancy,
3. Family history of metabolic diseases,
4. Prior obstetric history,
5. Substance use during pregnancy,
6. Presence of gestational diabetes (GDM), gestational hypertension, and pre-eclampsia,
7. Anthropometrics,

8. HIV immune status (CD4 and HIV RNA levels) and ARV history (where applicable).
9. Ultrasonography on all participants will provide accurate GA dating and an assessment for intrauterine growth restriction.
10. Randomization of HIV-infected women only
11. Maternal blood specimens will be collected for CD4 and HIV RNA levels for all HIV-infected women, and glucose from an OGTT (oral glucose tolerance test) (performed using a 75g glucose load)[7] for all women. A total of 21 mL whole blood will be collected, 9 mL of which will be required for the assays above. Additional blood available will be banked at -80°C in a dedicated study repository for possible future testing related to intermediary metabolism or analytes of fatty acid oxidation and lipid metabolism should preliminary data show a need for this.

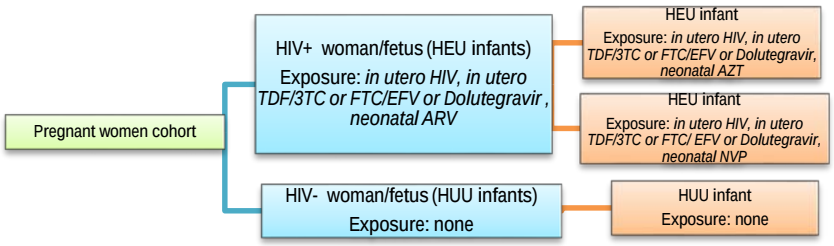
At Antenatal Study Visit 1, information on the above will be collected via questionnaires adapted from previous studies we have performed in Cameroon and Botswana (see Appendix) and from review of the participant's government health booklet. In Botswana, all citizens routinely carry their health records with them and both outpatient and inpatient records are well documented in these records.

Step 3: Antenatal Study Visit #2. At this visit we will perform the following:
1. Confirm understanding with the HIV-infected women of their randomization group
2. Repeat HIV testing of known HIV-uninfected women if a documented negative test is not available after 32 weeks GA (see Section 5.6, paragraph 3)
3. Repeat HIV RNA level
4. Anthropometric measurements

Step 4: Delivery/Birth Visit (within 72 hours of birth)
Location: Labour and Delivery, Postpartum Ward or BHP study site in a location where privacy can be offered
- For infants of mothers who have signed an informed consent to participate in the study:
  - Ensure that the infant is not greater than 72 hours old.
  - Ensure that the infant has no congenital anomalies or acute illness at delivery that would make it unlikely for the infant to survive to 12 months of life.

Prior to delivery, we will randomize all HIV-exposed infants 1:1 to receive either nevirapine (NVP) or zidovudine (AZT) (Figure 2). We will transport NVP infant prophylaxis to the delivery site for all mother-infant pairs that were randomized to NVP. AZT is the standard of care and is already available at labor and delivery sites. The mother will receive the assigned infant prophylaxis (NVP or AZT) from the labor and delivery nurse at delivery. After delivery, the study nurse and maternity ward nurse will confirm that the proper infant prophylaxis has been administered to the infant. Because dosing regimens are different between AZT and NVP, a placebo formulation for the evening dose of NVP would need to be created in order to blind the study. This would be difficult and costly to create, and therefore impractical for this study. Furthermore, the primary outcome is objective and unlikely to be biased by the behavior of the participant or physician based on knowledge of randomization grouping. Thus, participants and research coordinators will not be blinded to the group assignment but laboratory personnel will, and no placebo formulations will be used. Using a 1:1 randomization structure with alternating blocks of 6 and 8, group assignments will be electronically programmed into BHP's Electronic Data Capture (EDC) and management system. The EDC has been used as source documentation for other large clinical trials funded by the NIH, President's Emergency Plan for AIDS Relief, and Centers for Disease Control and Prevention and has been reviewed and accepted by external auditors as meeting research level security and conforming to source document requirements.

Figure 2. Schematic of Study Design and Nested Randomization Component in Relation to Exposure Groups



Maternal data which will be collected at the delivery study visit include:

1. Information on intrapartum complications such as pre-eclampsia/ eclampsia, placental abruption, severe hemorrhage, etc.
2. Anthropometrics (height and weight)

Infant data which will be collected at the delivery study visit include:

1. Birth weight, length, and head circumference
2. Congenital anomalies or other severe illnesses which would preclude survival of the child past 12 months of life
3. Phlebotomy for pre-feeding/fasting insulin and glucose levels totaling 5.5 mL

At the delivery visit a maximum of 6 mL would be drawn on the infant. The NIH recommends a limit of 3 mL/kg per single blood draw and a limit of 7 mL/kg in any 8-week period. In the Mma Bana study[6] where all HIV+ women received triple ARVs during pregnancy, 90% of infants weighed more than 2.5 kg. Infants conservatively gain approximately 0.6-0.9 kg per month, with a conservative estimated weight by 3 months of age of 4.8 kg in healthy term infants weighing 3 kg at birth and of 4.3 kg if the birth weight is 2.5 kg with a gain of 0.6 kg per month through three months.

Step 5: Interim Visits 1, 2, 4-6, 9-12, 18, 24, 30, 36 months of life (Postpartum Study Visits #1, #2, #3, #4, #5, #6, #7, and #8 respectively)
Location: BHP study site where private exam/interview rooms are available

Maternal data which will be collected at each visit include:

1. Anthropometrics (weight, height)
2. If HIV-negative, HTC to monitor for seroconversion
3. Abstract information from maternal health booklet or IDCC records including interim illnesses, use of any medications and IDCC results, if HIV-infected including interim CD4 and viral load results.

Infant/Child data which will be collected at each visit include:

1. Focused physical exam including infant/child weight, length and head circumference
2. Abstract information from the infant's health booklet including interim illnesses, interim medications, interim vaccinations
3. Phlebotomy for infant/child pre-feed/fasting insulin and glucose levels will be obtained at the 1, 18, 24, and 36 month visits. We have timed phlebotomy (1 and 18 months) to coincide with HIV PCR or ELISA testing schedules in HIV-exposed children so as to avoid unnecessary additional phlebotomy punctures. Phlebotomy for infant/child targeted metabolomic and mitochondrial studies will be obtained at the 1, 24, and 36 month visits. At the 1, 18, 24, and 36 month visits, we will collect no more than 8mL, 9mL 12mL, and 12mL of blood, respectively, which will yield adequate amounts for planned assays as well as approximately 1-2 additional aliquots of viable PBMCs (peripheral blood mononuclear cells) and plasma to be stored in the event preliminary data suggest the need for additional testing. Phlebotomy at the 1 month visit will follow current NIH guidelines for maximum phlebotomy limits as described above for Visit #2. (See below under "Blood Volumes" Section 5.8.2)
4. Infant HIV DNA PCR on HIV-exposed infants will be tested at 1 month, 2 months, 4-6 months, and any subsequent visits where the infant is still breastfeeding or has ceased breastfeeding since the last visit to confirm HIV infection status of the infant
5. Interview of mother to define actual infant feeding practices (see Appendix for Food Questionnaire) since last visit.

Step 6: Off-study Visit
Location: BHP study site or home visit, if permitted by enrolled women and home is the requested site of follow-up

- Mother-infant pairs will be placed off study when the infant reaches 3 years of life or sooner, if the infant dies or is reported to have moved out of the study area.
    - In the event of an infant death, prior to the infant reaching 36 months of life, information about the cause of death and caregiver health seeking behavior will be acquired from the mother/caregiver and abstracted from the infant's under-5 health booklet.

A summary of study measurements and timing are outlined in Table 1:

Table 1. Summary of Study Measures													
Variables	**Instrument/ Assay**	**Source**	**Time Points**										
OUTCOME VARIABLES			**Pre-natal 1**	**Pre-natal 2**	**Birth**	**1 mo**	**2 mo**	**4-6 mo**	**9-12 mo**	**18 mo**	**24 mo**	**30 mo**	**36 mo**
Pre-prandial or fasting insulin	Serum insulin ELISA at BHP laboratories	Infant serum			X	X				X	X		X
HOMA-IR	Plasma glucose and serum insulin ELISA at BHP laboratories	Infant serum and plasma			X	X				X	X		X
PREDICTOR VARIABLES													
MATERNAL													
Socio-demographics	Questionnaire*	---	X										
Comorbidities present before pregnancy	Questionnaire*	Medical record	X										
Family history of metabolic diseases	Questionnaire*	Medical record	X										
Prior obstetric history	Questionnaire*	Medical record	X										
Substance use during pregnancy	Questionnaire*	---	X		X								
Gestational Diabetes, Gestational Hypertension	75g load OGTT, Blood pressure via sphygmomanometer	Maternal plasma glucose	X										
Pre-eclampsia/ eclampsia	----	Medical record	X	X	X								
Anthropometrics	Maternal height for calculation of BMI Maternal weight for calculation of BMI	Standard scale, stadiometer	X X	X	X	X	X	X	X	X	X	X	X
HIV infection status	Rapid HIV ELISA at BHP laboratories or documentation if available	---	X	X^	X¥	X¥		X¥					
HIV immune status & viral suppression	CD4 level at BHP laboratories HIV RNA level at BHP laboratories	Maternal plasma	X X	X-	X$	X	X$ X	X	X§				
Antiretroviral history	Questionnaire*	Medical record	X		X	X	X	X	X	X	X	X	X
CHILD													
Intrapartum information	Medical Record	Medical record			X								
Anthropometrics	Child weight, length, head circumference for calculation of WAZ, LAZ, WLZ	Standard scale, stadiometer, tape measure			X	X	X	X	X	X	X	X	X
Gestational age at birth	Ultrasound dating	---	X										
HIV infection status	HIV PCR¶ HIV ELISA	Infant plasma				X	X	X		X			
Antiretroviral exposure	Questionnaire*	Medical record	X		X	X	X	X					
Feeding practices and diet	Solid Food Assessment Questionnaire*	---			X	X	X	X	X	X	X	X	X
Illnesses, Hospitalizations, Medical co-morbidities	Questionnaire*	Medical record			X	X	X	X	X	X	X	X	X
Metabolomics assays	Acylcarnitines and amino acids (intermediary metabolites) using mass spectrometry	Infant plasma				X					X		X
Mitochondrial functional assays	Mitochondrial oxygen consumption by measurement of 1)ATP production,2) basal and maximal mitochondrial respiration using the Seahorse® XF24	Infant viable PBMCs				X					X		X

BHP=Botswana Harvard Partnership; BMI=Body Mass Index; HOMA-IR=Homeostatic Model Assessment-Insulin Resistance; LAZ=Length-for-Age Z score; OGTT=Oral Glucose Tolerance Test; PBMCs=peripheral blood mononuclear cells; WAZ=Weight-for-Age Z score; WLZ=Weight-for-Length Z score
*** Standardized questionnaire adapted from previous Cameroon and BHP NIH-funded studies – see Appendix; ^ If not already done at Prenatal Visit 1 after 32 weeks; ¥If HIV-uninfected and still breastfeeding without a documented HIV(-) result in the last 3 months; $ From Medical Record; §If still breastfeeding; ¶ If mother shows evidence of seroconversion, infant PCR would be performed immediately**

1.3 Hypothesis and Study Aims

This study investigates the early longitudinal (first 3 years of life) impact of *in utero* and neonatal HIV/ARV exposure on child metabolic health, including insulin sensitivity, through these aims:

Aim 1: Investigate the association of *in utero* HIV/ARV and neonatal ARV prophylaxis with early longitudinal changes in insulin sensitivity among HEU children using HIV-unexposed uninfected (HUU) children as a comparator.

Hypothesis 1: HEU children exposed to *in utero* HIV/ARVs and neonatal ARV prophylaxis will exhibit increased insulin sensitivity at 1 month of life compared with HUU children, but these differences will reverse over time and by 3 years of age, HEU children will exhibit increased insulin resistance.

SubAim 1a: Assess differences in the effects of neonatal zidovudine (AZT) vs. nevirapine (NVP) prophylaxis on early longitudinal changes in insulin sensitivity.
Hypothesis 1a: Amongst HEU infants, neonatal AZT-exposed infants will exhibit increased insulin sensitivity at 1 month of life compared with neonatal NVP-exposed infants, but these differences will reverse over time and by 3 years of age, neonatal AZT-exposed infants will exhibit increased insulin resistance compared to neonatal NVP-exposed infants.

Aim 2: Assess whether specific intermediary metabolites (acylcarnitines and amino acids) reflect early changes in insulin sensitivity in HEU children.
Hypothesis 2: Short-chain acylcarnitines and branched-chain amino acids will be positively associated with insulin resistance in the first 3 years of life in HEU children.

Aim 3: Assess whether mitochondrial dysfunction contributes to early changes in insulin sensitivity and intermediary metabolism in HEU children.
Hypothesis 3: Mitochondrial dysfunction will be associated with insulin resistance and unfavorable alterations in intermediary metabolism in the first 3 years of life in HEU children.

2.0 Background and Significance

2.1 HIV-exposed uninfected infants: an expanding population worldwide. With the introduction of zidovudine (AZT) in 1994 and now widespread use of combination antiretroviral therapy (cART) for the prevention of mother-to-child transmission (PMTCT) of HIV, great strides have been made to reduce vertical transmission rates.[8-10] The overwhelming success of PMTCT has resulted in a diminishing population of perinatally HIV-infected children but has translated into a mounting number of HIV-exposed uninfected (HEU) children, particularly in countries with a high prevalence of HIV. In sub-Saharan Africa, an estimated 20% or more of all infants today are born HEU,[11] and in Botswana this number is approximately 30%, underscoring the public health importance of this population.

2.2 In utero HIV, antiretrovirals (ARVs), and neonatal ARVs: PMTCT exposures with potential lifelong implications. HEU fetal and infant exposures in Africa are unique in that they include exposure to intrauterine HIV, intrauterine ARVs, neonatal ARV prophylaxis, and in some cases, to maternal HIV/ARVs via breastmilk. Scale-up of PMTCT has resulted in a growing number of African countries implementing WHO's Option B+ recommendations[12] which provide HIV-infected (HIV+) women with triple ARVs - regardless of maternal immune status - throughout pregnancy, breastfeeding, and for life, thus shifting the landscape of *in utero* HIV/ARV exposures for HEU infants in Africa. In addition, all HIV-exposed infants receive neonatal ARV prophylaxis [either nevirapine (NVP) or AZT depending on country guidelines] for the first 4-6 weeks of life, representing another PMTCT exposure. These exposures comprise both an infectious and a drug exposure, making it often difficult to disentangle the effects between the two. Several short- and longer-term outcomes have been reported in HEU children in association with *in utero* HIV/ARV exposures including preterm birth[13-15] and small-for-gestational age (SGA) outcomes,[13] mitochondrial toxicity,[16-20] decreased postnatal growth,[21,22] and genotoxicity.[23,24] Though the benefits of ARVs during pregnancy far outweigh the potential risks, continued monitoring is necessary as the intrauterine interval is a critical developmental period.

2.3 Diabetes: a growing global epidemic. Today approximately 347 million people are living with diabetes worldwide, and this number is expected to soar to 552 million by 2035.[25] In sub-Saharan Africa, where an estimated 63% of the population remains undiagnosed, roughly 22 million people are living with diabetes,[25] a number projected to increase by 97% in 2030.[26] Childhood/adolescent insulin resistance and type 2 diabetes (T2DM) have increased at a disturbing rate,[27] with estimated prevalence rates reaching as high as 25-40% for insulin resistance[28] and 30% for T2DM[29] in many countries. Understanding early risk factors which stem from the fetal period for metabolic diseases is of significant public health importance, as it may be possible to design interventions which can successfully curb the growing worldwide problem of obesity and diabetes.

2.4 Fetal origins of insulin resistance. Developing theories on the origins of disease have suggested that fetal programming and the *in utero* milieu have a durable effect on the long-term metabolic health of an individual.[30,31] Both preterm birth and SGA outcomes can result from an altered intrauterine milieu and are independently associated with an increased risk for insulin resistance later in life.[32-35] In fact, SGA infants have been shown to exhibit a "thrifty" and more insulin sensitive phenotype at birth, only to reverse later in childhood to a more insulin resistant state compared to appropriate for gestational age (AGA) infants.[2,3,36]

2.5 Metabolic complications of in utero HIV/ARV exposure. Metabolic complications from *in utero* HIV/ARV exposures include poor intrauterine growth,[13] mitochondrial toxicity,[17,19,37,38] and altered intermediary metabolism,[4,39,40] all of which could perturb energy metabolism and impact the short- and long-term metabolic health of HEU children. **Moreover, our preliminary work has shown that *in utero* HIV/ARV and neonatal ARV exposure is associated with increased**

insulin sensitivity (see below Aim 1) and abnormal intermediary metabolism (see below Aim 2) which appear to impair the way in which HEU infants utilize metabolic fuel substrates at 6 weeks of life.[4]

Derangements in intermediary metabolism in HEU infants. Intermediary metabolism can be defined as metabolism occurring within the cell and includes pathways such as glycolysis, the Krebs cycle, fatty acid oxidation, and amino acid metabolism. (Figure 3) Intermediary metabolites are those involved in these pathways and include, but are not limited to, acylcarnitines (ACs) and amino acids (AAs), the latter of which include branched chain amino acids (BCAAs) and aromatic amino acids (AAAs). Studies have demonstrated that HEU infants exhibit higher rates of abnormal AC profiles than the general population.[39,40] The long-term significance of these abnormal ACs is still unclear, but we recently reported a positive association between a signature array of intermediary metabolites (short-chain ACs and BCAAs) and insulin resistance in a cohort of Cameroonian HEU and HIV-unexposed uninfected (HUU) infants.[4] Several adult non-HIV studies have also shown similar associations between levels of intermediary metabolites (ACs, BCAAs, and AAAs) and insulin resistance,[41-47] and one study of children reported an association between BCAAs and insulin resistance.[48]

Mitochondrial dysfunction from ARVs in HEU infants. The principal function of the mitochondria is to produce energy in the form of ATP. (Figure 1) Mitochondria obtain energy from fatty acids or glucose via oxidative phosphorylation (OXPHOS). ARVs, and in particular, nucleoside reverse transcriptase inhibitors (NRTIs) are known to cause mitochondrial toxicity through inhibition of mitochondrial DNA (mtDNA) polymerase-γ,[49] production of defective mtDNA, and inefficient repair of errors in mtDNA replication, leading to disruption of OXPHOS and eventual mitochondrial dysfunction.[50,51] Increased levels of mtDNA,[18,19,52,53] decreased levels of mitochondrial gene expression,[19] and abnormal mitochondrial morphology[37] have all been observed in HEU infants. In its most severe sequelae, mitochondrial dysfunction can result in clinically significant neurologic or muscular disease in HEU children.[17] However, other more insidious effects may include effects on insulin sensitivity. Impaired mitochondrial activity and mitochondrial abnormalities have been proposed by us **(see Aim 3)** and others to be associated with insulin resistance and T2DM.[54-57] Insulin-resistant offspring of parents with T2DM have impaired mitochondrial function, with mitochondrial ATP synthesis reduced by approximately 30%.[54] Mitochondrial dysfunction in skeletal muscle may be the underlying pathology of insulin resistance.[58,59] In addition, close relationships exist between mitochondrial dysfunction, elevated oxidative stress, and T2DM.[60]

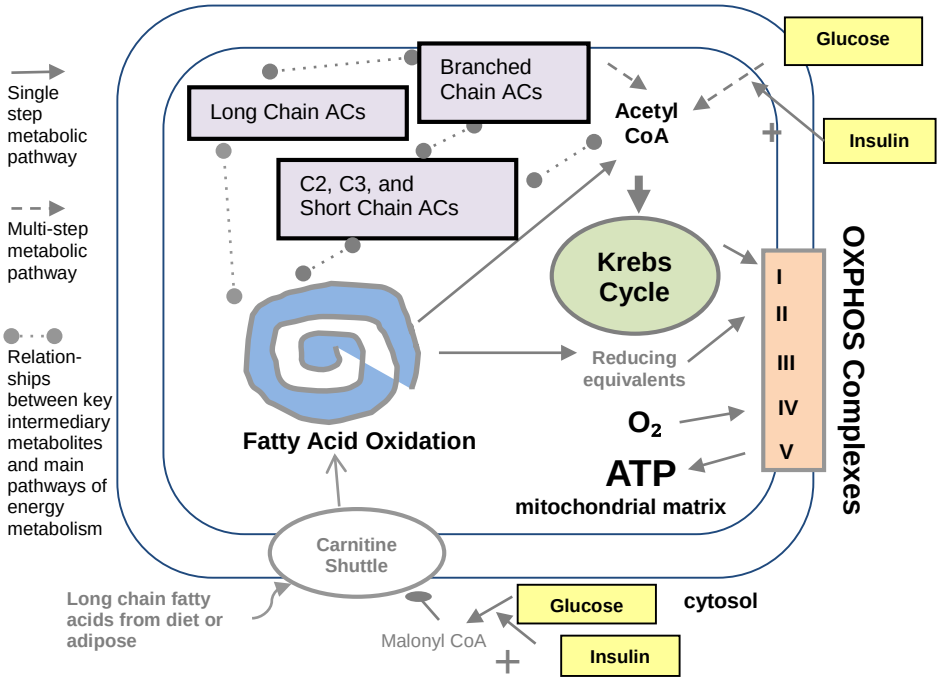
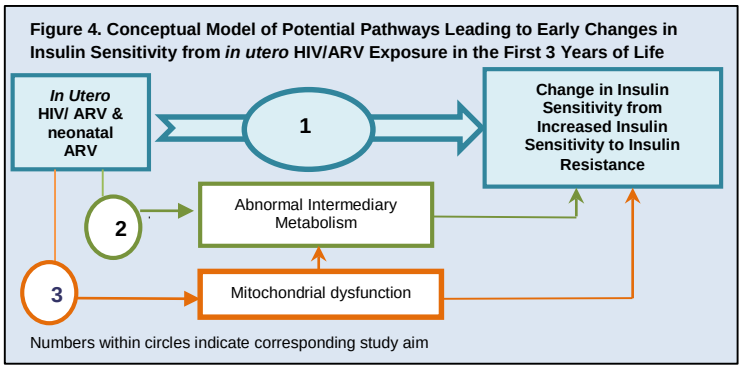


Figure 3. Relationships Between Key Intermediary Metabolites and Glucose Metabolism in the Mitochondria. ACs=acylcarnitines; ATP=adenosine triphosphate; OXPHOS=Oxidative Phosphorylation; Adapted from Jao J et al, J Clin Endocrinol Metab. 2015 Jul 2:JC20152198.

2.6 Proposed Conceptual Model of *In Utero* and Neonatal HIV/ARV Exposure Affecting Insulin Sensitivity

Our work draws upon the original theory posited by Barker and colleagues,[30,32] that fetal programming occurs whereby changes in the *in utero* environment trigger metabolic pathway plasticity that may permanently impair fuel handling and insulin sensitivity, resulting in adult diseases such as insulin resistance, obesity, and T2DM. *In utero* HIV/ARVs and neonatal ARVs represent exposures which may have deleterious long-term metabolic effects on the fetus/child. Specific pathways which we hypothesize to play a role in these metabolic effects are shown in Figure 4 where corresponding aims/hypotheses are indicated by the numbers within each pathway.



2.7 Improving Scientific Knowledge and Changing Clinical Practice. As HIV disproportionately affects resource-constrained settings, there is a pressing need to methodically evaluate the long-term safety of *in utero* and neonatal HIV/ARV exposure. Our data from Africa have shown that HEU infants have early metabolic alterations in insulin levels and intermediary metabolism which appear to favor increased insulin sensitivity at 6 weeks of life. What is unknown is whether these HEU children remain more insulin sensitive, normalize, or reverse to insulin resistance later in childhood, as is the case with SGA infants. Our study would fill this knowledge gap. In addition, we found that infants exposed to neonatal AZT demonstrated even greater shifts in fuel substrate utilization compared to those exposed to neonatal NVP. Currently there is still widespread use of neonatal AZT as well as NVP, and little data exist directly comparing the longitudinal metabolic effects of the two. If *in utero* and neonatal HIV/ARV exposures are found to contribute to derangements in intermediary metabolism such that insulin sensitivity is altered early in life and HEU infants are at increased risk for insulin resistance later in life, this would impact screening and prevention strategies for diabetes in this vulnerable population and argue for further research to identify prenatal and neonatal ARV regimens with superior PMTCT efficacy but minimal adverse metabolic consequences to the exposed fetus/child.

2.8 Research Infrastructure

Botswana-Harvard AIDS Institute Partnership (BHP) has been conducting maternal-child health research involving HIV-infected women and their infants in Botswana for over a decade. Established in 1996 as a collaboration between Botswana's Ministry of Health and Harvard School of Public Health AIDS Initiative, BHP maintains one of the largest HIV/AIDS research laboratories in Africa. Multiple studies enrolling more than 7,000 participants have been conducted at BHP, including HIV vaccine, PMTCT, and ARV trials (Phase I through Phase III), as well as observational studies, most of which have been NIH funded. BHP has research sites in Gaborone, Molepolole and Mochudi. All study visit data is captured electronically, with edits in place within electronic forms to ensure data accuracy and completeness at the time of visit. Participant specimens collected during the course of a visit are transported to BHP's research lab on day of collection and either processed immediately or stored in -70 ^{0}C freezers.

BHP has established a rapport with the clinical staff in the antenatal clinics and maternity wards, as well as pregnant women in Gaborone where more than 70% of women have at least 4 antenatal visits and greater than 98% deliver in a facility. This rapport has historically resulted in both timely study accrual, as well as high retention of mother-infant pairs in studies. We anticipate similar accrual and retention success for the current study.

The laboratory staff of BHP will process maternal glucose samples for OGTT, all infant HIV DNA PCRs and as well as infant/child insulin and glucose specimens. In addition, plasma and PBMCs will be processed from these specimens so that they may be shipped to their respective destinations for further planned assays: targeted metabolomics assays at the Ann & Robert H. Lurie Children's Hospital of Chicago/Northwestern University Feinberg School of Medicine and mitochondrial studies at the University of Hawaii. The study will build capacity in-country for insulin and glucose testing that will be performed locally.

3.0 Study Sites

All participants will be recruited and followed at BHP (Aim 1). No participants will be recruited or followed at any of the U.S.-based collaborator's institutions. However, specimens will be shipped to Ann & Robert H. Lurie Children's Hospital of Chicago/Northwestern University Feinberg School of Medicine for targeted metabolomics testing (Aim 2) and to the University of Hawaii for mitochondrial studies (Aim 3) as previously described. Below is a description of each site involved in this study.

Gaborone. The BHP study clinic is located at Princess Marina Hospital (PMH). Recruitment will occur in ANC sites in Gaborone and its surrounding area within a 100 km radius. For example, ANC sites within PMHs catchment area, as well as PMH itself will serve as recruitment sites, as will the BHP study clinic, located on the premises of PMH. All subsequent study visits for Gaborone enrolled mother-infant pairs will occur at the BHP study site.

Laboratory facilities.
Botswana Harvard HIV Reference Laboratory. Maternal viral load and CD4 testing, maternal OGTT, infant HIV DNA PCR testing, and infant/child insulin and glucose testing will be carried out at Botswana Harvard AIDS Research Institute Partnership's research laboratory facilities located in Gaborone in collaboration with the Botswana-Harvard HIV Reference Laboratory. In addition, all processing of samples (plasma for targeted metabolomics and PBMCs for mitochondrial studies) will be performed at the same facility. All laboratory samples will be transported daily from study sites

appropriately using standard operating procedures. Specimens to be shipped internationally will be shipped on dry ice and/or in liquid nitrogen shipper(s) at -80°C by BHP. BHP routinely ships plasma specimens every 2 weeks to the U.S. for the AIDS Clinical Trials Group (ACTG) and International Maternal Pediatric Adolescent AIDS Clinical Trials (IMPAACT) networks. Specimens for Aim 3 will be collected at 1, 24, and 36 months of life and processed as PBMCs in Botswana. Blood will be collected in EDTA tubes and PBMCs isolated over a Ficoll-Paque and washed three times with phosphate-buffered saline per IMPAACT/ACTG protocol.[61] Cells will be viably cryopreserved at a concentration of 1.0-1.5 million cells per 1.5 mL aliquot and stored at -140°C until the time of shipment.

Ann & Robert H. Lurie Children's Hospital of Chicago/Northwestern University Feinberg School of Medicine Metabolomics and Proteomics Core Laboratory. For each metabolomics testing occurring in the study (see Table 1), two 0.5 mL aliquots of plasma will be processed on site in Botswana, frozen, and maintained at -80°C until the time of metabolomics testing at Ann & Robert H. Lurie Children's Hospital of Chicago/Northwestern University Feinberg School of Medicine. We will measure intermediary metabolites (ACs and AAs) from infant plasma specimens at the PI's institution. Thirty-seven ACs from plasma will be measured using flow injection analysis (FIA) and multiple reaction monitoring (MRM) quantification with stable isotope labeled internal standards.[62] Full panel AAs in plasma will be measured using an underivatized liquid chromatography mass spectrometry (LC-MS/MS) method.[63]

University of Hawaii – Gerschenson Research Laboratory. For each mitochondrial functional assay occurring in the study (see Table 1), two 1.5 mL aliquots of PBMCs will be shipped every 3-6 months to the University of Hawaii on dry ice or in liquid nitrogen shipper(s). Mitochondrial functional assays will be performed at the University of Hawaii in Dr. Mariana Gerschenson's research laboratory which is equipped with a Seahorse XF96e machine to conduct these assays.

4.0 Recruitment and Enrollment of Study Participants

4.1 Inclusion Criteria

HIV-infected and uninfected pregnant women between 16-36 weeks GA are eligible for study enrollment. Other study inclusion criteria include:

- Women must be 18 years of older and able to provide informed consent for themselves and their infant to participate in the study.
- Participants must be Botswana citizens.
- Women must have evidence of HIV infection status. Women NOT documented as HIV seropositive must have documentation of HIV seronegativity during the present pregnancy at or after 32 weeks GA. Women who have an initial negative HIV test during the present pregnancy which was done at <32 weeks GA will need to undergo repeat testing on or after 32 weeks GA in accordance with national guidelines.
- HIV-uninfected women must be willing to undergo HIV pre-test counseling, rapid HIV testing and post-test counseling, referred to as HIV Testing and Counseling (HTC) during pregnancy.
- Women must be willing to remain in study area with their infant and attend scheduled study visits as described above until the child's 3rd birthday.
- For HIV-infected women, they must be on TDF/3TC or FTC/EFV or TDF/3TC or FTC/Dolutegravir at time of study enrollment or willing to initiate this treatment and continue throughout the period of breastfeeding, if not for their lifetime.
- At enrollment, all women must be willing to breastfeed exclusively for the first six months of life.

4.2 Exclusion Criteria

- Pre-existing maternal diabetes mellitus

4.3 Recruitment and accrual

Screening for study eligibility and enrollment for this study will occur at ANC clinics associated with PMH, or in the maternity wards of PMH. The ANC clinics have served as a viable site for study enrollment in past BHP studies, given the fact that over 90% of women in Botswana seek antenatal care.

Given experience with the recent Mpepu study where approximately 80 HIV+ pregnant women were enrolled per month, it is reasonable to estimate accrual of 20 HIV+ women per month who determine breastfeeding to be their safest infant feeding option as approximately 20-25% of HIV+ women opt to breastfeed in Botswana. Currently the Botswana MOH is considering changes for 2016 which will recommend that all virally-suppressed HIV-infected women breastfeed their

infants, so the number of eligible HIV+ woman/fetus dyads will likely increase throughout the course of the study. We do not anticipate any difficulty enrolling HIV- women willing to breastfeed (10 per month) for the infant's first 6 months of life. Therefore, we believe we can fully accrue participants in the first 18-21 months of the study, with follow-up through the child's 3rd year of life. We anticipate a <1.1% mother-to-child transmission rate[6] but higher mortality rate (5%) among HEU infants in the first 12 months of life. We believe that HUU infant mortality will be lower at 2.5% in the first year of life. From experience in past mother-infant studies conducted by BHP we anticipate that the loss to follow-up rate will be 6% for HEU and HUU infants. For these reasons, we will recruit 336 HIV+ and 163 HIV- woman/child dyads, with the expectation that longitudinal data/samples will be available from at least 300 HEU and 150 HUU children.

4.4 Informed Consent and Participant Reimbursement

All women, regardless of HIV status, will receive counseling on infant feeding in the language they are most comfortable speaking, generally Setswana, so that each woman can identify the infant feeding option that is safest, given their personal circumstances, for themselves and their infant. A scripted talk-off will be used by study staff during this counseling session (See Appendix for Infant Feeding Script). Women who elect to breast feed will receive information about the study. If women express interest in the study, they will undergo an informed consent process by trained study staff who have completed human subjects and good clinical practices training. Women will be given the opportunity to consult with family members or other individual who influence their decision after they are informed about the study and study staff will be appropriately trained to answer any questions the women may have about study participation. If the woman elects to participate in the study and have her infant participate, she will sign, or if illiterate mark in the presence of a witness, a written informed consent. Infants under the care of a guardian at study enrollment can be enrolled by the guardian with the guardian's permission and informed written consent.

Participants will be reimbursed 50 Pula (the equivalent of $5 U.S.) per study visit, congruent with other previous maternal-child studies at BHP, to be approved by the local governing IRB in Botswana.

5.0 Study Visits

5.1 Schedule of visits

The study will recruit HIV+ and HIV- pregnant woman/fetus dyads as early as 16 but no later than 36 weeks gestational age (GA) in Gaborone, Botswana and its surrounding area within a 100 km radius. A total of 300 HIV+ and 150 HIV- woman/child dyads will be evaluated through the child's third birthday. Within the 300 HIV+ woman/child dyads (HEU infants) we will randomize participants 1:1 at birth in alternating blocks of six and eight such that 150 receive neonatal AZT and 150 receive neonatal NVP prophylaxis in the first month of life. This nested randomized component within the cohort study will allow us to address sub-Aim 1a. Antenatal Study Visit #1 consists of the collection of maternal data as well as an oral glucose tolerance test (OGTT) to assess for gestational diabetes (GDM) between 24-32 weeks GA or at the earliest available time in women presenting after 32 weeks. Antenatal Study Visit #2 will occur between 34-39 weeks GA. The remaining study visits will occur at birth, 1, 2, 4-6, and 9-12 months postpartum, and approximately every 6 months thereafter up to 36 months postpartum. (Figure 1 above)

5.2 Missed visits

It is recognized that some mother-infant pairs may not be able to attend all study visits. To optimize attendance and minimize loss to follow-up, all women will be called within 3 days prior to their scheduled study visit and reminded about the visit. During participant phone contact, if any obstacles are known to attendance, the study staff will work with the woman to address obstacles, including offering a study driver to assist the woman with transportation or rescheduling the appointment to another convenient date within the window of the visit. While visits are schedule at specific time points (i.e. 1 month of life), there is an allowable window for all visits. The delivery visit can be conducted up to 72 hours after birth. The 1 month visit can be conducted as early as 21 days of life but no later than 53 days of life. The 2 month visit can be conducted between 54-90 days of life. The 4-6 month study visit may be between 91 days and 8 months of life. The 9-12 month visit may be conducted between 8 and 14 months of life. The 18, 24, and 30 month visits may be conducted within a 3 month window before or after each stated child age respectively. For example, for the 18 month visit, the window during which the child may still be seen is 16-20 months. The final study visit, at 36 months may be conducted 3 months before and up to 4 months after the child's 3rd birthday. We are allowing a larger window at the final study visit as this study visit is extremely important and this will allow for less missing follow-up/data. These available windows will minimize missed visits overall. However, if a mother-infant pair do not attend within the window of a study visit, that visit will be considered missed. Mother-infant pairs will continue in the study and at their next contact with study staff, data will the visit will be entered in the appropriate study visit window. Since maternal and infant interim health data is being

collected, if there has been a missed visit, all health data that has been documented in a woman's or infant's health booklet will be document at the next visit that the mother-infant pair physically attend.

5.3 Sick Visits

In the event that an enrolled woman presents to the study clinic requesting health care or health advice for herself or her sick infant, the clinical study staff will assist the mother as appropriate and refer them to the appropriate facility, government health clinic or hospital, for further management if necessary given the presenting symptoms. Sick visits will not be documented as a study visit. However, the study staff will document any health care provided in the woman's or infant's health booklet and data about the illness will be abstracted from the health booklet at the next scheduled visit.

As a benefit to the study participants, all study participants will receive a study mobile phone number that the participant can call 24 hours a day, 7 days a week in the event of an acute illness for which they need clinical guidance. Contact between a study participant and the on-call study clinician will not constitute a study visit.

5.4 Off-study

Mother-infant pairs will be placed off study:

- Upon completion of the protocol when the infant reaches 36 months of life (within 3 months before or 4 months after this time point as stated above)
- In the event that the mother requests to withdraw from the study
- In the event that the mother does not complete her first antenatal study visit
- In the event that the mother has multiple (2 or more) viable gestations seen on the ultrasound after entering the study
- In the event that the mother has a miscarriage or abortion (fetal demise less than 20 weeks GA), fetal death at greater than or equal to 20 weeks GA (IUFD), or stillborn
- In the event that the mother took ART for less than 4 weeks prior to delivery
- In the event that the mother states that she will be moving out or is unable to stay in the study area
- In the event of a maternal seroconversion before delivery
- In the event of a maternal death before delivery
- In the event that the infant is found to be HIV-infected
- In the event of an infant death
- In the event that the study is discontinued prior to full protocol completion by NIDDK, governing Institutional Review Board (IRB), the Botswana Government or other government agencies as part of their duties to ensure that research participants are protected.

In addition, mother-infant pairs who are placed off study for the following reasons will be replaced and, thus, will not count against accrual goals for the study:

- Mother did not complete first antenatal study visit
- Multiple viable gestations seen on ultrasound after entering the study
- Miscarriage or abortion – fetal demise <20 weeks GA
- Fetal death at ≥20 weeks GA (IUFD) or stillborn
- Maternal seroconversion before delivery
- Mother took ART for less than 4 weeks prior to delivery.

5.5 Maternal or Infant Death

In the case of maternal or infant death after enrollment (including stillbirth, if a woman has enrolled during pregnancy), a Death CRF will be completed within 3 business days of study staff becoming aware of the death. If a woman or infant dies in a health facility, events prior to and at the time of death will be collected from hospital records and a verbal autopsy CRF will be completed. If death occurs outside the hospital setting, a verbal autopsy CRF will be completed upon interview of the mother or guardian, in the event of an infant's death, or a relative in the event of a maternal death. If the mother of an infant dies, infant follow-up will continue throughout the study period. The purpose of the study will be explained to the guardian of the infant. In the event of a death, the study team will assist the mother or family members with referral to social services for additional support.

5.6 Prevention of Mother-to-Child HIV Transmission

It is of paramount importance that woman participating in the study be at the lowest possible risk of transmitting HIV to their infant over the course of the study. For HIV-infected women enrolled in the study who have identified breast feeding as the safest infant feeding option for themselves and their infant, MTCT can be virtually eliminated as long as maternal viral suppression is achieved throughout the study. Viral suppression requires compliance with a triple ARV regimen which, according to our inclusion criteria described above, will be in place for all HIV-infected pregnant women since all HIV-infected pregnant women will be receiving TDF/3TC or FTC/EFV or TDF/3TC or FTC/Dolutegravir. During the course of counseling on feeding choices, study staff will review with HIV-infected women their last documented IDCC viral load and discuss the risk of transmission based upon that viral load. Additionally, a viral load will be drawn at Antenatal Visits #1 and #2. If the final maternal viral load prior to delivery is found to be detectable, we will notify the participant, and, with her permission, share the result with the antenatal clinic staff prior to delivery as well as the labor/delivery staff at delivery, so that applicable infant feeding and prophylaxis may be considered according to the prevailing Botswana HIV Treatment Guidelines. If a woman with a detectable viral load opts to switch to formula feeding, she will still be eligible for free infant formula from the government under national HIV care and prevention guidelines and the mother-infant pair can continue to participate in the study. Throughout the course of the study, study staff will interview HIV-infected women for ARV adherence and will review IDCC documentation of viral load. If an HIV-infected woman reports non-adherence to ARVs or IDCC documentation reveals a detectable viral load, the woman will be counseled on the increased risk of MTCT and will be asked to make an informed infant feeding choice again. If an ARV non-adherent woman or woman with documented virologic failure opts to switch to formula feeding based upon updated risk based counseling, the mother-infant pair will be referred to her local clinic to secure free infant formula.

It is recognized that while HIV-infected women must choose the safest infant feeding choice given their personal circumstances and that a choice of breastfeeding may protect their HEU infant from mortality associated with infectious causes, including diarrheal disease, breastfeeding may still pose a risk of infant infection. For that reason, we will perform HIV DNA PCR testing of HEU infants at the 1 month, 2 month, 4-6 month, and any subsequent study visits where the infant is still breastfeeding or has stopped breastfeeding since the last visit. In addition, we will coordinate with a government health facility (eg hospital if the infant has been hospitalized) to perform the HIV DNA PCR in cases where the infant is hospitalized or cannot attend a study visit. In the event that an infant has a positive HIV DNA PCR, the study team will counsel the mother on the infant's HIV status, immediately refer the mother and infant to the closest IDCC site with Pediatric care capability and, if the HIV-positive infant is ≥ 6 weeks of age, Cotrimoxazole will be initiated by the study staff in accordance with Botswana national treatment guidelines.

High risk of MTCT also exists if a women experiences HIV seroconversion during pregnancy or breastfeeding. For that reason, all women who, at the time of enrollment, do not have evidence of a negative HIV-test on or after 32 weeks gestational age will receive HIV pretest counseling, a rapid HIV test and HIV posttest counseling from the study clinical staff. Furthermore, women enrolled in the HIV-uninfected arm who are breastfeeding their infant will undergo HIV testing and counseling (HTC) at each subsequent study visit as per the schedule outlined in Table 1.

HIV-uninfected women may undergo HTC at the following study visits if there is no recent (within the last 3 months) documentation of an HIV negative result: Antenatal Visit #1, Antenatal Visit #2, Birth Visit, Postpartum 1 mo Visit, Postpartum 2 mo Visit, or Postpartum 4-6 mo Visit and will be provided with their current HIV status during the study visit. In the unfortunate event that a previously HIV-uninfected mother is noted to have HIV seroconverted, their infant will have a HIV DNA PCR drawn at the same study visit, infant feeding counseling will be conducted with the woman if the infant is breastfeeding and the mother's informed feeding choice will be documented on a CRF. Finally, the mother will be urgently referred to IDCC clinic for initiation of ARV treatment, in accordance with national guidelines.

In the event that study staff becomes aware of a case of MTCT, the governing IRBs and study sponsor will be notified of the event within 3 business days. The notification will include the circumstances leading up to the event and re-evaluation of existing study protocols and SOPs to prevent further MTCT.

5.7 Adverse Events and Reporting

During the course of the study, it is possible that a woman in the HIV-uninfected arm of the study might acquire HIV or that an HIV-exposed uninfected breastfeeding infant could become HIV-infected. Additionally, it is possible that participants on the study may die during the course of the study, although there is no expectation that deaths would be study related. A serious adverse event is defined as any event temporally associated with a participant's participation in research that meets any of the following criteria:

- Results in death (including stillbirths)
- HIV seroconversions

All serious adverse events will be reported to the study sponsor, HRDC, and Partners Human Research Committee IRB within 3 business days of the study site becoming aware of the event. All other adverse events (non-serious) will be reported to the IRBs on an annual basis. In preparing adverse event reporting, the study team, with the assistance of the Principal Investigator will seek to incorporate any possible preventative measure that can be introduced using the circumstances of each adverse event as an opportunity to introduce quality improvement.

5.8 Laboratory

5.8.1. Testing and Panels

Maternal Rapid HIV ELISA: Maternal rapid HIV testing will be performed at ≥ 32 weeks GA in either women with no documented HIV infection status or women with a documented HIV negative test done during the present pregnancy but was performed prior to 32 weeks GA.

CD4+/CD8+: Maternal CD4+ and CD8+ cell counts and subset percentage evaluations will be assayed at the approved BHP Research Laboratory in Gaborone throughout the course of the study for enrolled women at delivery. CD4+/CD8+ testing results will be electronically transmitted to the correct participant's electronic data capture (EDC) record within 24 hours of the assay result.

Plasma HIV-1 RNA: All HIV-1 RNA testing will be performed using approved assays at the BHP Research Laboratory in Gaborone in Botswana. Viral load results will be electronically transmitted to the correct participant's electronic data capture (EDC) record within 24 hours of the completion of the viral load testing.

Maternal OGTT: All pregnant women will undergo OGTT between 24-36 weeks GA after enrollment in order to screen for gestational diabetes. We will use a 75g oral glucose load which the participant will drink as per WHO guidelines as described above.[7] Glucose will be tested at the following time points for this test: fasting, 1 hour and 2 hours post glucose load. Glucose testing will be performed by the BHP Research Laboratory and transmitted to the correct participant's EDC record within 24 hours of the assay result.

HIV DNA PCR: Whenever an HIV DNA PCR is required and it is the only sample required, it will be acquired as a dried blood spot (DBS) on filter paper following Botswana national standards for collection of DBS in the testing of HIV DNA PCRs. At study visits requiring other blood specimens, blood for the HIV DNA PCR will be obtained via venipuncture by study staff. All HIV DNA PCR results will be processed by BHP Research Laboratory. Any positive results will be communicated in real-time by phone or in person to the study site and the study's Principal Investigator following existing operating procedures for the notification of positive HIV DNA PCRs and the site will be instructed to obtain a second blood specimen for confirmatory testing. All HIV DNA PCR results will be electronically transmitted to the correct participant's EDCrecord within 24 hours of the completion of HIV DNA PCR.

Plasma for HIV ELISA at infant 18 months: This will be performed in HIV-exposed children by the BHP Research Laboratory.

Blood for serum insulin and plasma glucose: Insulin and glucose will be performed on the infant at the delivery, 1, 18, 24, and 36 month visits by the BHP Research Laboratory in Gaborone throughout the course of the study and testing results will be electronically transmitted to the correct participant's EDC record within 24 hours of the assay result.

Blood Samples for Targeted Metabolomics: For each metabolomics testing occurring in the study (see Table 1), two 0.5 mL aliquots of plasma will be processed on site in Botswana, frozen, and maintained at -80°C until the time of metabolomics testing at Ann & Robert H. Lurie Children's Hospital of Chicago/Northwestern University Feinberg School of Medicine. Specimens will be shipped on dry ice at -80°C by BHP. BHP routinely ships plasma specimens every 2 weeks to the U.S. for the AIDS Clinical Trials Group (ACTG) and International Maternal Pediatric Adolescent AIDS Clinical Trials (IMPAACT) networks. Dr. Yu's laboratory is a certified clinical genetics laboratory which provides confirmatory testing for newborn screen abnormalities of AAs, organic acids and carnitine/ AC profiles. We will measure intermediary metabolites (ACs and AAs) from infant plasma specimens at the PI's institution. Thirty-seven ACs from plasma will be measured using flow injection analysis (FIA) and multiple reaction monitoring (MRM) quantification with stable isotope labeled internal standards.[62] Full panel AAs in plasma will be measured using an underivatized liquid chromatography mass spectrometry (LC-MS/MS) method.[63]

Blood Samples for Mitochondrial Studies: Blood will be collected in EDTA tubes and PBMCs isolated over a Ficoll-Paque and washed three times with phosphate-buffered saline per IMPAACT/ACTG protocol.[61] Cells will be viably cryopreserved at a concentration of 1.0-1.5 million cells per 1.5 mL aliquot and stored at -140°C until the time of shipment.

Two 1.5 mL aliquots of PBMCs will be shipped quarterly to the University of Hawaii on dry ice. Mitochondrial functional assays will be performed at the University of Hawaii in Dr. Mariana Gerschenson's research laboratory which is equipped with a Seahorse XF96e machine to conduct these assays. Mitochondrial function will be assessed via the MitoStress Test using Seahorse XF96e[64] (Seahorse Bioscience, North Billerica, MA) technology. We will measure mitochondrial respiration [oxygen consumption rate (OCR)] and glycolysis [extracellular acidification rate (ECAR)] in PBMCs using XF96e cell culture microplates coated with poly-L-lysine. Cells will be allowed to adhere for 30-60 min in running media (80 µL). Preliminary data demonstrates that cells can be seeded at 200,000 cells/well and dosed with oligomycin (1 µM), FCCP (2 µM), antimycin a (1 µM), and rotenone (1 µM).

5.8.2 Blood volumes

Blood volumes are an important consideration for this protocol, and will be minimized to the greatest extent possible at every visit. We will follow NIH Clinical Center guidance for maximal allowed blood volumes allowed in research. Infant blood will be collected by trained pediatric nurses. At the delivery, 1 month, and 2 month visits combined, a total of 13.75 mL of blood will be drawn. We will draw no more than 8 mL at any blood draw during the delivery, 1 month and 2 month study visits. The NIH recommends a limit of 3 mL/kg per single blood draw and a limit of 7 mL/kg in any 8-week period. In the Mma Bana study[6] where all HIV+ women received triple ARVs during pregnancy, 90% of infants weighed more than 2.5 kg. Infants conservatively gain approximately 0.6-0.9 kg per month, with a conservative estimated weight by 3 months of age of 4.8 kg in healthy term infants weighing 3 kg at birth and of 4.3 kg if the birth weight is 2.5 kg with a gain of 0.6 kg per month through three months. If, for any reason, infants weigh less than 3.0 kg at the 2 month visit, priority will be placed on HIV DNA PCR testing, and if blood volume can still be drawn for the two remaining tests, it will not exceed a volume of 3 mL/kg. This control will provide added security to avoid the potential for exceeding the specified limits. At the 18, 24, and 36 month visits, we will collect 9mL, 12mL, and 12mL of blood respectively which will yield adequate amounts for planned assays as well as approximately 1-2 additional aliquots of viable PBMCs and plasma to be stored in the event preliminary data suggest the need for additional testing.

5.9 Clinical assessments and data collection

For this study, written informed consent will be documented on a paper form. Other study case report forms (CRFs) may be collected on either paper form or electronically using an encrypted study computer that can only be used by study staff with appropriate sign-on authority. If data is originally collected on paper CRFs, these CRFs will be the source document. If data is originally collected on the EDC, the EDC is the source document. BHPs EDC has been used as source documentation for other large clinical trials and observational studies funded by organizations such as the National Institute of Health (NIH), the President's Emergency Plan for AIDS Relief, and the Centers for Disease Control and Prevention (CDC) and has been reviewed and accepted by external auditors as meeting research level security and conforming with source document requirements. The EDC has an electronic audit trail that automatically documents any CRF modifications. The EDC also has security permission features in the administration module that require each EDC system user to have a profile. A user's profile requires designation of access to specific study functions, such as data entry or maintenance applicable to their position. This functionality can be restricted to specific CRFs, if applicable.

Women: Using paper or EDC CRFs, sociodemographic, co-morbid medical conditions, HIV-testing history and results, ARV treatment during this pregnancy and historically (if a woman was diagnosed with HIV prior to study enrollment), CD4, HIV RNA levels, estimated delivery date, results of OGTT, obstetric history will be entered at the prenatal visit. Specifics about labour and delivery for this pregnancy will be entered into the applicable CRFs at the delivery visit. The lab module of the EDC will be employed to prepare specimen labels and lab requisition for specimens collected at the designated study visits. At subsequent visits, focused physical exams, interim illnesses, interim medications taken, any changes in antiretroviral medications and adherence with ARVs (for HIV-infected women) breastfeeding safety reassessment and counseling, and subsequent CD4s and HIV RNA levels from clinic visits will be acquired either by interviewing the woman or by abstraction of medical information for her health records and recorded on CRFs. HIV rapid test results will be recorded on a CRF after completion of HTC testing and counseling. Specimen labeling and the lab requisition forms for maternal laboratory testing as outlined in Table 1 will be obtained from the EDC.

Children: Using paper or EDC CRFs, infant birth data including date of birth, gestational age at birth, birth weight, birth length, birth head circumference, birth exam findings, birth medications including prophylactic ARVs if the infant is HEU, birth immunization and actual feeding will be documented.. A specimen label and lab requisition for will be generated from the EDC to document collection of infant/child blood specimens at the specified time points discussed above. Additionally, at subsequent visits results of a focused physical exam, interim illnesses, adherence with prophylactic ARVs in the first month of life for HEU infants, interim medications, interim immunizations and actual feeding practices will be documented using CRFs. If a blood specimen is required (eg HIV DNA PCR testing for HEU infants who are breastfeeding at any study visit or plasma specimens for immunology aims), a specimen label and lab requisition form will be generated from the EDC to document the collection of the specimen.

Note: All laboratory results for tests performed at the BHP Research Laboratory will be electronically accessible within 24 hours of assay completion.

5.10 Data Management

The study's CRFs will be deployed in the EDC and incorporate inter-form and intra-form data validation logic. This systems approach ensures that the EDC CRFs are embedded with validation checks that display corrective error messages to the user in real-time to avoid the capture of implausible or incongruent data. The study team member will not be able to designate data collection for the study visit as "complete" in the EDC unless all required CRFs have been completed. BHP's Data Management Development team and the research staff have extensive experience with the use of real-time, automated, embedded validation logic and skip logic. These tools provide a high degree of data quality and preclude the study staff from concluding the study survey with the participant prior to obtaining all information necessary to ensure that the interaction results in a "usable" study questionnaire.

Study staff will devote a large portion of their two week study training period to reviewing each CRF question through prepared "scenarios" to ensure that all study team members understand the type of information being sought. The training session also empowers study staff to discuss question clarity and propose EDC CRF adaptions. Such careful and collaborative review ensures all study team members are seeking and documenting the same study data for all study questions.

On a weekly basis, the study's Data Manager and the study's PI will review a percentage of data to ensure data quality. All errors or omissions will be brought to the attention of the responsible study team member for correction at source. Data issues are tracked and compiled for immediate retraining of an individual team member or periodic retraining of all study team members. Additionally, the EDC development team reviews all data issues to determine if any issues can be prevented through software logic. All EDC changes are managed according to BHP's change management and software version control protocol.

The clinic study staff will enter data electronically as source. In the event of system failure, temporary outage, or study visits conducted off site, the team will revert to paper CRFs identical to the EDC CRFs. Once the system is restored, the paper CRFs (and source document) will be immediately captured into the EDC. CRFs initiated on paper during system down time will serve as source documents. Additionally, data abstracted from a medical record into the EDC will be copied or scanned and retained as a source document.

On a periodic basis, study's Principal Investigator will visit the study site to monitor consenting and data entry procedures and review compliance with study operating procedures.

5.11 Data Movement

The EDC data will be deployed on computers using Full-Drive Encryption (FDE) with daily transmission to the central database at the BHP Data Management Center (DMC) in Gaborone over and encrypted virtual private network. Prior to transmission, data is queued in an encrypted state. The EDC database is backed up according to BHP's backup procedures (GFS rotation). Backup files are encrypted and stored on encrypted drives before transfer to removable media or pushed directly over VPN. Secure and lockable rooms will be used to house all workstations and servers and to store mobile devices when not in use.

All information will be collected and transmitted electronically except consent forms and laboratory labels, laboratory requisition forms and laboratory packing lists. However, each of these paper items has an electronic representation in the EDC. A paper copy of the informed consent forms will be offered to the participants. The consent form with participants' signature and Omang number (national personal identity) will be collected and secured in locked file cabinets at the study sites. Laboratory labels with bar codes, as well as laboratory requisition forms and packing lists, will be used for sample tracking. The sample labels and documents will contain a computer-generated unique study subject identifier, sample date, date of birth, coded HIV status, and the type of sample. No personally identifiable information (PII) can be derived from the labels.

5.12 Data Security

All components of the distributed data systems use authentication and encryption to render electronically stored subject identity and personal health information unusable, unreadable, or indecipherable to unauthorized individuals for "data in use" (e.g. data being analyzed by statisticians), "data in motion" [e.g. data being transferred between data entry points and the Data Management Center (DMC) in Gaborone] and "data at rest" (e.g. data in storage at DMC in Gaborone).

Full Drive Encryption will be implemented at the hardware layer of all devices storing protected health information. A three-factor scheme will be used to authenticate users through the hardware layer to the application layer where personal health information is available. The applications will have user profiles to control access to certain data and reports. The application and database layers will use a combination of hashing and encryption for sensitive and personal data. Mobile devices and the staff operating them will not be equipped with the encryption keys to decrypt sensitive data fields.

5.13 Study Monitoring Plan

The Principal Investigator will take primary responsibility for monitoring and auditing study operations to ensure that the protocol is being conducted as specified and that all standard operating procedures (SOPs) are being properly performed. Data integrity and quality will be ensured as outline in section 5.10 based on programming logic embedded in the electronic CRFs. The PI or designated individual will validate the completeness of all consents. On a quarterly basis, audits of study SOPs will be performed.

6.0 Study Timeline

AIM	TASK	Y1	Y2	Y3	Y4	Y5
Aim 1	Regulatory approvals, study start-up					
	Recruitment					
	Follow-Up					
	Data Analysis & Dissemination of Results					
Aim 2	Metabolomics Assays					
	Data analysis & Dissemination of Results					
Aim 3	Mitochondrial Assays					
	Data analysis & Dissemination of Results					

7.0 Statistical Considerations

7.1 Aim 1 and Sub Aim 1a Statistical Analysis plan

Aim 1: Investigate the association of *in utero* HIV/ARV and neonatal ARV prophylaxis with early longitudinal changes in insulin sensitivity among HEU children using HIV-unexposed uninfected (HUU) children as a comparator.

SubAim 1a: Assess differences in the effects of neonatal zidovudine (AZT) vs. nevirapine (NVP) prophylaxis on early longitudinal changes in insulin sensitivity.

Baseline characteristics of HIV+ and HIV- women and HEU and HUU infants will be compared using the t test, Wilcoxon or chi-square test, as appropriate. WAZ, LAZ, and WLZ will be calculated using both INTERGROWTH and WHO standards.[65,66] HOMA-IR will be calculated.[1] Both insulin and HOMA-IR will be quarter-root transformed to normalize the distribution. First we will perform preliminary cross-sectional analyses at each of the time points where the primary outcome is measured (birth, 1, 18, 24, and 36 months) to evaluate differences in the primary outcome between HEU and HUU children. Then we will fit linear regression models at each cross-sectional time point to evaluate the association between *in utero* HIV/ARV exposure and the primary outcome while adjusting for confounders. Finally, linear mixed effects models will be used to evaluate the longitudinal association between *in utero* HIV/ARV exposure group and the primary outcome while incorporating the correlated structure of the data due to repeated measures over time. We will introduce an interaction term between the exposure group and time to assess differences in the trajectory of the primary outcome over time by exposure group such that the *p*-value and β coefficient for the interaction terms will show whether the effect reverses over time. Missing data will be handled by multiple imputation techniques.

Sub Aim 1a Statistical Analysis Plan. We will perform the analyses as outlined above for Aim 1 in a sub-group of only HEUs which will include HEU-AZT and HEU-NVP infants. We will begin by comparing baseline characteristics between HEU-AZT and HEU-NVP groups and continue with the same methods of analysis as outlined for Aim 1 above.

Aim 1 Sample Size Justification. Based on previous data reporting a difference in slopes between SGA and AGA infants for insulin over time (birth to 3 years)[2,3] we anticipate a difference in slope for fasting insulin of approximately 0.48/year between the HEU and HUU infants with a standard deviation (SD) of 3.55.[4] Fixing the type I error at 0.05, and assuming an intra-class correlation of 0.20 among repeated measures,[5] a sample size of 300 HEU and 150 HUU will yield >90% power to detect a difference in insulin slopes of at least 0.48 between HEU and HUU children. Furthermore, for sub-Aim 1a, a sample size of 150 HEU-AZT and 150 HEU-NVP infants will yield 90% power to detect a difference in insulin slopes of at least 0.48 between HEU-AZT and HEU-NVP children, ensuring more than adequate power for both Aim 1 and Sub-Aim 1a.

7.2 Aim 2 Statistical Analysis Plan

Aim 2: Assess whether specific intermediary metabolites (acylcarnitines and amino acids) reflect early changes in insulin sensitivity in HEU children.

We will perform both longitudinal and cross-sectional multivariate analyses to assess whether particular intermediary metabolites (increased ACs and BCAAs) are associated with early longitudinal changes (between 1 and 36 months of age) in insulin sensitivity.

Longitudinal analyses: Because we will measure a large number of intermediary metabolites, many of which are potentially highly correlated and represent similar pathways, we will first perform a principal component analysis (PCA) using an orthogonal rotation to reduce the complexity of all intermediary metabolites at each time point. This will allow us to re-group these metabolites into a smaller number of components. PCA is a variable reduction technique which converts a set of correlated variables into a set of uncorrelated variables defined as principal components (PCs). The number of PCs is less than the number of original variables and will be chosen based on scree plots, eigenvalues >1, and theoretical congruence with prior knowledge of intermediate metabolic pathways. Each PC will then be assigned a score representing the level of activation of its corresponding metabolic pathway, and these PC scores will then be used as new variables in the multi-variate model. Initial multivariate modeling will be performed to explore the effect of exposure group on the change in intermediary metabolites over time using mixed effect models while adjusting for confounders. Next, we will evaluate the contribution of intermediary metabolites measured at 1 month of life on the trajectories of fasting insulin and HOMA-IR (repeated measures of the outcome) over time using mixed effects models in HEU infants only. We will then repeat this model with group exposure [first with two groups (HEU vs. HUU), then with three groups (HEU-AZT vs. HEU-NVP vs. HUU)] added along with other covariates, and we will test for interactions between groups and intermediary metabolites at 1 month of life. Finally, a correlation analysis will be performed to assess the relationship between 1) changes in intermediary metabolites and 2) changes in fasting insulin and HOMA-IR measured from 1 month to 3 years. If a significant correlation is observed, mixed effects models with insulin as the dependent variable and intermediary metabolites as time-dependent variables will be used to confirm the association while controlling for group exposure and other covariates.

Cross-sectional analyses: To complement the longitudinal analyses outlined above, we will also perform cross-sectional analyses, considering the primary outcome at several fixed time points (1, 18, 24, and 36 months of age) separately. We will use the results of the PCA above in multivariate modeling for each cross-sectional analysis using linear regression modeling to evaluate the association between *in utero* HIV/ARV and insulin with PC scores of 1 month intermediary metabolites introduced as covariates.
Missing data will be handled by multiple imputation techniques.

***Aim 2 Sample Size Justification*.** Specifically, with 150 infants in each exposure group we will have 90% power to detect differences in slopes of insulin of 0.48 or larger using two-tailed tests at the 0.05 alpha level. In addition, 150 infants in each group will provide more than 85% power to detect correlations of 0.25 or larger between changes in metabolites and changes in insulin within each exposure group.

7.3 Aim 3 Statistical Analysis Plan

Aim 3: Assess whether mitochondrial dysfunction contributes to early changes in insulin sensitivity and intermediary metabolism in HEU children.

We will follow the same principals of analysis as outlined in Aim 2 above. Initial multivariate analyses will be performed using mixed effects models to assess the relationship between group exposure and changes in mitochondrial function parameters measured over time (1, 24, and 36 months) where group exposure is the predictor and mitochondrial function parameters are the outcome. Separate models will be run for the three mitochondrial function parameters listed above. Next, we will evaluate the contribution of mitochondrial function measured at 1 month of age on the changes in insulin and HOMA-IR over time measured from 1 to 36 months in HEU infants where measures of mitochondrial function will be used

as predictors in these mixed effects models. We will then repeat these models with group exposure [first with two groups (HEU vs. HUU) and then with three groups (HEU-AZT vs. HEU-NVP vs. HUU)] added along with other covariates, and we will test for interactions between group exposure and mitochondrial function at 1 month of age. Finally, to explore potential indirect and direct pathways between insulin/ HOMA-IR, intermediary metabolites and mitochondrial function over time which we have hypothesized in Figure 1, we will perform marginal structural modeling [weighted Generalized Estimating Equations (GEE)][67] in the following two ways: 1) using only intermediary metabolites and mitochondrial function parameters (measured at 1 month) to assess whether these intermediary metabolites or mitochondrial function parameters affect the association between *in utero* HIV/ARV group exposure and the change in insulin sensitivity (fasting insulin and HOMA-IR measured repeatedly at 1, 18, 24, and 36 months), 2) using all time points of intermediary metabolites and mitochondrial function parameters (1, 24, and 36 months) to assess whether the change in intermediary metabolites or the change in mitochondrial function affects the association between *in utero* HIV/ARV group exposure and the change in insulin sensitivity (fasting insulin and HOMA-IR measured repeatedly over time.) Marginal structural models/ weighted GEE have the added advantage of modeling repeated measures of both a covariate and outcome and are particularly robust when handling issues of feedback mechanisms whereby an outcome may be associated with subsequent time-varying covariates and confounding by indication may exist.

***Aim 3 Sample Size Justification*.** The sample size calculation for Aim 1 is also sufficient for Aim 3 since the primary outcome remains the same. In addition, with 150 infants in each group, we will have 80% power to detect at least a difference of 700 pmol AUC for mitochondrial maximal respiration between groups assuming an α=0.05 and a standard deviation=2188 pmol using data from non-insulin resistant children.[68]

8.0 Protection of Human Subjects

Enrollment of HIV-infected and HIV-uninfected women and their infants with collection of demographic and health data, as well as blood specimens as outlined in this proposal will require approval from Botswana's Health Research and Development Committee and Partners Human Research Committee as well as all other governing IRB bodies (Ann & Robert H. Lurie Children's Hospital of Chicago and University of Hawaii).

8.1 Human Subjects Considerations

8.1.1 Human Subjects Involvement and Characteristics

Women and children are the target population of this study. HIV-infected and HIV-uninfected women and their infants will be recruited. The health status of HIV-exposed children will be monitored throughout the course of the study, with both HIV-infected and HIV-uninfected mothers having access to study staff 24-hours a day, 7 days a week to discuss any health concerns utilizing a dedicated study mobile telephone. All infants who are reported to have any potential health problems will be referred to the closest government health facility by study staff. Although study procedures should ensure that no infants become HIV infected from maternal vertical transmission, in the event that an infant does experience HIV seroconversion, the mother-infant pair will be referred to the Botswana Masa Programme for antiretroviral (ARV) initiation and the study staff will immediately initiate Cotrimoxazole in accordance with national HIV Care guidelines.

Inclusion criteria for this trial will require that pregnant women must be18 years of age or older, able to provide informed consent for their enrollment, a citizen of Botswana, willing to enroll their infant in the study, committed to breastfeed their infant for the first 6 months of life, and be willing to attend study visits for the child's first 3 years of life. Incarcerated individuals will not be enrolled. Women with multiple gestation pregnancies or documented diabetes prior to pregnancy will not be enrolled. For women enrolled in the HIV-infected cohort, they must test positive or have a previously documented positive test for HIV at the time of enrollment and be receiving or willing to initiate TDF/3TC or FTC/EFV or TDF/3TC or FTC/Dolutegravir, Botswana's first-line cART as per national treatment guidelines. For HIV-uninfected women who have no documentation of their HIV status after 32 weeks gestational age in pregnancy, they must be willing to undergoing HIV counseling and testing performed by the study staff. Any HIV-uninfected mother who consents to participate in the study also must be willing to undergo HIV counseling and testing at subsequent study visits if no documentation of HIV status is available in the last 3 months (see section 5.6) to ensure that they have not seroconverted during the course of the study. Mother-infant pairs will not be eligible for study enrollment if the infant has a congenital anomaly or other life threatening illness that makes survival to 12 months of life unlikely.

It is anticipated that full accrual can be achieved within 18 months at PMH located in Gaborone, an existing research site for Botswana Harvard AIDS Institute Partnership (BHP).

8.1.2 Sources of Materials

For consented study participants, data sources will include documentation in the participant's medical records; current participant recall/interview; and laboratory testing as per study protocol.

For consented study participants, data sources will include documentation in the participant's medical records; current participant recall/interview; and laboratory testing as per study protocol.

Specifically, for mothers:

- Current vital signs including weight
- Demographic and socioeconomic data
- Medical and obstetric diagnoses and history, hospitalizations, weight, height, medication use (including maternal ARV and antibiotic use during pregnancy, labor, and postpartum), and prior laboratory results recorded during index pregnancy including CD4 cell count, HIV-1 RNA level, complete blood count, chemistry and syphilis screening results, all of which are standard of care for pregnant women in the national prenatal health program. [Note: 94% of Botswana women access prenatal care through the national program.]
- CD4 and HIV RNA levels will be collected from blood samples on all women at enrollment
- A 75g Oral Glucose Tolerance Test (OGTT) will be performed between 24-32 weeks GA or at earliest available time if enrolled later than 32 weeks to screen for gestational diabetes
- Additional plasma will be banked for future research studies
- Accurate dating will be obtained via ultrasound at enrollment
- For women enrolling as HIV-, HIV status will be confirmed using an HIV rapid antigen test at delivery, after providing pretest counseling, so long as there is no documentation of HIV testing on or after 32 weeks gestational age.
- Delivery/intrapartum information including duration of labor and any complications
- Adherence to ARV medications among HIV+ women
- Interim illnesses and medications received between Visit 1 and Visit 2 using questionnaires and the woman's health booklet to abstract data.

Specifically, for infants/children we will collect:

- Vital signs and physical exam including length, weight and head circumference at birth and each scheduled study visit
- GA at birth
- Detailed feeding information as reported by the infant's mother
- Any diagnoses from outpatient visits to government health facilities, hospitalizations, medications (including prophylactic and therapeutic ARVs and use of antibiotics)
- HIV-1 infection status via neonatal HIV PCR performed at 1 and 4-6 months of life and HIV ELISA performed at 18 months of age
- Pre-prandial (birth and 1 month) and fasting (18, 24, and 36 months) insulin and glucose
- Blood plasma from the 1 month, 24 month, and 36 month study visit will be stored and shipped to Ann & Robert H. Lurie Children's Hospital of Chicago for targeted metabolomics testing
- Viable PBMCs from the 1 month, 24 month, and 36 month study visit will be stored and shipped to the University of Hawaii for mitochondrial functional assays

8.1.3 Potential Risks to Human Subjects

We believe this study's risk can be categorized as Category One Research under 45 CFR § 46 Subpart D: research involving greater than minimal risk but presenting the prospect of direct benefit to the individual subjects, based on the definitions of minimal risk in 45 CFR § 46.405. The Institutional Review Boards (IRBs) recognized that: (a) the risk is justified by the anticipated benefit to the subjects including routine health reviews (screening for gestational diabetes and ultrasound dating) in the first year of life and anticipatory infant development guidance provide to women participating in the study; (b) the relation of the anticipated benefit to the risk is at least as favorable to the subjects as that presented by available alternative approaches; and (c) adequate provisions will be made for soliciting the assent of the children and permission of their parents or guardians, as set forth in §46.408.

The study is not likely to pose any physical, social, or legal harm to participants. However, potential risks include:

- Psychological stress that might arise from a new diagnosis of HIV-1 (although most of the mothers in the study will already know their HIV status), as over 90% of pregnant women in Botswana receive HIV-testing during antenatal care, or from stigma associated with participation at sites potentially associated with HIV care.

- There is risk of mother-to-child HIV transmission (MTCT) among women who breastfeed. However, all HIV+ women will be receiving or initiating cART consisting of current WHO 1st line regimens under Option B+: TDF/3TC, FTC/EFV, or FTC/Dolutegravir as part of enrollment criteria. They will have received this for at least 4 weeks by the time of delivery. HIV+ women who are breastfeeding will have their treatment adherence reviewed at each visit, and receive counseling to promote adherence. Our experience in other studies at BHP is that MTCT rates during breastfeeding have been below 1% when women are taking triple ARVs during breastfeeding.[6]

- There is a minor risk of local bruising and bleeding with phlebotomy.

8.2 Adequacy of protection against risks

8.2.1 Recruitment and Informed Consent

Mothers will undergo an informed consent process and will sign a written informed consent for their and their child's participation in this study. We will employ an informed consent form containing all of the required elements as outlined by CFR 45, 46.116. Informed consent forms for BHP studies are translated into Setswana, and back-translated into English. These translations are approved by both IRBs; Botswana's Health Research and Development Committee and Partner's Human Subjects Research Committee. In general, Setswana-speaking nurses or recruiters conduct the informed consent process with potential participants. In a private setting, the study staff member verbally reviews the contents of the entire informed consent form with the participant (regardless of her literacy level). For more complicated studies, this process can take 1-2 hours, allowing sufficient time for participants to ask questions. Schematics/diagrams are used when useful. Sometimes, informed consent occurs over two separate visits. At the end of the discussion regarding the study (and full review of the informed consent form), the participant is given the opportunity to read the form, to ask questions, or discuss the study with family members or significant person in their life, prior to providing written informed consent. Study staff will verbally ask prospective participants questions about the study purpose, procedures, risks, benefits, etc.—the most important elements of the study from a human subjects perspective—to ensure that the volunteer understands these prior to signing of the consent form.

8.2.2 Protection Against Risk

Confidentiality: All subjects enrolled in the study will be assigned a unique participant identification (ID) number. The participant ID number will be used for identification purposes on all laboratory specimens, evaluation forms, and reports retained in the research records. Any electronically stored personally identifiable information (PII) will be encrypted and accessible only through password protected computers. All consent forms which will contain participant name and national identity number (Omang), will be stored at Gaborone in a locked filling cabinet in a locked facility with 24-hour alarm service. Only the PI and the clinical study staff will have access to the locked filing cabinet containing consents.

All research staff at the clinical research sites are required to sign confidentiality forms pledging to hold research information in confidence. Furthermore, all study staff who work with human subjects undergo training in maintaining confidentiality (with SOPs specific to this topic).

Stigma: The risk of stigma is considered to be minor from participation in this study, and is mainly associated with willingness to be seen at a BHP study clinic. This is minimized by the fact that both HIV-infected and uninfected women will participate in the study with their infants and will attend the same study site. In previous BHP studies, women who have joined have not later had concerns about being seen at BHP sites.

MTCT: HIV infected women who will breast feed (BF) in the study will be at some risk for transmitting HIV to their infants. In the Mashi Study, a prior BHP study, we found no appreciable risk of BF MTCT in the first month of life (identical birth to 4 week positivity between FF and BF arms). [48] Beyond 4 weeks, late MTCT occurred in Mashi in the setting of ZDV prophylaxis – 2.7% from 4 weeks to 4 months, and 1.7% from 4 to 7 months. In the Mma Bana Study, a study where all HIV-infected women were taking triple antiretrovirals throughout breastfeeding, only 2 (0.3%) cases of MTCT among 709 breastfeeding women occurred. [35] The longer period of breastfeeding may actually provide a survival benefit, protecting infants against pneumonia or diarrheal disease. At the very least Mashi Study demonstrated similar 18-month HIV-free survival between feeding arms. All women in the study will be appropriately counseled about the risks/benefits of breast vs. formula feeding to help each participant decide what the preferred method is for her individual circumstances. This counseling will occur at each subsequent study visit for HIV-infected women with tailored counseling based on the woman's reported ARV adherence and IDCC documented viral load. HIV-infected women will still have access to free formula under Botswana's national guidelines, should they opt to discontinuing breastfeeding during the course of the study. In addition, HIV-exposed breastfeeding infants will be tested at all study visits and any sick visits, a testing

schedule which exceeds that of Botswana national guidelines and will allow for early identification of MTCT, in the rare event that such occurs. Lastly, all HIV-uninfected women will undergo HIV testing and counseling at each study visit as per the schedule outlined above and in Table 1, in an effort to identify any incident infections and provide women with the most current assessment of their risk of breastfeeding their infant based upon HIV-status.

8.3 Potential benefits of the proposed research to the subjects and others

There is an indirect benefit to society in helping to better understand the metabolic safety of *in utero* and neonatal ARVs. No mother or infant in the study will receive less than the current best standard of care in Botswana. Breastfeeding to 6 months may improve overall HEU infant survival. All infants will likely benefit from improved overall medical care and access to study clinicians. Women enrolled in the study will have access to ultrasound dating of their pregnancy which more accurately estimates delivery dates and reduces postdate deliveries. In addition, women will receive screening for gestational diabetes which is not routinely performed in Botswana. Those diagnosed with gestational diabetes will receive appropriate treatment and management that will improve the health of the mother and fetus. In addition, women enrolled in the study will have 7 days per week, 24 hours per day access to a study clinician who can assist with health maternal and infant health concerns and can coordinate care in the setting of acute illness. Family planning counseling and referral to clinics will be undertaken at all study visits, a service that exceeds national standard. Enrolled mothers will be provided with anticipatory guidance about the developmental stages which their infant will experience before the next clinic visit. This type of guidance is not routinely available in Botswana.

8.4 Importance of the knowledge to be gained

There is important societal benefit from this study. The study has broad applicability for Botswana and other regions of sub-Saharan Africa. In Botswana, nearly 1/3 of all infants are born HIV-exposed but remain uninfected as a result of the government's policy of providing HIV-infected pregnant women with triple ARVs. HEU infants experience increased morbidity, mortality, and perturbations in intermediary metabolism. If *in utero* and postnatal HIV/ARV exposures are found to contribute to derangements in intermediary metabolism such that insulin sensitivity is altered early in life and HEU children are at increased risk for insulin resistance later in life, this would impact screening and prevention strategies for diabetes in this vulnerable population and argue for further research to identify pre- and postnatal ARV regimens with superior PMTCT efficacy, but with minimal adverse metabolic consequences to the exposed fetus or infant.

8.5 Data and safety monitoring plan

The PI has primary responsibility for the overall conduct of the study, including the safety of human subjects. The PI will ensure appropriate (1) conduct of the informed consent process (e.g. that informed consent is obtained before proceeding with study procedures); (2) enrollment of study subjects; (3) collection and analysis of data; (4) implementation of study procedures to ensure consistent monitoring of subjects for possible adverse events (see below); (5) review of any adverse events and reporting funding agency and all regulatory bodies involved; and (6) maintenance of the privacy and confidentiality of study subjects. The PI maintains ultimate responsibility for the project and for the safety of study participants. The PI will be in contact with the research team on a regular basis to review the progress of the study and address any human subject issues that occur.

The main aim of this study is to evaluate early changes in insulin sensitivity in the first 3 years of life in HEU infants/children using HUU infants/children as a comparator group. While there is always the potential to find changes in insulin sensitivity in study participants that may be clinically significant in the diabetic range [fasting insulin >20μIU/mL or Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) >8] we do not anticipate this given our results from our similar cohort study in Cameroon where median fasting insulin levels at 6 weeks of age ranged from 2.7 -4.9 μIU/mL and no infant had a fasting insulin >12μIU/mL.[4] In addition, there is a randomized component where amongst HEU infants where we will randomize postnatal infant prophylaxis regimens 1:1 to AZT vs. NVP. It is important to note that both of these are well-recognized as WHO options for standard of care in infant prophylaxis regimens worldwide.[69] Despite statistical differences observed between groups, neither of these regimen groups within our Cameroon cohort exhibited clinically significant fasting insulin or HOMA-IR results in the diabetic range at 6 weeks of age. Therefore, we do not anticipate a risk for changes in insulin sensitivity which are clinically significant or in the diabetic range at the early age of 3 years in our proposed study in Botswana and believe our study to be more in line with an observational study where randomization will give us more tight control of the exposure groups (HEU exposed to postnatal AZT vs. HEU exposed to postnatal NVP vs. HUU infants) in order to best assess sub-Aim 1. As a result, we will not require an outside data safety monitoring board (DSMB).

Nonetheless, because early longitudinal studies of insulin sensitivity or intermediary metabolism have not yet been published in HEU and HUU infants/children in Africa and because few norms have been published on infant/child insulin

and HOMA-IR in Africa, we will put in place the following precautions in order to ensure the safety of our participants with regards to the monitoring of our primary outcome as well as any other adverse events should they arise:

- Procedures for Identifying, Reviewing, and Reporting Adverse Events and Unanticipated Problems:
    - Reports of HOMA-IR levels (calculated from pre-prandial (birth and 4 week) and fasting (18, 24, and 36 months) insulin and glucose) will be reviewed by the PI every 6 months. We will flag levels at a lower threshold than the diabetic range and use a threshold of 4 for HOMA-IR which indicates insulin resistance in older children and adults.[70] If levels exceed those in the insulin resistant range, we will review the entire case and if necessary, recall the study participant for a repeat insulin and glucose and have the participant fully evaluated.
    - Study staff can be reached 24 hours a day, 7 days a week, so that participants may present with potential adverse events (including those referred for clinically significant insulin or HOMA-IR levels) as they arise.
- Plan for reporting both anticipated and unanticipated adverse events:
    - Adverse events will be reported to the local Botswana's Health Research and Development Committee and the Partners Human Research Committee IRB.
    - The report will include a description of the event, when and how it was reported, as well as any official chart records or documentation to corroborate the event or the reporting of the event. All adverse events will be graded as mild, moderate, or severe. Any serious and/or unanticipated adverse event will be reported to the sponsor, HRDC, and Partners Human Research Committee IRB within 3 business days of the study site becoming aware of the event. All other adverse events (non-serious) will be reported to the IRBs on an annual basis.

Infant Feeding Choices

Infant Feeding Choices

How you decide to feed your baby is your choice. Before you decide how you will feed your baby it is important that you understand the risks and the benefits of the different feeding methods.

If you are HIV positive, feeding your baby with infant formula is the surest way to protect your baby from becoming infected with HIV after birth. However, babies who are formula fed are more likely to become ill from infections like diarrhea and respiratory diseases. Babies who are formula fed are also more likely to die from these infections than babies who are breastfed, especially early in life. By 2 years of age, the difference between formula and breastfeeding is smaller, but still present. In a previous study in Botswana, about 9 out of 100 babies died by 2 years if they formula fed, compared with about 7 out of 100 babies who breastfed. Formula feeding has been shown to be a higher risk to infants at times when there are outbreaks of diarrheal illnesses in the community. If you choose to formula feed your baby, you can reduce the chance that your baby will become ill from diarrhea by making sure that the formula is prepared safely using clean and boiled water and using clean bottles or a cup and spoon for each feeding. If you choose to formula feed, the Botswana government will provide you with free formula until the baby is 12 months of age.

If you are HIV positive and you breastfeed your baby, your baby may become infected with HIV during breastfeeding. However, the risk of HIV transmission can be reduced if mothers take three medications at the same time that are effective in treating HIV during pregnancy and breastfeeding. These medications are called antiretrovirals. Research has shown that HIV transmission during breastfeeding can be lowered to less than 1 in 100 babies when the mother takes three antiretrovirals from pregnancy and throughout breastfeeding. If you choose to breastfeed your baby, you also should not mix breastfeeding with infant formula or other foods or liquids during the first 6 months; rather, you should breastfeed exclusively for the first 6 months (giving only breast milk). All HIV-infected women who prefer to breastfeed will be able to receive free antiretroviral medications from the Botswana government during pregnancy and breastfeeding, to protect their babies.

If you feel that you can always safely prepare formula for your baby without breastfeeding at all, and can bring your baby in for good medical care quickly any time that he/she becomes ill, then formula feeding your baby may be the best option for your baby. If you are not sure that you can do this but you do think that you can take three antiretrovirals medications every day, without missing doses, then breastfeeding exclusively for the first 6 months while taking this medication and continuing breastfeeding through twelve months of life while give your baby appropriate food starting at 6 months may be the best option for you and your baby.

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